INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700  800/521-0600
THE BOVINE CALPASTATIN GENE PROMOTER AND A NOVEL N-TERMINAL REGION OF THE PROTEIN ARE TARGETS FOR cAMP DEPENDENT PROTEIN KINASE ACTIVITY

by

Mei Cong

A Dissertation Submitted to the Faculty of the DEPARTMENT OF BIOCHEMISTRY In Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 1998
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Mei Cong entitled "The bovine calpastatin gene promoter and a novel N-terminal region of the protein are targets for cAMP dependent protein kinase activity." and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOCTOR OF PHILOSOPHY.

Antin, Parker B. 4/9/88
Goll, Darrel E. 4/10/88
Miesfeld, Roger 4/11/88
Vierling, Elizabeth 4/9/88
Hartshorne, David J.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University library to be made available to borrowers under rules of the library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Request for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: [Signature]
ACKNOWLEDGMENTS

A special thank to Dr. Goll for giving me the chance to come to the United State and learn molecular biology. I would also like to acknowledge Dr. Antin who is such a wonderful advisor and have been giving me maximum guidance for all these years. Dr. Antin always encourages me to be optimistic and let me to regain confidence when I was so frustrated at the beginning of my research. I will remember his advice for years to come. May thanks to other members who served on my supervisory committee: Dr. David Hartshorne, Dr. Roger Miesfeld, Dr. Elizabeth Vierling, Dr. Jennifer Hall, Dr. Mark Dodson, and Dr. Ronold Allen.

I would have never finished without the support of each member in Dr. Antin’s lab, especially Tania Yatskievych, Sharon Pascoe and Haida Shaw, and Valery Thompson in Dr. Goll’s lab. I would also like to thank Debbie for her helpful discussions.

I would like to thank Andrea Ladd, Mark Bales for their helpful discussions and suggestions.

Financial support from Animal Science department is gratefully acknowledged.

This thesis is dedicated to my husband Mengjun for his love and support; to my mom, dad and my little sister for being so understanding.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>12</td>
</tr>
<tr>
<td>CHAPTER ONE: INTRODUCTION</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER TWO: REVIEW OF LITERATURE</td>
<td>16</td>
</tr>
<tr>
<td>I. Calpains and the calpain system</td>
<td>16</td>
</tr>
<tr>
<td>II. Function of the calpain system</td>
<td>22</td>
</tr>
<tr>
<td>III. Calpastatin cDNA and deduced protein structure</td>
<td>25</td>
</tr>
<tr>
<td>IV. Diversity of calpastatin molecules</td>
<td>29</td>
</tr>
<tr>
<td>V. Calpastatin protein binding and inhibition to the calpains</td>
<td>32</td>
</tr>
<tr>
<td>VI. Calpastatin genomic structure</td>
<td>35</td>
</tr>
<tr>
<td>VII. Phosphorylation of calpastatin</td>
<td>37</td>
</tr>
<tr>
<td>VIII. Effect of β-agonists on muscle growth and hypertrophy and the role of calpastatin</td>
<td>43</td>
</tr>
<tr>
<td>CHAPTER THREE: MATERIALS AND METHODS</td>
<td>48</td>
</tr>
<tr>
<td>I. 5’RACE (Rapid amplification of complementary DNA 5’ ends)</td>
<td>48</td>
</tr>
<tr>
<td>II. Screening of bovine heart cDNA library</td>
<td>51</td>
</tr>
<tr>
<td>III. Total RNA isolation</td>
<td>52</td>
</tr>
<tr>
<td>IV. Northern blot analysis</td>
<td>52</td>
</tr>
<tr>
<td>V. Coupled <em>in vitro</em> transcription and translation</td>
<td>53</td>
</tr>
<tr>
<td>VI. Construction of recombinant calpastatin proteins</td>
<td>54</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS - continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VII. Expression and extraction of recombinant calpastatin proteins from <em>E. coli</em></td>
<td>57</td>
</tr>
<tr>
<td>VIII. Western blot analysis</td>
<td>59</td>
</tr>
<tr>
<td>IX. Separation of polyclonal antibodies from yolk</td>
<td>60</td>
</tr>
<tr>
<td>X. Protein kinase A phosphorylation assay</td>
<td>61</td>
</tr>
<tr>
<td>XI. Screening of bovine heart genomic library</td>
<td>61</td>
</tr>
<tr>
<td>XII. Southern blot Analysis</td>
<td>63</td>
</tr>
<tr>
<td>XIII. Primer extension</td>
<td>65</td>
</tr>
<tr>
<td>XIV. Construction of recombinant plasmids for promoter analysis</td>
<td>65</td>
</tr>
<tr>
<td>XV. Cell culture, transient transfection and CAT assay</td>
<td>67</td>
</tr>
<tr>
<td>XVI. Protein concentration assay</td>
<td>69</td>
</tr>
<tr>
<td>XVII. PCR-based mutagenesis to generate pCS-102mutAT</td>
<td>70</td>
</tr>
</tbody>
</table>

CHAPTER FOUR: DISCOVERY OF A NOVEL N-TERMINAL XL REGION OF THE CALPASTATIN PROTEIN AND ITS PHOSPHORYLATION BY PROTEIN KINASE A

I. Isolation of full length calpastatin cDNAs and the discovery of a novel N-terminal XL region of the calpastatin protein ................................................................. 72

II. Identification and *in vitro* functionality of an upstream translation initiation codon ................................................................. 82

III. Identification of the XL region on calpastatin *in vivo* .......... 85
### TABLE OF CONTENTS - continued

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV.</td>
<td>cAMP dependent phosphorylation of the N-terminal XL region by protein kinase A</td>
<td>92</td>
</tr>
<tr>
<td>V.</td>
<td>Discussion</td>
<td>95</td>
</tr>
</tbody>
</table>

**CHAPTER FIVE: THE BOVINE CALPASTATIN GENE PROMOTER IS A TARGET FOR cAMP DEPENDENT PROTEIN KINASE ACTIVITY**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Isolation of bovine calpastatin genomic DNA clones and determination of the bovine calpastatin gene 5’ structure</td>
<td>102</td>
</tr>
<tr>
<td>II.</td>
<td>Determination of the transcription initiation site</td>
<td>106</td>
</tr>
<tr>
<td>III.</td>
<td>Sequence analysis of the calpastatin gene promoter</td>
<td>110</td>
</tr>
<tr>
<td>IV.</td>
<td>Transient transfection analysis of the calpastatin gene promoter</td>
<td>118</td>
</tr>
<tr>
<td>V.</td>
<td>Effect of dibutyryl cAMP on calpastatin promoter activity</td>
<td>121</td>
</tr>
<tr>
<td>VI.</td>
<td>Site directed mutagenesis mapping of cAMP responsive elements</td>
<td>127</td>
</tr>
<tr>
<td>VII.</td>
<td>Discussion</td>
<td>132</td>
</tr>
</tbody>
</table>

**CHAPTER SIX: CONCLUSIONS AND DISCUSSION**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>The Bovine heart calpastatin gene and protein are both targets for cAMP-dependent protein kinase activity</td>
<td>134</td>
</tr>
<tr>
<td>a.</td>
<td>Discovery of a new N-terminal region of the bovine calpastatin protein</td>
<td>134</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS - continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>b. Identification of the calpastatin gene promoter</td>
<td>135</td>
</tr>
<tr>
<td>c. <em>In vitro</em> analysis of regulation of the calpastatin gene promoter by PKA</td>
<td>136</td>
</tr>
</tbody>
</table>

II. Effects of calpastatin phosphorylation by PKA on the regulation of calpain activity. 137
   a. Possible roles of PKA phosphorylation in domain 1 137
   b. Possible roles of PKA phosphorylation in XL region 139

III. Model for β-adrenergic agonist effects on muscle growth 141

LIST OF REFERENCES 144
LIST OF TABLES

TABLE 2.1, Phosphorylation of calpastatin I from rat heart, kidney and erythrocytes by various protein kinases .................. 41
TABLE 2.2, Changes in efficiencies of phospho-calpastatin I and of native calpastatin II following dephosphorylation .......... 42
TABLE 4.1, Calpastatin amino acid composition ............................................. 83
TABLE 5.1, Sequencing primers for calpastatin promoter region .......... 110
TABLE 5.2, Effect of dibutryl cAMP on calpastatin promoter activity ............................................................................ 122
TABLE 5.3, cAMP responsive elements in mammalian gene promoters ........................................................................... 125
TABLE 5.4, PCR primers for constructing calpastatin-TKCAT .......... 130
LIST OF ILLUSTRATIONS

FIGURE 2.1, Schematic structure of \( \mu\)- and m-calpain......................18
FIGURE 2.2, Calpastatin RNA gel blot analysis. ..............................26
FIGURE 2.3, Structure of different types of calpastatin molecules ......28
FIGURE 2.4, Partial structure of the human calpastatin gene..........36
FIGURE 2.5, Selectivity of rat calpastatin I and II as inhibitors of
\( \mu\)- and m-calpains...........................................39
FIGURE 2.6, Ion exchange chromatography of calpastatin activity
of rat heart, kidney, skeletal muscle, and erythrocytes...40
FIGURE 3.1, Schematic diagram of 5' RACE protocol....................50
FIGURE 3.2, Schematic diagram of calpastatin cDNA and strategies
to make recombinant calpastatin proteins ..................55
FIGURE 4.1, Isolation of full length bovine calpastatin cDNAs .......74
FIGURE 4.2, cDNA and deduced amino acid sequence of bovine heart
calpastatin.........................................................76
FIGURE 4.3, Comparison of 5' cDNA calpastatin sequences from
bovine and human heart.........................................77
FIGURE 4.4, Hydropathy plot of amino acid sequences deduced
from cloned cDNAs for bovine heart muscle.................79
FIGURE 4.5, RT-PCR confirmed that an upstream ATG exists in
bovine heart, liver and muscle calpastatin cDNAs...........81
FIGURE 4.6, In vitro transcription and translation confirms that the
upstream ATG is functional in vitro..........................84
FIGURE 4.7, Expression of full-length calpastatin fusion protein in
E. coli..............................................................86
FIGURE 4.8, Expression of calpastatin domain 1-4 fusion protein in
E. coli..............................................................87
FIGURE 4.9, Western blot analysis of partially purified calpastatin
fusion proteins, pCalp786 and pCalp569..................88
LIST OF ILLUSTRATIONS - continued

FIGURE 4.10, Immunoblot analysis of bacterially expressed calpastatin, whole adult heart, and liver lysates .......... 91

FIGURE 4.11, Expression of calpastatin domain XL+L fusion polypeptide in E. coli ........................................ 93

FIGURE 4.12, PKA kinase assay of bacterially expressed calpastatin proteins ............................................. 94

FIGURE 5.1, Genomic organization and lambda phage clones spanning the 5' region of the bovine calpastatine gene 104

FIGURE 5.2, Nucleotide sequences of four exons of the 5' bovine calpastatin gene .................................. 105

FIGURE 5.3, Primer extension analysis of calpastatin mRNA ......................................................... 108

FIGURE 5.4, RT-PCR and product sequencing confirmed the 5' boundary of exon 1 .................................... 109

FIGURE 5.5, Schematic diagram of plasmid construct p4139 ......................................................... 111

FIGURE 5.6, Calpastatin promoter region GC content ................................................................. 113

FIGURE 5.7, Sequence of the bovine calpastatin gene 5' flanking region ........................................ 116

FIGURE 5.8, Intron 3 sequence immediately upstream of exon 4 ........................................ 117

FIGURE 5.9, Effect of positive and negative elements on activities of heterologous promoters ................. 120

FIGURE 5.10, cAMP responsiveness the bovine calpastatin promoter ............................................... 123

FIGURE 5.11, Effect of deletion of CRE2 in exon 1 on induction of CAT expression by dibutyryl cAMP .... 126

FIGURE 5.12, Effect of CRE element mutation on basal and induced expression of CAT fusion constructs .... 129

FIGURE 5.13, Effect of dibutyryl cAMP on promoter activities ...................................................... 131

FIGURE 6.1, Model for β-agonist effects on muscle growth ......................................................... 143
ABSTRACT

To investigate the regulation of calpastatin gene expression, bovine heart calpastatin cDNAs and 5' regions of the calpastatin gene were isolated. Analysis of 5' terminal cDNA sequence identified a new translation initiation site that is in-frame and 204 nucleotides upstream of the previously designated start site. Conceptual translation from this upstream AUG produces a protein containing 68 additional N-terminal amino acids. This "XL" region contains three potential protein kinase A (PKA) phosphorylation sites but shares no amino acid sequence homology with other regions of calpastatin or with any known protein. Immunoblot studies demonstrated that heart and liver contain a calpastatin protein with an apparent size of 145 kDa on SDS PAGE that comigrates both with full length bacterially-expressed calpastatin, and with calpastatin produced by coupled in vitro transcription-translation from the upstream AUG. An antibody raised against the XL region recognized the 145 kDa band, demonstrating that the upstream AUG is utilized and that the 145 kDa band represents full length calpastatin protein in vivo. The organization of the calpastatin 5' genomic region was determined by comparing calpastatin cDNA and genomic sequences. The region encompassing exons 1-4 contains large introns and spans at least 60 kb. Calpastatin promoter
sequence analysis revealed that calpastatin belongs to the family of "house keeping" genes which lack TATA boxes and are GC rich in their proximal promoter regions. Transient transfection assays demonstrated that sequence within 272 nucleotides upstream of transcription initiation of the calpastatin gene is sufficient to direct moderate level transcription. Promoter sequences further upstream act both to inhibit and to stimulate transcriptional activity. Exposure of transfected cells to dibutyryl cAMP resulted in a seven to twenty fold increase in calpastatin promoter activity for constructs containing at least 272 nucleotides of upstream promoter sequence. Deletion and mutation analyses identified a cAMP responsive element at nt-76. These findings demonstrate that the calpastatin gene and protein are both targets for cAMP-dependent kinase activity. β-Agonist treatment can increase both calpastatin gene transcription and protein phosphorylation.
Chapter One

Introduction

To meet consumer satisfaction, tremendous effort has been made in the field of meat science to improve beef production. Although most of the attempts to increase rates of muscle growth have focused on increasing the rate of muscle protein synthesis, decreasing the rate of muscle protein degradation can also contribute to muscle growth.

β-Agonists are structural analogues of catecholamines, such as adrenalin, potent growth stimulators that markedly stimulate muscle hypertrophy within a few weeks of administration (Emery et al., 1984; McElligott et al., 1989; Reeds et al., 1986). Observations of animals after drug administration revealed increased protein content with absence of increased rates of protein synthesis, suggesting that proteases controlling the rate of protein degradation play important roles in muscle growth.

The calpain system consists of a group of calcium-dependent neutral proteases (μ- and m-calpain) and a calpain-specific inhibitor, calpastatin. Calpain and calpastatin are colocalized within the cytoplasm and can regulate many calcium-dependent cellular processes, including cell
proliferation and differentiation. Calpains are also believed to be major players in regulating muscle growth and protein degradation. β-Agonist treatment of animals can stimulate calpastatin mRNA levels and calpastatin inhibitory activity, suggesting that both the calpastatin gene and protein are targets for β-agonist action. The molecular basis of this action is unknown.

This dissertation investigates the molecular basis by which β-agonists regulate calpastatin function. Results show that β-agonists affect calpastatin gene transcription as well as postranslational phosphorylation and provide a molecular model for the mechanisms by which β-agonist treatment alters calpain activity, leading to muscle hypertrophy.
Chapter Two

Review of Literature

I. Calpains and the calpain system.

The calpain system was first discovered in 1969 as a calcium activated factor that could remove Z-disks from myofibrils (Goll, et al., 1990). m-Calpain was the first member to be purified and partly characterized (Dayton et al., 1975; Dayton et al., 1976; Reville et al., 1976). Since then, a myriad of studies have shown that m-calpain is one of a group of proteins that constitute the calpain system.

The calpain system consists of three relatively well characterized proteins: 1) \( \mu \)-calpain, a proteinase requiring 3-50 \( \mu \)M \( \text{Ca}^{2+} \) for half maximal activity in \textit{vitro}; 2) m-calpain, a proteinase requiring 400-800\( \mu \)M \( \text{Ca}^{2+} \) for half maximal activity in \textit{vitro}; and 3) calpastatin, a protein that specifically inhibits the proteolytic activity of the two calpains but of no other proteases tested thus far (Johnson, 1990; Goll et al., 1992b). The \( \mu \)- and m- calpains are nonlysosomal, cysteine proteinases that have been found ubiquitously in all tissue in the animal kingdom. They each contain an 80-kDa large subunit and a 28-kDa small subunit. The 28-kDa subunit is
identical in both calpain molecules, while the 80-kDa subunits of \( \mu \)-calpain and \( m \)-calpain are different gene products (genes on human chromosome 11 and 1, respectively; Ohno et al., 1990), but share 55-65% amino acid homology within a given species (Suzuki, 1990).

The structure of the 80-kDa subunits on the basis of amino acid sequence can be divided into four domains (Fig. 2.1): Domain I at the N-terminus has no sequence homology with any polypeptide sequenced thus far. Sequence homology of domain I among different species is 72-86%. Domain II has a catalytically active site consisting of cysteine-histidine-asparagine, characteristic of cysteine proteases such as papain or cathepsins B, L, and S, but not other cysteine proteases (Arthur et al., 1997). The sequence homology of domain II among different species is 85 to 93%. Domain III shows no homology to any known polypeptide, although it has two potential EF hand \( \text{Ca}^{2+} \)-binding sites. The apparent function of domain III is thought to link domain IV to the active site in domain II (Suzuki, 1990). The sequence of domain IV at the C-terminus shows marginal homology with calmodulin (24-44% identity and 51-55% similarity for \( \mu \)- and \( m \)- calpain, respectively) and contains five sets of potential EF-hand \( \text{Ca}^{2+} \)-binding sites. The fifth \( \text{Ca}^{2+} \)-binding site is involved in association of
Large subunit of \( \mu \) - and m-calpains, 80 kDa

Figure 2.1: Schematic structure of \( \mu \) - and m-calpains. The structure of the 80 kDa subunits can be divided into four domains: domain I has no sequence homology with other peptides; domain II has catalytically active site containing cysteinase activity; function of domain III is unknown, although it is likely to link domain II to domain IV; domain IV contains five Ca\(^{2+}\)-binding sites. The 28 kDa small subunit contains two domains: N-terminal domain V may serve as a tether to other molecules and structures. Domain VI is a Ca\(^{2+}\)-binding domain. Both large and small subunit autolyze rapidly from their N-termini in the present of Ca\(^{2+}\), and generate 76 and 78 kDa (\( \mu \)-calpain large subunit), 78 kDa (m-calpain large subunit), and 18 kDa (small subunit) final autolysis products. Autolysis sites are indicated by arrows.
the 28/80 kDa subunits and is not a functional Ca\(^{2+}\)-binding site (Blanchard et al., 1997). The 28-kDa subunit is identical in the \(\mu\)- and m-calpain molecules and is encoded by a single gene on chromosome 19 in human (Ohno et al., 1990). The N-terminal region of this subunit, domain V, consists of contiguous glycine residues together with hydrophobic residues and is thought to serve as a tether to other molecules and structures (Imajoh et al., 1985). The C-terminal part of this 28 kDa subunit, domain VI, is frequently called the calmodulin-like domain because it has five EF hand Ca\(^{2+}\)-binding motifs (Emori et al., 1986) (Fig. 2.1).

Both \(\mu\)- and m- calpain autolyze rapidly in the presence of Ca\(^{2+}\) (Suzuki et al., 1981a, 1981b). Autolysis involves both the large and small subunits and produces polypeptides of 78 (\(\mu\)- and m-) or 76 (\(\mu\)-) kDa and 18 kDa, respectively. Autolysis occurs in several steps: for 80 kDa subunit of \(\mu\)-calpain, the N-terminal 14 amino acids are removed to produce a 78 kDa intermediate product followed by removal of additional 12 amino acids to produce the 76 kDa autolysis product (Zimmerman and Schlaepfer, 1991); for the 80 kDa subunit of m-calpain, 9 amino acids are removed from the N-terminus followed by removal of additional 10 amino acids to produce the 78 kDa autolysis fragment (Brown and Crawford, 1993); for the 28 kDa small subunit, the N-terminal 26, 37 and 28 amino
acids are sequentially removed to produce a final autolysis product of 18 kDa (McClelland et al., 1989). Autolysis does not change the specific proteolytic activity of either calpain, but rather reduces the \( \text{Ca}^{2+} \) concentration required for their half-maximal activities by 3-10 fold (Goll et al., 1992b). It has been proposed that this autolysis is initiated by association with membrane phospholipids, which in turn leads to enzymatic activity (Mellgren et al., 1989). Although this membrane activation theory has been widely accepted, accumulating evidence suggests that calpain autolysis can occur without interacting with cell membranes (Goll et al. 1992a; Saido et al., 1994). Thus while calcium-activated autolysis is likely to play a role in the regulation of calpain activity, its physiological significance remains to be fully determined.

Calpastatin is also ubiquitously distributed in mammalian and avian cells. Calpastatin requires \( \text{Ca}^{2+} \) to bind to and inhibit \( \mu \)- and m-calpain (Otsuka et al., 1987). The calcium concentration required for calpastatin to bind to either the unautolyzed or the autolyzed form of the calpains \textit{in vitro} is less than that required for proteolytic activity of the calpains (Kapprell et al., 1989). Consequently, regulation of calpain activity in cells must involve a complex series of interactions with \( \text{Ca}^{2+} \) and calpastatin.
In addition to \( \mu \)- and \( m \)-calpain and calpastatin, a skeletal muscle-specific calpain has also been discovered (called skm-calpain, or p94, Sorimachi and Suzuki, 1992). The cDNA clone from chicken encodes a polypeptide of 821 amino acids with an apparent molecular mass of 94 kDa. Skm-calpain shows 51-54\% homology to the 80 kDa large subunit of \( \mu \)- and \( m \)-calpains (Sorimachi et al., 1989). Studies of skm-calpain expression (Sorimachi et al., 1993) showed that its mRNA is abundant (10-fold higher than \( \mu \)- and \( m \)-calpains) and expressed only in fully differentiated myotubes and myofibers. Attempts to detect this protein failed (Sorimachi et al., 1993), perhaps as a consequence of its extremely rapid turnover with a half life of less than 1 hour (Sorimachi et al., 1992, 1993). Ubiquitous \( \mu \)- and \( m \)-calpains as well as other proteases also undergo autolysis at the NH\(_2\)-terminus, but only to a limited extent.

Recently, skm-calpain was found to be the defective gene in limb-girdle muscle dystrophy type 2A (LGMD2A), which belongs to a group of inherited diseases whose genetic etiology has yet to be elucidated (Richard et al., 1995). The autosomal recessive forms (LGMDs) constitute a genetically heterogeneous group, with LGMD2A mapping to chromosome 15q15.1-q21.1. The gene encoding skm-calpain large subunit is located in this region. Fifteen nonsense, splice site, frameshift, or missense skm-
calpain mutations cosegregate with the disease in LGMD2A families. This is the first case in which a defect enzyme is responsible for muscle dystrophy (Richard et al., 1995). Other muscle dystrophies arise from defects in structural proteins such as dystrophin, merosin and adhalin (Campbell, 1995).

II. Functions of the calpain system.

Protein makes up approximately 20% of muscle weight in mature, domestic animals. Accumulation of muscle protein occurs as a result of the imbalance between rates of protein synthesis and breakdown. The rates of muscle protein synthesis and degradation are not constant and are subject to regulatory controls (Goldberg et al., 1974). Changes in the rate of either synthesis or degradation can lead to an increased or decreased rate of muscle protein accumulation. Although most of the attempts to increase rates of muscle growth have focused on increasing the rate of muscle synthesis, decreasing the rate of muscle degradation can also contribute to muscle growth. In fact, available evidence indicates that loss of muscle protein that occurs during several myopathies such as denervation atrophy and several of the muscle dystrophies, is due primarily to an increased rate of muscle protein degradation, with little or no change in rate of muscle
protein synthesis. Therefore, protein degradation rates may play a major role in regulating the amount of muscle protein in animals.

Intracellular protein degradation in mammalian cells is catalyzed by proteolytic enzymes. Two proteolytic systems, the multicatalytic protease (MCP, or proteosome) system and the calpain system, have thus far been demonstrated to be active at physiological conditions in cells. Although the proteosome system is abundant, the small size of its center core (less than 30 Å° in diameter), makes it impossible to be accessed by myofibrils which average 10 -100 μm in diameter and 1mm long (Tanaka et al., 1988; Koohmariae, 1992). Consequently, although the proteasome may degrade actin and myosin after they have been disassembled from the myofibril (Mykles and Haire, 1991), the calpain system is likely responsible for initiation of myofibril protein degradation (Goll et al., 1990, 1992a).

It has been reported that most calpain and calpastatin molecules are not free in the cytoplasm but are associated with subcellular structures (Kumamoto et al., 1992). In skeletal muscle, most of the calpains are adsorbed to the myofibrils, and the concentration of calpain and calpastatin at the Z disk, the structure that calpains destroy, is about twice that of other regions of myofibrils. In skeletal muscle cells, calpains are believed to initiate myofibril protein degradation by making specific cleavages that
release thick and thin filaments from the surface of myofibrils; and large polypeptide fragments from some myofibril proteins (Goll, et al., 1991; Goll, et al., 1992a). Consequently, the calpains could have an important role in regulation of the rate of myofibril protein turnover. In nonmuscle cells, the calpains seem to be involved in disassembly of the cell cytoskeleton, especially at those points where the cytoskeleton interacts with the cell membrane (Goll, et al., 1992a).

In addition to regulating the rate of muscle growth, since calpain activities are regulated by calcium ions, which are essential for cellular signal transduction, the calpain system is believed to regulate signal transduction by modulating the activity and the structure of various proteins. It has been reported that the calpain system has several functions in a living cell, including degradation of transcription factors such as c-Jun and c-Fos (Harai et al., 1991), regulation of cell proliferation (Cottin et al., 1994), and cleavage of the cytoplasmic domains of adhesion receptors (Du et al., 1995). m-Calpain is also involved in myoblast fusion (Balcerzak et al., 1995). Addition of antisense oligomers targeting to m-calpain blocked myoblast fusion (about 70%) while having no effect on myoblast proliferation.
III. Calpastatin cDNA and Deduced Protein Structure.

Virtually all cells that express calpains also express calpastatin. Calpastatin is encoded by a single gene in birds and mammals. Calpastatin cDNAs were first isolated by screening rabbit lung and heart cDNA libraries with a synthetic oligonucleotide probe based on partial amino acid sequence of the purified protein (Emori et al., 1987). Nucleotide sequence analysis revealed the presence of a single long open reading frame of 2154 nucleotides that encoded 718 amino acid residues (Mr, 76964). This open reading frame is preceded by an in-frame termination codon at nt -135. The deduced amino acid sequence contained all the determined partial amino acid sequences of the peptides derived from purified calpastatin, indicating that this open reading frame actually encoded calpastatin. Northern analysis using calpastatin cDNAs as probe revealed three mRNAs with sizes of 3.8kb, 3.0kb, and 2.5kb, resulting from different poly(A) addition sites (Fig.2.2).

Since then, partial calpastatin cDNAs from human, mouse, monkey, pig, rat, and bovine have been cloned and sequenced (Asada et al., 1989; Ishida et al., 1991; Killefer and Koohmarri, 1994, Maki, 1990). There is greater than 65% amino acid sequence identity among the calpastatins sequenced thus far. A search for domain structures revealed that the
Figure 2.2: Calpastatin RNA gel blot analysis. (A) Schematic diagram of rabbit calpastatin mRNA and probes used for northern blot. Filled bar indicates calpastatin coding region, asterisks indicate polyadenylation sites. (B) Poly(A)$^+$ RNA from rabbit heart was electrophoresed on a 1% agarose gel, blotted and then hybridized with three probes. lane 1: probe 1 covers the total amino acid coding region; lane 2: probe 2 is an 841-bp hindIII fragment (nucleotide 2479-3220); lane 3: probe 3 is a fragment corresponding to nucleotides 3221-3544. The mRNA species and the locations of 28S and 18S rRNA markers are shown. (Emori et al., 1987)
calpastatin protein consists of four repeating, marginally homologous domains (23-36% homology) of approximately 140 amino acids each (domains 1 to 4), plus an N-terminal domain, called domain L (Fig.2.3). For each repetitive domain, highly conserved residues are clustered into three regions, termed subdomains A, B, and C. A highly conserved sequence, Glu-Lys-Leu-Gly-Glu-Xaa-Glu-Xaa-Thr-Ile-Pro-Pro-Xaa-Tyr-Arg, is present in the middle of each repeat.

No significant sequence homology has been found between calpastatin and any protein in GenBank and EMBL databases. The cystatin superfamily of cysteine protease inhibitors, which include the kininogens, has a consensus sequence of Gln-Val-Ala-Gly (Barrett et al., 1986). This sequence is not found in calpastatin. Kininogen is also known to inhibit calpain, although it shows no sequence similarity to calpastatin. Thus, calpastatin belongs to a unique group of protease inhibitors quite independent of the cystatin superfamily.

It was also been observed that the calculated molecular weight for calpastatin is significantly lower than that deduced from SDS-PAGE. It is now clear that calpastatin is an acidic protein (pI 4.7), and has a biased amino acid composition: rich in hydrophilic residues such as Lys, Glu, Ser,
Figure 2.3: Structure of different types of calpastatin. The numbers indicate the amino acid boundaries between each domain. Three subdomains (A, B and C) are marked with solid black boxes within domains 1-4. Question mark (?) indicates the undetermined N-terminus.
and Asp, but lacking aromatic residues. This unusual amino acid composition may explain the abnormal behavior of calpastatin in SDS-PAGE which results in an overestimation of molecular weight by 50-60% (Takano et al., 1988).

IV. Diversity of Calpastatin Molecules.

Early attempts to purify calpastatin protein produced inconsistent and variable results (see Goll et al., 1990; Maki et al., 1990, for summaries), with apparent molecular masses ranging from 34kDa (Takahashi-Nakamura et al., 1981; Yamoto et al., 1983) to 200kDa (Nishiura et al., 1978). Molecular weights above 200-kDa that have been reported for calpastatin probably are the result of using size exclusion chromatography to estimate calpastatin size. Since conformation of the calpastatin polypeptide in solution resembles a random coil rather than the spherical shape assumed in size exclusion, estimation of molecular weight by exclusion chromatography probably overestimates its molecular weight. Since calpastatin is very sensitive to proteolytic degradation (Mellgren and Carr, 1983; Yamato et al., 1983; Otsuka and Goll, 1987), it was unclear whether this diversity in molecular masses reported for calpastatin was the result of proteolytic degradation during purification, or due to a number of calpastatin isoforms having different molecular masses.
Three distinct types of calpastatin proteins have been discovered so far: 1) muscle type calpastatin (also called large calpastatin), which contains a functionally unknown domain L at the N-terminus and four inhibitory domains, designated 1 to 4; 2) erythrocyte type calpastatin (also called small calpastatin), which lacks domains L and 1; and 3) sperm type calpastatin, which is a 17.5 kDa calpastatin peptide with deduced amino acid sequences matching domains 3 and 4.

Comparison of muscle type calpastatin cDNAs from the same tissue in different species showed that there are multiple forms of calpastatin cDNA in the coding region (Asada et al., 1989, Lee et al., 1992, Killefer and Koohmaraie, 1994). Human calpastatin has two deletions of 22 and 13 amino acid residues in domain L and domain 1, while from bovine skeletal muscle calpastatin has one 22 amino acid deletion in domain L. Two types of calpastatin sequences at the N-terminal coding region were also obtained from rat; one had a sequence similar to mouse and human, while the other had a deletion of 38 amino acid residues in domain L. This alternative splicing leads to different sizes of calpastatin proteins during translation.

In contrast to muscle type calpastatin, calpastatin purified from human erythrocytes had a molecular mass of 70-kDa as measured with SDS-PAGE. Protein sequencing showed that the N-terminus of erythrocyte
calpastatin was Ser-Asp-Aln-Ala-Leu-Glu-Ala-Ser-Ala-Ser-Leu (Imajoh et al., 1987), which corresponds to human hepatic calpastatin sequence beginning at Ser287(Fig. 2.3) This Ser is the first residue of domain 2 in human hepatic calpastatin, and is located immediately after a methionine residue that is coded by acaATGa, a Kozak consensus sequence for translation initiation. Hence, calpastatin from human erythrocytes is a smaller protein molecule lacking domains L and 1 that is likely produced through use of a downstream translation initiation site.

A 17.5 kDa glycoprotein with a predicted amino acid sequence identical to the C-terminal 186 amino acids of human hepatic calpastatin has recently been identified in human sperm (Wang et al., 1994). Serum obtained from an infertile woman contained antibodies against a 17.5 kDa sperm protein. Polyclonal antibodies were raised against this protein and used to screen a human testis expression library. A cDNA clone was isolated and amino acid sequence analysis showed that it matched to the C-terminus of calpastatin. In situ hybridization studies showed that this small calpastatin is expressed during spermatogenesis at the final stages of differentiation to spermatozoa (Wei et al., 1995). This 17.5 kDa calpastatin was not isolated, so the N-terminal sequence is unknown. However, human
hepatic calpastatin contains a Met residue at position 506, which could be a translation initiation site for this sperm calpastatin.

Overall, these results suggest that at least some of the multiple calpastatin peptides identified in western blot analysis of different tissues may in fact be different isoforms of calpastatin that have been produced by either alternative splicing mechanisms or from different translation start sites, rather than from degradation of the proteolytically susceptible calpastatin peptide, as has been widely assumed.

V. Calpastatin Protein Binding and Inhibition to the Calpains.

Calpastatin was first discovered during the initial studies on purification of m-calpain (Dayton et al., 1976), in which it was found that muscle extracts having calpain activity also contained an inhibitor of this activity (Goll et al., 1990). Initial studies established that this inhibitor was a heat-stable protein, as no activity was lost even by boiling for 15 min (Okitani et al., 1976). Subsequently, it was shown that this inhibitor was resistant to a wide variety of denaturing agents such as urea, SDS, or trichloroacetic acid (Otsuka and Goll, 1987).

It was recognized early that Ca^{2+} is required for calpastatin to bind to and inhibit the calpains (Cottin et al., 1981; Imajoh and Suzuki, 1985, Otsuka and Goll, 1987). Use of calpastatin affinity columns indicated that
the Ca\(^{2+}\) concentration required for the calpains to bind to calpastatin depended on the calpain molecule. The bound calpain could be released in an undegraded form by chelating Ca\(^{2+}\) with EDTA (Kapprell and Goll, 1989; Otsuka and Goll, 1987). Studies showed that the Ca\(^{2+}\) concentration required for the calpains to bind to calpastatin is significantly lower than that required to initiate their proteolytic activity, except for unautolyzed \(\mu\)-calpain. Furthermore, evidence suggests that Ca\(^{2+}\) binding to calpain results in a conformational change in the calpain molecule that permits calpastatin binding. Taken together, these results suggest that if calpain and calpastatin are colocalized in cells, as immunolocalization results suggest, an increase in Ca\(^{2+}\) concentration would initiate calpastatin binding before the calpains could initiate proteolytic activity. Therefore, cells must contain some way to translocate calpastatin away from the calpains or to reduce the Ca\(^{2+}\) concentration required for calpain proteolytic activity without affecting the Ca\(^{2+}\) concentration required for calpastatin binding. The mechanism is still unknown.

Initial studies investigating the binding of autolytic fragments of the calpains to calpastatin indicated that calpastatin bound to both domain IV and VI of the calpain large and small subunits (Nishimura and Goll, 1991). Subsequent studies using expressed subdomains of the calpastatin molecule
showed that calpastatin binds to the calpains at three sites: 1) subdomain A bound specifically to domain IV of calpain (Takano et al., 1995; Ma et al., 1994; Yang et al., 1994) in a Ca\(^{2+}\)-dependent manner; 2) subdomain B containing the highly conserved sequence binds at or near the active site (domain II) of calpain. This binding is Ca\(^{2+}\) dependent; 3) subdomain C binds in a Ca\(^{2+}\)-dependent manner to domain VI of the calpain molecule. This tripartite binding ostensibly contributes to the tight binding and efficient and specific inhibition of the calpains by calpastatin. Deletion of either subdomain A or C can greatly reduce but does not altogether ablate the ability of calpastatin to inhibit the calpains. Furthermore, Kawasaki et al. (1993) found that subdomain B didn't block binding of the calpains to the cell membranes, although it reduced the rate of autolysis of the calpains. Calpastatin fragments containing subdomains A and C, however, blocked binding of the calpains to the cell membrane, but did not effect the rate of autolysis. It has been mentioned earlier that the Ca\(^{2+}\) concentrations required \textit{in vitro} for calpastatin binding are much higher than the Ca\(^{2+}\) concentrations normally existing in living cells (50-400nM) (Becker et al., 1989; Berlin and Konishi, 1993; Harkins et al., 1993; Kurebayashi et al., 1993). It is unclear whether calpastatin subdomain A and C bind to calpain \textit{in vivo}, or just subdomain B binds.
Overall, the following mechanism is consistent with the current information on calpastatin binding and inhibition of the calpains: binding of \( \text{Ca}^{2+} \) to one or more of the \( \text{Ca}^{2+} \)-binding sites on calpain results in a conformational change needed to "open" the active site and initiate proteolytic activity (Goll et al., 1992b). "Opening" the active site also makes this site available to bind subdomain B of the calpastatin, resulting in inhibition.

VI. Calpastatin Genomic Structure.

The calpastatin gene has been mapped to the long arm of chromosome 5 at 5q 14-22 in human. Partial sequencing of the calpastatin gene (Lee et al., 1992; Maki et al., 1991) has shown that amino acids 1-307 in human hepatic calpastatin are encoded by 14 exons (Fig. 2.4). Domain L is split into 6 exons. Domain 1 is split into 4 exons and each of the three highly conserved regions (called subdomain A, B, and C) within the repetitive domain is located on a separate exon. The exon that contains the translation initiation codon proposed by Emori et al (1987) has also been identified. Comparison between the cDNA sequence and the genomic sequence identified an intron-exon boundary, where cDNA diverged from the genomic sequence 39 nucleotides upstream of this translation start site. Lee et al.(1992) therefore designated the exon containing the apparent
Figure 2.4: Partial structure of the human calpastatin gene. Exons are presented by boxes, where translated regions are filled. Black and gray boxes represent coding sequences in domain L and domain 1, respectively. Lu, Lv, Lw, Lx, Ly, Lz represent exons encoding domain L, while 1A, 1B, 1C and 1D represent exons encoding domain 1. US and 2A represent exons encoding untranslated region and N-terminal region of domain 2, respectively. Arrow head indicate potential translational initiation site ATG used for protein translation in rabbit.
translation initiation site as exon 2, presuming that the upstream region was encoded by a single exon. Cloning of the calpastatin gene in other species has not been reported, and a calpastatin promoter has not been identified in any species.

VII. Phosphorylation of Calpastatin.

Calpastatin and the calpains are located exclusively within the cytoplasm, and calpastatin can inhibit both μ- and m-calpain activities in vitro. However, several important issues remain unresolved: 1) the in vitro Ca^{2+} concentration required for binding of calpastatin to the calpains is much higher than that which normally exists in living cells; 2) there are two isoforms of calpains within the cells; how does a common inhibitor regulate more than one isoforms of calpains?

Pontremoli et al. (1991) provided evidence for the existence in rat skeletal muscle of two calpastatin forms having different specificities for each of the calpain isozymes. Calpastatin I was more effective against μ-calpain, while calpastatin II was more effective as an inhibitor of m-calpain (Fig. 2.5). Salamino et al.(1994) subsequently isolated two distinct forms of calpastatin corresponding to calpastatin I and calpastatin II from rat heart, kidney and skeletal muscle where both isoforms of the calpains exist within the same tissue. It was noted that the levels of calpastatin II activity
were strikingly correlated to those of m-calpain. On the other hand, in rat erythrocyte lysates where no m-calpain can be detected, a single calpastatin activity was found that co-eluted with calpastatin I on ion exchange chromatography (Fig. 2.6). No calpastatin II activity could be detected. These observations suggest that a mechanism exists for adjusting the amount of calpastatin II relative to the amount of m-calpain.

Three major kinases, protein kinase A (PKA), protein kinase C (PKC), and histone phosphorylating protein kinase (PKind), were used for in vitro phosphorylation studies (Salamino et al., 1994). Results showed that PKA and PKind were very active in phosphorylating calpastatin I, whereas PKC was much less effective. Calpastatin isolated from rat erythrocytes was resistant to any phosphorylation (Table 2.1). Phosphorylation of calpastatin I by PKA could alter calpastatin inhibitory efficiency from μ-calpain to m-calpain, whereas dephosphorylation of native calpastatin II by phosphatase altered its activity from m-calpain to μ-calpain (Table 2.2). However, since the calpastatin inhibitory efficiency, $K_i$, within the cells is less than 1 nM, which is remarkably lower than that shown in Table 2.2, this data may not be true.
Figure 2.5: Selectivity of rat muscle calpastatin I and II as inhibitors of $\mu$- and m-calpains. Calpastatin I and II were purified from rat skeletal muscle. $\mu$- Calpain (closed symbols) or m-calpain (open symbols) were incubated with the indicated quantities of calpastatin I (A) or calpastatin II (B) and the residual calpain activity assayed.

from Pontremoli et al., 1991
Figure 2.6: Ion exchange chromatography showing calpastatin activity of rat heart, kidney, skeletal muscle, and erythrocytes. Crude extracts from heart (3g of tissue), (▲), kidney (2.8g of tissue), (Δ), skeletal muscle (3.5g of tissue), (○), or erythrocytes (2 ml of packed cells), (●), were loaded on a DE-32 chromatography column equilibrated with 50 mM sodium acetate, pH6.7, containing 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol. The absorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3M.
Table 2.1
Phosphorylation of calpastatin I from rat heart, kidney and erythrocytes by various protein kinases

<table>
<thead>
<tr>
<th>Protein Kinase</th>
<th>³²P-Incorporation (pmol) in calpastatin I from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>PKA</td>
<td>92±12</td>
</tr>
<tr>
<td>PKC</td>
<td>14±3</td>
</tr>
<tr>
<td>Pkind</td>
<td>82±10</td>
</tr>
</tbody>
</table>

Note: Calpastatin I purified from heart (10.6 µg), kidney (9.7 µg) or erythrocytes (12.4 µg) was incubated with purified protein kinase A (PKA), protein kinase C (PKC), or histone phosphorylating protein kinase (Pkind), which is fully active in the absence of any effector. The specific radioactivity of [γ⁻⁳²P]ATP was 5x10⁵ counts/min/nmol. (from Salamino et al., 1994)

In summary, two forms of calpastatin molecules are found to coexist with µ- and m-calpain. These two forms can be interconvertible through a phosphorylation-dephosphorylation mechanism by cAMP-dependent PKA and phosphatase. The different inhibitory efficiencies which distinguish one calpastatin from another may indicate a very sensitive mechanism that allows the cell to control the activity of both calpains precisely and efficiently. However, the molecular regulation of this posttranslational modification has not been studied.
Table 2.2
Changes in efficiencies of phospho-calpastatin I and of native calpastatin II following dephosphorylation

<table>
<thead>
<tr>
<th>Inhibitor type</th>
<th>Calpastatin inhibitory efficiency, $K_i(\mu M)$, against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart $\mu$-calpain  m-calpain $\mu$-calpain  m-calpain</td>
</tr>
<tr>
<td>Native calpastatin I</td>
<td>0.022  0.088  0.027  0.092</td>
</tr>
<tr>
<td>Native calpastatin II</td>
<td>0.109  0.033  0.103  0.030</td>
</tr>
<tr>
<td>Phospho-calpastatin I</td>
<td>0.114  0.031  0.106  0.036</td>
</tr>
<tr>
<td>Phospho-calpastatin I,</td>
<td></td>
</tr>
<tr>
<td>treated with alkaline PPase</td>
<td>0.030  0.096  0.033  0.11</td>
</tr>
<tr>
<td>Native calpastatin II,</td>
<td></td>
</tr>
<tr>
<td>treated with alkaline PPase</td>
<td>0.025  0.091  0.037  0.118</td>
</tr>
</tbody>
</table>

(from Salamino et al., 1994)
VIII. Effect of β-agonists on Muscle Growth and Hypertrophy and the Role of Calpastatin.

β-adrenergic agonists are the most potent growth promoting agents in many species of animals (Hanrahan, et al., 1986). β₂-agonists bind to the type II β-adrenergic receptor and activate intracellular signaling cascades that are mediated by the cAMP-dependent protein kinase A pathway. The β₂-adrenergic receptor contains seven transmembrane loops, which is characteristic of G-linked receptors. Binding of ligand (epinephrine or β-agonists) to the β₂ receptor activates the G protein complex which stimulates adenylate cyclase to produce cyclic AMP (cAMP). cAMP in turn binds to the regulatory subunit of cAMP-dependent protein kinase A, causing release of the catalytic subunit which then phosphorylates target proteins.

β-agonists produce a dramatic increase in skeletal muscle mass and a large reduction in body fat content (Beermann et al., 1987; Reeds et al., 1986). A 10-20% increase in muscle weight was observed after treating rats with the β-agonist clenbutarol for only 1-2 weeks (Emery et al., 1984; McElligott et al., 1989; Reed et al., 1986). Lambs fed cimaterol for approximately 2 months showed a 25-30% increase in the weights of
several muscles compared with control lambs (Beermann et al., 1986; 1987).

Several studies suggest that muscle growth in response to β-agonist treatment is true muscle hypertrophy rather than hyperplasia, in which satellite cell division precedes protein accretion. Hypertrophy is primarily the result of increased fiber size and protein content without the incorporation of new satellite cells. Consequently, although the total RNA content was not altered following β-agonist treatment, the ratio of DNA to protein decreased (McElligott et al., 1989, Salleo et al., 1980).

Adult muscles maintain a constant size under normal conditions. Therefore, in order to increase the size of muscle, β-agonists effect the “set point” responsible for maintaining a balanced state. The primary issue that must be addressed is to ascertain the mechanism of action of β-agonists. There are two possible mechanisms of action: 1) β-agonist may directly bind to the skeletal muscle membrane receptors and activate a series of events leading to muscle protein accumulation; 2) β-agonist may promote muscle growth indirectly by activating non-muscle cell receptors, leading to the production of hormone(s) or other factors. These factors may then act on the muscle cells to stimulate muscle growth.
Although the mechanism by which β-agonists induce muscle growth is not known, evidence suggests that the ability of β-agonists to stimulate muscle growth under conditions of altered endocrine status argues against the involvement of hormones and factors such as insulin, IGF1, gonadal steroid hormone, etc. (Beermann et al., 1987; Hanrahan et al., 1986; Reeds et al., 1986; Smith et al., 1981). The mechanism of β-agonist action on muscle cells has been studied and evidence suggests that β-agonists act directly on muscle fibers. Protein synthesis, degradation, amino acid uptake and protein accretion have been measured in muscle cell cultures incubated in the presence of β-agonists. Results showed that addition of the β-agonist clenbutarol significantly stimulated fusion in neonatal muscle cultures and also increased culture protein content. No protein accumulation was observed in clenbutarol-treated satellite cells (McMillan et al., 1992). Cimaterol at 1μM, but not at higher concentrations, inhibited protein degradation in L8 and L6 muscle cell cultures while cimaterol had no effect in MM14D mouse muscle cell cultures. Protein synthesis was not affected in either cell type (Forsberg et al., 1986).

*In vivo* effects of β-agonist on protein synthesis and degradation have also been addressed. Early results gave conflicting information concerning the effect of β-agonists on protein synthesis and degradation,
mostly due to difficulties in accurately measuring the rate of protein synthesis (Reeds et al., 1987). Koohmaraie et al.(1991) treated lambs with β-agonists and the weight of biceps and several protease activities were measured postmortem. Results showed that treated lambs had more protein with no changes in total DNA amount, indicating that the increase in muscle mass was due to hypertrophy rather than hyperplasia. In addition, the ability of the muscle to undergo postmortem proteolysis had been dramatically reduced by β-agonist feeding, demonstrating directly that β-agonist treatment decreased the rate of muscle protein degradation.

The effects of β-agonists on the calpain system have also been investigated. β-Agonists can induce changes in the calpain system in both bovine and ovine muscle (Koohmaraie et al., 1991,1994; Higgins et al., 1988; Kretchmar, 1990; Parr et al., 1992). When lambs were treated with β-agonists, the activities of m-calpain and calpastatin increased 62.8% and 24.6%, respectively, relative to control lambs (Koohmaraie et al., 1991). In steers, a 37% increase in muscle mass following β-agonist treatment was coincident with a 96% increase in calpastatin mRNA levels and a 76% increase in calpastatin-specific activity (Parr et al., 1992). m-Calpain-specific activity increased 27%. These changes appear specific to hypertrophic muscles, as muscles that do not respond to β-agonists show no
change in calpains or calpastatin. Based upon changes observed in the calpain system following β-agonist treatment, it has been proposed that increased calpastatin activity following β-agonist treatment inhibits calpain activity and thereby reduces protein degradation, leading to muscle hypertrophy. Reduced protein breakdown has been observed following β-agonist induced hypertrophy (Reeds et al., 1986; Bohorov et al., 1987). An important unanswered question is how observed increases in calpastatin mRNA levels as well as protein activities following β-agonist treatment relate to muscle hypertrophy. One possible mechanism is that β-agonists could increase the rate of calpastatin gene transcription, consequently increasing the amount of calpastatin protein. Alternatively, β-agonist treatment can increase the activity of m-calpain, and one possible mechanism is that calpastatin protein could be postranslationally modified by cAMP-dependent phosphorylation to inhibit the elevated activity of m-calpain. Experiments in this proposal will seek to determine whether calpastatin mRNA and calpastatin protein are targets for β-agonists.
Chapter Three

Materials and Methods

I. 5’RACE (Rapid Amplification of Complementary DNA 5’ Ends):

5’ RACE was performed as described (Frohman, 1993) using total RNA from adult bovine heart and primers derived from a published partial calpastatin cDNA sequence (Killefer, et al., 1994). Two nested calpastatin-specific primers, CPB8: 5’- CCTGTATCTGAUGAGTGCTTGGG -3’, CPB3: 5’- GGTAGGCTTTTTGGCTCTGTGTG -3’, were used (Fig. 3.1). Single stranded cDNA template was generated by incubating 5μg of bovine heart total RNA and the CPB8 primer at 37°C for 2 h with 200 units of M-MuLV Reverse Transcriptase (Boehringer Mannhein), 1mM dNTP, 10 unit RNasin, and 1 x RT buffer. Following removal of excess primers using Centricon-100 spin filters (Amicon Corp), poly (A) was added to 5’ cDNA ends by incubation in a solution containing 10 units terminal deoxy transferase (TdT, Promega), 0.2 mM dATP, 100 mM cacodylate buffer (pH6.8), 1 mM CoCl₂ and 0.1 mM DTT at 37 °C for 5 min, followed by heating to 65°C for 5 min to stop the reaction. Second strand cDNA was
synthesized using the 52 nt hybrid primer $Q_T$ (\(Q_T : 5'-$\)

CCAGTGAGCAGAGTGACGAGGACTCGAGCT

CAAGCTTTTTTTTTTTTTTTTTTTTTTTT- 3’), and two rounds of PCR were then performed using the primer $Q_o$ (\(5'- CCAGTGAGCAGAGTGACG -3'\)) and the calpastatin-specific primer CPB3, followed by the primer $Q_l$ (\(5'- GAGGACTCGAGCTCAAGC -3'\)) and CPB1. PCR reaction products were separated on a 1% agarose gel, excised and cloned into pGEM-T vector.
Figure 3.1  Schematic diagram of 5'RACE protocol
II. Screening of Bovine Heart cDNA Library:

A bovine heart Uni-ZAP XR library was obtained from Stratagene. Approximately $1.7 \times 10^5$ recombinant phage were plated on 150 mm NZ-yeast broth (NZY) plates. Plates were incubated overnight at 37°C and placed at 4°C for 2 h. Plaque lifts were made, denatured and UV-crosslinked to nitrocellulose membranes (Schleicher & Schuell). Membranes were probed with $^{32}$P-labeled random primed 5' RACE cDNA product. Hybridization was performed overnight at 42°C in a solution containing 800 mM NaCl, 20 mM pipes buffer (pH 6.5), 50% deionized formamide, 0.5% SDS, heat-denatured sperm DNA(100μg/ml), and $^{32}$P-labeled probe at 10$^6$ cpm/ml. Membranes were washed twice at room temperature with 0.1% SDS, 0.1xSSC and air-dried. Positive signals were visualized on Kodak X-OMAT™-AR film. Clones giving positive signals were plaque purified and rescreened two additional times to eliminate false positives. The plaque-purified cDNA phage clones were converted into respective pBluescript plasmid counterparts by in vivo excision according to the manufacturer’s protocols to produce double-stranded DNA pBluescript plasmids containing cDNA inserts.
III. Total RNA Isolation:

Total RNA was isolated according to a published protocol (Chomczynski, et al., 1987). Briefly, tissue was homogenized with a tissue homogenizer and solubilized in solution D (10ml/gram tissue) containing 4M guanidium thiocyanate, 25mM sodium acetate (pH 7), 0.5% sarcosyl and 0.1M β-mercaptoethanol. 0.1 Volume of 2M sodium acetate (pH 4.0), 1.0 volume of water saturated phenol, and 0.2 volume of chloroform-isoamyl alcohol (49:1) were added sequentially and mixed well. The solution was then shaken vigorously for 10 sec and placed on ice for 15 min. The aqueous phase containing total RNA was isolated by centrifuging at 10,000×g for 20 min at 4°C and transferred to a fresh tube. Total RNA was precipitated with 1.0 volume of isopropanol at -20°C for 1 h and resuspended in 0.3 volume of solution D. Subsequently, RNA was reprecipitated again with an equal volume of isopropanol at -20°C for 1 h. Pelleted RNA was washed twice with 75% ethanol and resuspended in ddH₂O.

VI. Northern Blot Analysis:

20μg of bovine heart total RNA were denatured and electrophoresed on a 1% formaldehyde-containing agarose gel. After electrophoresis,
RNAs were transferred to a Genescreen hybridization transfer membrane (DuPont). The membrane was prehybridized at 42°C for 2 h with a solution containing 50% formamide, 10 x Denhardt’s solution, 1M NaCl, 1% SDS, 50 mM Tris-HCl (pH7.5), 10% dextran sulfate, 0.1% sodium pyrophosphate, and 100μg/ml preheated sperm DNA. The probe used for hybridization was a 413 nt PCR product from the middle of the bovine heart cDNA (5’ primer: 5’- CAATTTTCTGACAGTTTCGGGC -3’, 3’ primer: 5’- TCCATCAGCTTTTGGCTTGG -3’). Probes were added at 10^6 CPM/ml and hybridized to the membrane overnight at 42°C. The membrane was finally washed at room temperature with 2×SSC and 0.1% SDS for 15 min, and 0.1×SSC, 0.1% SDS for 15 min at 42°C. Signals were visualized on Kodak BIOMAX-MR film.

V. Coupled In Vitro Transcription and Translation:

Coupled in vitro transcription and translation experiments were performed according to manufacturer’s protocols (Promega). Briefly, 25μl TNT® rabbit reticulocyte lysate, 2μl TNT® reaction buffer, 1 μl TNT® T7 RNA polymerase, 1μl amino acid mixture minus Methionine (1mM), 4μl 35S-Methionine (1000Ci/mmoll) at 10mCi/ml (Amersham Intl.), 1μl Rnasin® ribonuclease inhibitor (40U/μl), and 1μg DNA template were assembled
and ddH$_2$O was added to a final volume of 50µl. The reaction was incubated at 30°C for 90 minutes and the translation products were gel analyzed and visualized on X-Omat AR film (Kodak).

VI. Construction Of Recombinant Calpastatin Proteins:

Calpastatin cDNA fragments representing full length protein, domain XL+L, or domain 1-4 were generated by the polymerase chain reaction (PCR). Synthetic oligonucleotides were derived from the bovine calpastatin sequence. 5’ Primer F1 (5’-CTCTCGGAACACATCGTC-3’) corresponded to nucleotide 104-125 and primer F2 (5’-GGAAAGTCAGcCATGGACACTGC-3’) corresponded to 783-805nt (c is a point mutation to generate an NcoI site). Two 3’ primers B1 (5’-ccgctcgagcTTCCAGATGGTTTACTTGG-3’, sequence in small letters is XhoI linker) and B2 (5’-ccgctcgagcgaTCAGCAAATACCCTG-3’) are located at nt 768-786 and nt 2597-2612 relative to the transcription initiation site (Fig. 3.2).
Figure 3.2: Schematic diagram of calpastatin cDNA and strategies to make recombinant calpastatin proteins. Calpastatin domains are shown by boxes. Black box represent the XL region. Arrows indicate the primers used for generating PCR products. Gray boxes represent in vitro expressed calpastatin fragments.
PCR amplifications were carried out as follows: 50 ng of calpastatin cDNA template were added to a 100 µl PCR reaction containing 0.2 mM dNTP (Pharmacia Biotech), pfu DNA polymerase buffer containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-Cl (pH 7.75), 2 mM MgSO₄, 0.1% Triton X-100, and 100µg/ml BSA, 0.25µM of each forward and backward primer. The solution was overlaid with two drops of mineral oil, denatured at 97°C for 5 min, and then maintained at 72°C to add 0.5µl pfu DNA polymerase (Stratagene). PCR was performed with 30 cycles of denaturation (94°C, 30 sec), annealing (52°C, 30 sec), and elongation (72°C, 1 min). After the final cycle, the temperature was maintained at 72°C for 15 min to allow completion of elongation.

PCR products were gel-purified using Qiaquik Gel Extraction Kit (Qiagen) and treated with NcoI and XhoI, and subcloned into the same sites of pCAL-n Vector (Stratagene) to generate fusion proteins having a 26 amino-acid Calmodulin-binding unit at their N-termini. Recombinant plasmids expressing full length, domains 1-4, or domain L plus region XL were designated pCalp786, pCalp569 and pCalp217, respectively, and transformed into BL21(DE3) competent cells.
VII. Expression and Extraction of Recombinant Calpastatin Proteins

From *E. coli*:

Recombinant Protein Expression and Lysate Preparation.

2 ml of LB broth (plus 50 μg/ml ampicillin) were inoculated with a single colony and incubated at 37°C with shaking until an optical density at 600 nm (OD₆₀₀) of 0.6-1.0 was reached. Subsequently this broth was used to inoculate 50 ml of LB-ampicillin in a 250 ml flask at 37°C with shaking. When OD₆₀₀ of 0.6-1.0 was reached, 1 ml of the sample was removed for use as an uninduced control and 100 mM IPTG (Gibco/BRL) stock solution was added to a final concentration of 1 mM to induce protein expression. After 2.5 h incubation, *E. coli* cells were harvested and centrifuged at 5000 × g for 5 minutes at 4°C. Cell pellets were then resuspended 1:5 (w/v) in binding buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM β-mercaptoethanol, 1.0 mM magnesium acetate, 1.0 mM imidazole, and 2.0 mM CaCl₂. Cells were lysed using 1 mg/ml lysozyme and 0.1% triton X-100 and stored at -20°C overnight.

Frozen lysates were thawed at room temperature the next day and homogenized with a polytron to shear genomic DNA. Crude lysates were
obtained by centrifugation and then applied to a calmodulin affinity column.

**Calmodulin Affinity Column Preparation.**

The calmodulin affinity resin was supplied in a storage buffer containing 20% (v/v) ethanol, 0.1 mM CaCl₂, 20 mM Tris-HCl (pH7.5), and 500 mM NaCl. Prior to use, the resin was equilibrated with 5 bed volumes of the binding buffer. The calmodulin affinity resin was allowed to settle and the supernatant was decanted, following which the resin was washed twice with CaCl₂ binding buffer and finally an equal volume of the CaCl₂ buffer was added. Equilibrated calmodulin resin was degassed and chilled to 4°C. A 10 ml disposable polystyrene column (Pierce) was used for affinity chromatography. The column was filled with 3 ml of degassed H₂O and put into a 16 × 125 mm test tube. The end of the column was gently tapped to dislodge any air bubbles. A porous polyethylene disc was then floated on top of the liquid within the column and depressed evenly to the bottom of the column. The column was then emptied of water and a desired volume of degassed gel slurry containing 2 ml of resin was added. Resin was allowed to settle for 30 min following which a second disc was depressed to just above the settled resin level, leaving approximately 1-2
mm of space between the top of the resin and the bottom of the top disc. The entire procedure was performed at 4°C.

**Protein Purification.**

Recombinant proteins were isolated using the calmodulin affinity column according to manufacturer's protocols (Promega). Briefly, cell lysate was loaded on the top of the column. The column was washed with 5-10 column volumes of Ca^{2+}-binding buffer to remove unbound material, and bound proteins were eluted from the column matrix with 10 column volumes of elution buffer containing 50 mM Tris-HCl (pH8.0), 10 mM β-mercaptoethanol, and 2 mM EGTA.

**VIII. Western Blot Analysis:**

Western blots were performed as described by Towbin et al.(1979). Whole tissue extracts from bovine heart and liver, a crude ammonium sulfate fraction of whole heart homogenates, or recombinant proteins representing full length calpastatin or domains 1-4, were electrophoresed on 8% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell) using a TE 22 Mighty Small Transphor apparatus (Hoefer Scientific Instruments). Transfer was performed at 4°C for 2 hours at 300 mA
constant current. The membrane was then incubated in 5% non-fat dry milk in 1x TTBS (0.1% Tween 20, 100 mM Tris-HCl pH 7.5, and 0.9% NaCl) buffer at room temperature for 1 h, washed in 1X TTBS and then incubated in 1X TTBS -1% BSA plus 1F7, a monoclonal antibody that recognizes an epitope in the C-terminal portion of bovine calpastatin, or a chicken antiserum prepared against amino acids 17-33 of the XL region. Following washes and incubation with horseradish peroxidase-conjugated goat anti-mouse (American Qualex) at a dilution of 1:10,000 in 1X TTBS-1%BSA, or anti-chicken IgG (Promega) at a dilution of 1:2000, peroxidase was visualized using SuperSignal substrate (Pierce).

IX. Separation of Polyclonal Antibodies from Yolk:

Egg yolk was separated and added to 4 volume of solubilization buffer containing 10 mM phosphate (pH7.5), 0.1M NaCl, and 0.01% NaN₃. Subsequently, PEG 6000 was added to a final concentration of 3.5% and stirred until dissolved. Following centrifugation at 14,000×g for 10 min, supernatant was separated by filtering through absorbent cotton. PEG 6000 was then added to 12% with stirring to precipitate antibodies. Solution was recentrifuged at 14,000×g for 10 min and precipitated antibodies were
dissolved in solubilization buffer to original volume of yolk. Precipitation with PEG 6000 and centrifugation at 14,000×g were repeated two additional times, following which polyclonal antibodies were resuspended in half of the original yolk volume of solubilization buffer.

X. Protein Kinase A Phosphorylation Assay:

Protein kinase A (PKA) was a generous gift from Dr. Mike Walsh (University of Calgary). 0.1 mg/ml of the partially purified recombinant proteins pCalp786, pCalp569 or pCalp217 were added to a reaction solution containing 20 mM NaHEPES (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 1 μg/ml PKA. The phosphorylation reaction was initiated by adding 26.25 μCi γ-³²P ATP and 0.25 mM ATP. Following incubation at 30°C for 30 min, reactions were boiled 5 min at 95°C and loaded onto a 7.5-20% gradient SDS-PAGE gel.

XI. Screening of bovine heart genomic library:

Approximately 1.1 x 10⁶ recombinant phage of a bovine heart FIX II library (Strtagen) were plated on 150 mm NZ-yeast broth (NZY) plates. Plates were incubated overnight at 37°C and placed at 4°C for 2 h. Plaque lifts were made, denatured and UV-crosslinked to nitrocellulose
membranes (Schleicher & Schuell). A 139bp PCR product from the 5' end of the calpastatin cDNA clone was used as the hybridization probe (5' primer: 5' - CTCTCGGAACACATCCATCGTC -3', 3' primer: 5' - CCCAAGGCTTGTGCTTTTTTTC -3'). Hybridization was performed overnight at 42°C in a solution containing 800 mM NaCl, 20 mM pipes buffer (pH6.5), 50% deionized formamide, 0.5% SDS and heat-denatured sperm DNA(100µg/ml), and 32P-labeled probe at 10^6 cpml/ml. Membrane was washed at room temperature with 0.1% SDS, 6 x SSC for 5 min, 0.1% SDS, 0.1 x SSC for 2 min and positive signals were visualized on Kodak X-OMAT™-AR film.

Clones giving positive signals were plaque purified and rescreened two additional times to eliminate false positives. The plaque-purified phage clones were isolated and phage DNAs purified using a Lambda DNA Midiprep kit (Qiagen). Genomic DNA fragments hybridizing to the 5' end of calpastatin cDNA by southern blot analysis were gel isolated and subcloned into pBluescript plasmid. Extensive subcloning and nucleotide sequencing defined the promoter region as well as the structure of the 5' region of the bovine calpastatin gene.
XII. **Southern Blot Analysis:**

**Preparation of double stranded and single stranded labeled DNA probes.**

Double stranded DNA labeling was carried out using Prime-It II Random Primer Labeling kit (Stratagene). Briefly, 25ng of DNA template was mixed with 10μl random oligonucleotide primers and ddH₂O to a 34μl total reaction volume. The reaction was heated at 100°C for 5 min and centrifuged briefly. Subsequently 5μl of [α-³²P] dCTP at 10Ci/ml (Amersham), 1μl Exo-Klenow enzyme (5U/μl), and 10μl of 5× dCTP primer buffer containing dATP, dGTP, dTTP were added into the reaction and incubated at 37°C for 10 min. The reaction was stopped by adding 2μl of stop mix and the radiolabeled DNA was separated from the unincorporated nucleotides using NucTrap probe purification columns (Stratagene).

Single-stranded probe was synthesized according to the standard protocol (Ausubel et al., 1994). Briefly, 100 ng single-stranded oligonucleotide primer was mixed with 10μl [γ-³²P]ATP (Amersham), 7.5μl 10×polynucleotide kinase buffer, 40U T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 min, following which the kinase was inactivated by heating at 65°C for 5 min. The radiolabeled
oligonucleotide primer was separated from the unincorporated oligonucleotides using NucTrap probe purification columns (Stratagene).

Southern Blot Analysis.

DNA fragments digested with appropriate restriction enzymes were loaded and run on an agarose gel. The gel was then pretreated at room temperature with 0.25N HCl for 15 min, 0.2N NaOH and 0.6N NaCl for 30 min, and 0.025M NaPO4 (pH6.5) for 1 hour. DNA was thereafter transferred from the agarose gel to GeneScreen membrane (DouPont) for 2 h using a Posiblot Pressure Blotter (Stratagene) at 75 mmHg. The DNA on the membrane was then denatured with 0.4N NaOH for 1 min, renatured with 2×SSC, and crosslinked to the membrane using STRATALINKER UV Crosslinker (Stratagene).

The membrane was prehybridized at 42°C for 2 h with prehybridization solution containing 6×SSC, 20mM NaH₂PO₄, 0.4% SDS, 100μg/ml preboiled salmon testes DNA (Sigma). Radiolabeled probe was added at 10⁶ CPM/ml and hybridized at 42°C overnight. Blots were washed in solution I containing 6×SSC and 0.1% SDS for 5 min and washing solution II containing 0.2×SSC and 0.1% SDS for 10 to 20 min. Signals were visualized on Kodak BIOMAX-MR film.
XIII. Primer Extension:

An oligonucleotide (CPB522; 5'-
AUGGCGACGAUGGAUGTGTTCC -3’ ) near the 5’ end of the calpastatin cDNA was labeled with $\gamma (^{32}\text{P})\text{dATP}$ (7000mCi/mmol, ICN). The annealing reaction was performed at 85°C for 5 min followed by incubation at 55°C for 90 min in a solution containing 10µg of total RNA from bovine heart tissue, 10 pmol of $\gamma (^{32}\text{P})\text{dATP}$-labeled CPB522, 250 mM KCl, 250 mM Tris-HCl (pH8.3 at 45°C). Reverse transcription was carried out at 45°C for 45 min in a reaction containing 1 mM dNTP, 12.5 unit AMV reverse transcriptase, 50 mM Tris-HCl (pH8.3), 50 mM KCl, 10 mM MgCl$_2$, 0.5 mM spermidine, and 10 mM DTT. Primer extension products were separated on a 6% polyacrylamide-urea gel and visualized by autoradiography.

XIV. Construction Of Recombinant Plasmids For Promoter Analysis:

A genomic Hind III fragment containing 1667 bp of 5’ flanking region, 158 bp of exon 1 and approximately 2400 bp of intron 1 cloned into pBluescript KS served as template for constructing pCS-1667. PCR
amplification was performed using the forward primer KS located within pBluescript vector and a reverse primer CPB36, which is located in exon 1 and contains an additional Xba I site at its 5' end. Since the proximal promoter region has high GC content, DMSO was added to the PCR amplifications to a final concentration of 5%. The PCR products were gel purified and restricted with Hind III plus Xba I and cloned into the pCAT-Basic reporter vector (Promega). The resulting plasmid clone, pCS-1667, contains 1667 nt upstream of transcription initiation plus 130 nt sequence from exon 1 linked to the CAT reporter gene. pCS-1667 was used as a template to generate pCS-1242, pCS-944, pCS-671, pCS-272, pCS-102 and pCS-31.

PCR amplifications were carried out as follows. 50ng of calpastatin pCS-1667 was added to a 100 µl PCR reaction containing 0.2 mM dNTP (Pharmacia Biotech), pfu DNA polymerase buffer, 0.25µM of each forward and backward primers, and 5% DMSO. The solution was overlaid with mineral oil, denatured at 97°C for 5 min, the temperature was then maintained at 72°C while 1.0µl pfu DNA polymerases (Stratagene) was added. PCR was continued with 30 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and elongation (72°C, 1min). After the final
cycle, the temperature was held at 72°C for 15 min to allow completion of elongation.

XV. Cell Culture, Transient Transfection And CAT Assay:

Cell Culture and Transient Transfection.

Mouse fibroblast NIH3T3 cells were grown to 70% confluence in complete medium containing Dulbecco's Modified Eagle Medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum, 50μg/ml Gentamicin, 1:100 antibiotic-Antimycotic (Gibco/BRL). All cultures were maintained at 37°C in a humidified atmosphere containing 7% CO₂. Cells were harvested with Trypsin-EDTA (Gibco/BRL) and the cell concentration was calculated using a Spotlight Hemacytometer (Baxter). One day prior to transfection, 5 x 10⁵ NIH3T3 cells were plated onto a 35 mm tissue culture plate in complete medium. The following day, for each transfection 2μg of plasmid DNA were combined with 12μg of LipofectAMINE (Gibco/BRL) in 200μl of DMEM at room temperature for 45 min to allow DNA-liposome complexes to form. 800 μl DMEM was then added to the tube and the solution was applied to NIH3T3 cells. Cells were incubated with the complexes for 5 h in a CO₂ incubator and 1ml of
complete medium was added without removing the transfection mixture. Transfection medium was replaced with fresh complete medium at 24 h following the start of transfection and cells were incubated for another 24 h. For some experiments, dibutyryl cAMP (Sigma) was added to culture medium at a final concentration of 1mM, 24 h after transfection. Forty-eight h following transfection, cultures were rinsed with 2 ml chilled TBS (50 mM Tris-HCl, 90 mM NaCl, pH7.5). 750 μl of chilled STE (40 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH7.4) was then added to detach cells from the plate. Cells were pelleted, brought up in 200 μl of lysis buffer (0.25M Tris-HCl pH7.8, 0.5% Triton-X100), placed on ice for 10 min and sonicated. Protein concentrations of extracts were determined using the BCA protein assay kit (Pierce).

**CAT Assay.**

CAT activities were measured essentially by the method previously described (Gorman, 1983). Briefly, 70 μl of cell extract per reaction was incubated at 37°C for 1 h with 1 μl of 14C-chloramphenicol (25 μCi/ml, Amersham Corp.), 8 μl of 40 mM acetyl-CoA (Pharmacia) and 100 μl of 250 mM Tris-HCl (pH7.5). Reactions were terminated by extraction with 1 ml of ethylacetate. Ethylacetate was transferred to a separate tube, dried
and the extracted chloramphenicol dissolved in 20 μl ethyl acetate. Unacetylated and acetylated 14C-chloramphenicol were resolved by thin-layer chromatography (TLC, VWR) in 90% chloroform/10% methanol and quantitated using an Instant Imager (Packard). CAT activities were normalized to protein content within extracts, and each construct was assayed in at least three independent transfection experiments.

XVI. Protein Concentration Assay:

Protein concentration was determined using BCA Protein Assay Reagent Kit (Pierce). A set of protein standards was prepared by diluting 2 mg/ml BSA stock standard with an appropriate dilution buffer (cell lysate buffer). To start the measurement, 10μl of each standard or unknown cell lysate sample was pipetted into a microtiter plate well (Becton Dickinson). 10 μl of the diluent (cell lysate buffer) was used for the blank. 200 μl of the working reagent containing 50 parts of BCA reagent A with 1 part of reagent B provided by the manufacturer was added to each well. The plate was then covered and incubated at 37°C for 30 min. Absorbance was measured at 540nm on a TiterTek Multiskan plate reader (Flow Laboratories). A standard curve was then prepared by plotting the average blank corrected A540 reading for each BSA standard versus its
concentration in μg/ml. The protein concentration for each unknown sample was determined using the standard curve.

XVII. PCR-Based Mutagenesis to Generate pCS-102mutAT:

Mutagenic primer pCATmutAT (5'-
ccaaGCTTTGAGCCAAAAACGGCAGGGCGGGGAGTCTGCGG-3'), where nucleotide ‘ccaa’ is the 5’ tail to generate a Hind III site, small letters a and t within the primer are mutations which are designed to mutate the potential CRE (GTCA) to ATCT. 3’ Primer pCATb (5’-TGTTCTTTACGATGCCATTGGG-3’) is located within the CAT coding sequence. PCR amplifications were carried out using this mutagenic primer and pCATb, using the same PCR program for generating promoter constructs. The PCR products were gel purified and restricted with Hind III plus Xba I and cloned into the pCAT-Basic reporter vector (Promega). The resulting plasmid clone, pCS-102mutAT was sequenced to confirm the mutation.
Chapter Four

Discovery of A Novel N-terminal XL Region of the Calpastatin Protein and Its Phosphorylation by Protein Kinase A

This chapter will be presented in five sections in the following order: (1) isolation of full length calpastatin cDNAs and the discovery of a novel N-terminal XL region of the calpastatin protein; (2) identification and in vitro functionality of an upstream translation initiation codon; (3) identification of the XL region on calpastatin protein in vivo; (4) cAMP dependent phosphorylation of the N-terminal XL region by protein kinase A; (5) discussion. Results given are representative of all experiments unless otherwise noted.
I. Isolation of Full Length Calpastatin cDNAs and the Discovery of a Novel N-terminal XL Region of the Calpastatin Protein.

Initial studies on calpastatin cDNAs from rabbit liver or heart (Emori et al., 1987) and pig heart (Takano et al., 1988) indicated that calpastatin polypeptide consists of a domain L at the N-terminus and four repeating domains of approximately 140 amino acids each. Although the designated translation initiation site proposed from rabbit calpastatin cDNA sequence is not in a perfect Kozak consensus sequence, a termination codon located 135 nucleotides 5' of this AUG precluded a potential start site further upstream. However, this stop codon does not exist within any published calpastatin sequences from other species. Moreover, although sequence for partial calpastatin cDNAs have been published in several species, a full length calpastatin cDNA has not been reported, raising the possibility that a functional translation initiation codon might exist further upstream.

To identify 5' bovine calpastatin mRNA sequence, 5' RACE (Frohman, 1993) was performed using 10μg of total RNA from adult bovine heart and primers derived from a published partial calpastatin cDNA (Killefer et al., 1994). A 152 bp cDNA fragment containing
additional upstream sequence was isolated and cloned into the pGEM-T vector.

This 5'RACE product was used to screen a bovine heart cDNA library. From approximately $1.7 \times 10^5$ plaques of a bovine heart cDNA library, three cDNA clones with sizes of 4296nt, 3430nt, 2544nt were obtained. Nucleotide sequence analysis revealed that each contained a unique poly A adenylation site and also differed in their 5' termination point (Fig. 4.1A). Overlapping regions of all clones were identical except for a 66 nt deletion within cDNA #3 that likely represents an alternatively spliced exon (Lee et al., 1992).

Three calpastatin mRNA species have been identified in rabbit heart (Emori et al., 1987) and bovine muscle (Killefer and Koohmaraie, 1994). To verify that the three calpastatin cDNA clones obtained represent three calpastatin mRNAs in bovine heart, Northern blot analysis was performed using a PCR probe representing the protein coding sequence. Three calpastatin mRNA species were identified (Fig. 4.1B), the size of which corresponded approximately to the size of cDNAs cloned from the bovine heart cDNA library.

Full length calpastatin cDNA sequence and the deduced amino acid sequence are shown in Fig. 4.2. The sequence contains 4551 nucleotides
Figure 4.1: Isolation of full length bovine calpastatin cDNAs. (A) Schematic diagram of 5' RACE product used for cDNA library screening. (B) Structure of three calpastatin cDNAs obtained from a bovine heart cDNA library. cDNAs differ in the amount of 3' untranslated region and in their 5' termination point. cDNAs #2 and 3 extend 5' beyond an AUG that is in frame and 204 nucleotides upstream of the previously designated initiation of translation. (C) Northern blot of bovine heart RNA showing three bands obtained using calpastatin cDNA as probe. Size of these three bands corresponds to the size of cDNAs #1-3.
comprising a 128 bp 5' untranslated region, a 2358bp coding region and a 2065bp 3' untranslated region. Three polyadenylation sites were identified at nt2676, nt3466 and nt4551, respectively.

Published characterizations of partial calpastatin cDNAs from human, rabbit, pig and cattle have proposed a translation initiation site that yields a predicted protein of 639 to 718 amino acids, depending on the species (Emori et al., 1987; Takano et al., 1988; Asada et al., 1989; Killefer et al., 1994; Kretchmar, 1990). Designation of this translation start site derived from the rabbit cDNA (Emori et al., 1987), in which a stop codon was identified 135 nt upstream of the proposed AUG. This upstream stop codon is not present in any of the bovine cDNAs that we isolated. 5' cDNA sequence comparison between bovine heart and human showed extremely high homology (Fig. 4.3), raising the possibility that human calpastatin may also contain the upstream ATG. Additionally, cDNAs # 2 and 3 contain a single long open reading frame and extend 5' to a potential translation initiation site that is in-frame and 204 nt upstream of the previously designated start site (Fig. 4. 1A, Fig. 4.2). This upstream AUG is in excellent “Kozak consensus” context for translation initiation (GCCAUGG), whereas the previously designated downstream site (AGUAUGA) is a poor translation initiation site (Kozak, 1986).
Figure 4.2. cDNA and deduced amino acid sequence of bovine heart calpastatin. cDNA sequence was derived by combining cDNA #1, which extends farthest 3', with the 5’ sequence of cDNA #2. Extreme 5’ mRNA sequence and the transcription initiation site were determined by primer extension and comparison with genomic sequence spanning this region. Domain boundaries are indicated; amino acid sequence of the XL region is shown in bold. Dashed underlined region denotes the sequence absent from cDNA #3. Single underlined sequence denotes regions that differ from a previously published bovine skeletal muscle calpastatin cDNA (Killefer and Koohmaraie, 1994). Asterisks indicate polyadenylation sites.
Figure 4.3. Comparison of 5' cDNA calpastatin sequences from bovine and human heart. Residues identical to bovine heart are marked with dots. The plus signs (+) indicate gaps. The number on the top indicates the residue numbers corresponding to the transcription start site of bovine calpastatin. Sequence underlined is the previously proposed translation initiation site.
Conceptual translation from the upstream AUG produces a protein containing 786 amino acids with 68 additional amino acids at the N-terminus (region “XL”, extended domain L). The XL region shares no homology with other regions of calpastatin or with any known protein. Amino acid composition analysis shows that the XL region is very basic, with a predicted pI of 10.07, and has a very hydrophilic nature (Fig. 4.4), consistent with the characteristics of L domain and suggesting that XL region is an extension of domain L. The XL region contains 18 Ser and 6 Thr residues which suggest that it may be subject to modification by phosphorylation and glycosylation. Indeed amino acid sequence analysis showed that Thr\(^{16}\) or Ser\(^{17}\), Ser\(^{29}\) and Thr\(^{35}\) are in appropriate contexts for phosphorylation by protein kinase A, Thr\(^{45}\) is in a context favorable for casein kinase II phosphorylation and Ser\(^{13}\) is a potential site of protein kinase C phosphorylation.

The overall predicted 786 residue sequence of bovine heart calpastatin protein contains five domains: domain L containing the 68 additional amino acid XL-region sequence at the N-terminus, and four repeats of approximately 140 amino acid residues containing the highly conserved inhibitory sequence of LGxxxxTIPPxYRxLL, which is located in the middle of each repeat.
Figure 4.4: Hydropathy plot of amino acid sequences deduced from cloned cDNAs for bovine heart muscle.
To verify that the novel translation start site discovered from bovine heart cDNA library screening represents an authentic calpastatin amino terminal cDNA sequence, rather than an artifact created during preparation of the cDNA library, RT-PCR was performed using a 5' primer located immediately upstream of the newly discovered translation start site and a 3' primer located further downstream (Fig. 4.5. A). RT-PCR studies showed that sequence containing the putative upstream translation initiation site is contained within calpastatin mRNA. Two PCR products were obtained (Fig. 4.5B, lane 2), a major band with a predicted size of 341bp representing mRNA with the 66nt deletion of an alternatively spliced exon, and a minor band running at 407bp arising from mRNA containing this alternatively spliced exon. Similar results were obtained with muscle and liver, confirming that this upstream AUG is present within calpastatin mRNA in several bovine tissues (Fig. 4.5B, lane 3, 4).
A.

 CPF422: 5' -CTCTCGGAACACATCCATCTGC-3'
 CPB1: 5' -GTTCCTTTGGGCTTACTTCTTGGG-3'

B.

Figure 4.5. RT-PCR confirmed that an upstream ATG exists in bovine heart, liver and muscle calpastatin cDNAs. Lane 1 is a 123bp DNA marker. RT-PCR products from bovine heart, muscle and liver are shown in lane 2, 3, 4, respectively.
II. Identification and in vitro Functionality of an Upstream Translation Initiation Codon.

Sequence analysis of bovine calpastatin cDNA clones has revealed a potential upstream translation initiation site. This start site is in a better "Kozak consensus" context than the downstream previously designated one and generates 68 additional amino acids at the N-terminal end. To verify that the upstream translation initiation site is functional, coupled in vitro transcription-translation experiments were performed using calpastatin cDNA #1, which contains only the downstream ATG, or cDNA #2, which contains both the upstream and downstream ATGs (Fig. 4.6A). cDNA #2 produced two products, one major band arising from the upstream ATG ran on SDS-PAGE at an apparent molecular weight 145 kDa (Fig. 4.6B), which is almost twice the predicted molecular weight of 84 kDa, and a minor band representing the translation product generated from downstream ATG. cDNA #1 produced a protein of 135 kDa, comigrating with the minor band generated by cDNA #2 and reflecting initiation at the downstream AUG. The anomalous migration of calpastatin on SDS-PAGE has been previously reported (Takano et al., 1988; Emori et al., 1988; Maki et al., 1988). The deduced calpastatin protein is acidic, with a predicted pI of 5.07, and has a biased amino acid composition (Table 4.1):
rich in hydrophilic residues such as Lys, Glu, Ser, and Asp, which comprise 45\% of all amino acid residues, but extremely poor in aromatic residues (Phe 0.76\%, Tyr 0.89\%, and Trp 0.25\%). This unusual amino acid composition may explain the abnormal behavior of calpastatin in SDS-PAGE, leading to an over-estimation of its molecular weight by 50 to 60\%.

Table 4.1
Calpastatin Amino Acid Composition

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No.</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-polar:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>77</td>
<td>9.80</td>
</tr>
<tr>
<td>Val</td>
<td>33</td>
<td>4.20</td>
</tr>
<tr>
<td>Leu</td>
<td>47</td>
<td>5.98</td>
</tr>
<tr>
<td>Ile</td>
<td>13</td>
<td>1.65</td>
</tr>
<tr>
<td>Pro</td>
<td>84</td>
<td>10.69</td>
</tr>
<tr>
<td>Met</td>
<td>8</td>
<td>1.02</td>
</tr>
<tr>
<td>Phe</td>
<td>6</td>
<td>0.76</td>
</tr>
<tr>
<td>Trp</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Polar:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>38</td>
<td>4.83</td>
</tr>
<tr>
<td>Ser</td>
<td>91</td>
<td>11.58</td>
</tr>
<tr>
<td>Thr</td>
<td>51</td>
<td>6.49</td>
</tr>
<tr>
<td>Cys</td>
<td>5</td>
<td>0.64</td>
</tr>
<tr>
<td>Tyr</td>
<td>7</td>
<td>0.89</td>
</tr>
<tr>
<td>Asn</td>
<td>12</td>
<td>1.53</td>
</tr>
<tr>
<td>Gln</td>
<td>22</td>
<td>2.80</td>
</tr>
<tr>
<td>Acidic:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>62</td>
<td>7.89</td>
</tr>
<tr>
<td>Glu</td>
<td>88</td>
<td>11.20</td>
</tr>
<tr>
<td>Basic:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>114</td>
<td>14.50</td>
</tr>
<tr>
<td>Arg</td>
<td>16</td>
<td>2.04</td>
</tr>
<tr>
<td>His</td>
<td>10</td>
<td>1.27</td>
</tr>
</tbody>
</table>
In vitro transcription and translation confirms that the upstream ATG is functional in vitro. (A) cDNA clones used for coupled in vitro transcription-translation. cDNA #1 contains a single downstream ATG, while cDNA #2 contains an additional ATG 204 nt further upstream. (B) Autoradiograph showing SDS-PAGE analysis of coupled in vitro transcription-translation reactions, containing no DNA (lane 1), cDNA #1 (lane 2), or cDNA #2 (lane 3) as template. While the largest translation product produced from cDNA #1 migrated with an apparent molecular mass of 135 kDa, cDNA #2 produced a predominant product with an apparent molecular mass of 145 kDa.
III. Identification of the XL Region on Calpastatin Protein in vivo.

In vitro transcription/translation studies using the full length calpastatin cDNA have shown that the novel upstream AUG is a preferred translation initiation site, generating a polypeptide with apparent molecular mass of approximately 145 kDa.

To determine whether the upstream AUG is functional in vivo, full length calpastatin cDNA (pCalp786) or calpastatin cDNA coding for a polypeptide spanning domains 1 to 4 (pCalp569), were fused in-frame at their N-termini to a 5 kDa calmodulin binding domain and expressed in E.coli. Cell lysates were prepared at 0 h and 2.5 h following IPTG induction and subjected to SDS-PAGE (Fig. 4.7, 4.8). A polypeptide with an apparent molecular mass of 150 kDa was present in pCalp786 expressing cells after 2.5 h of induction (Fig. 4.7, lane 3), and a 120 kDa polypeptide was present in the cell lysates from pCalp569 expressing cells (Fig. 4.8, Lane 3). Immunoblot analysis with monoclonal antibody 1F7, which recognizes an epitope in the C-terminal region of calpastatin, confirmed that the affinity purified fusion proteins with apparent molecular masses of 150 kDa and 120 kDa are calpastatin polypeptides (Fig. 4.9).
Figure 4.7. Expression of full-length calpastatin fusion protein in E. Coli. Plasmids containing full length calpastatin coding sequence were transformed into BL21(DE3) cells and protein expression was induced with IPTG. Lane 2 and 3 were loaded with cell lysate samples prepared following 0h and 2.5 h of IPTG induction. Partially purified full length calpastatin fusion protein was loaded in Lane 4. The 150 kDa marker indicates the migration distance of the full length calpastatin fusion protein. Standard protein markers were loaded in lane 1.
Figure 4.8. Expression of calpastatin domain 1-4 fusion protein in *E. coli*. Plasmids containing calpastatin domains 1-4 coding sequence were transformed into BL21(DE3) cells and protein expression was induced by IPTG. Standard protein markers were loaded in lane 1. Lanes 2 and 3 were loaded with cell lysate samples prepared following 0h and 2.5 h of IPTG induction. Partially purified fusion protein was loaded in Lane 4. The 120 kDa marker indicates the migration distance of the calpastatin domain 1-4 fusion protein.
Figure 4.9. Western blot analysis of partially purified calpastatin fusion proteins, pCalp786 and pCalp569. Lanes 1 and 2 contain partially purified pCalp786 and pCalp569, respectively. Monoclonal antibody 1F7 which recognizes an epitope in the C-terminus of calpastatin, was used for calpastatin detection.
To determine whether the upstream AUG is utilized \textit{in vivo}, western blot analyses were performed using heart or liver homogenate, or an ammonium sulfate fraction of crude bovine heart homogenate. Monoclonal antibody (Mab) 1F7 identified a 145 kDa protein in whole heart extracts that runs slightly faster than bacterially expressed full length calpastatin protein (pCalp 786, Fig. 4.10) containing an additional 5 kDa calmodulin binding domain. A 145 kDa band that comigrates with the protein from bovine heart, plus a second band of 135 kDa, were detected in whole liver extracts (Fig. 4.10, lane 3). This lower band could represent translation initiation at the downstream AUG or a proteolytic cleavage product of the full length protein. Mab 1F7 recognized a band of 145 kDa plus two smaller bands in an ammonium sulfate fraction of crude heart muscle homogenate (Fig. 4.10, lane 4). These lower bands migrate more rapidly than calpastatin protein initiated at the downstream AUG, and their origin is unclear; they may arise from proteolysis (Mellgren et al., 1986) or they may be products of alternative splicing. The 110 kDa and 120 kDa polypeptides represent the calpastatin protein isolated biochemically and studied extensively by several laboratories (Kaprell and Goll, 1989; Shannon and Goll, 1985; Takano et al., 1991).
To further confirm that calpastatin contains the XL region *in vivo*, a polyclonal antibody was prepared against a 17 amino acid peptide from the XL region (aa #17-33). This antibody recognized only the 145 kDa band in the crude ammonium sulfate fraction (Fig. 4.10, lane 5), but not the lower bands that migrated more rapidly on the SDS-PAGE. Taken together, these results demonstrate that the upstream AUG is utilized and that the XL region is present within calpastatin protein *in vivo*. 
Figure 4.10 Immunoblot analysis of bacterially expressed calpastatin, whole adult heart, and liver lysates. Mab 1F7, produced against a C-terminal calpastatin epitope, recognized a protein of approximately 150 kDa, representing bacterially expressed calpastatin protein produced from cDNA clone #2 containing the upstream AUG, plus a 5 kDa calmodulin domain (lane 1). Mab 1F7 recognized a band of 145 kDa in whole heart and liver homogenates (lanes 2, 3). A smaller band of 135 kDa was also detected in liver. Three bands were recognized by Mab 1F7 in a crude ammonium sulfate fraction of whole heart homogenate (lane 4), one band migrating at 145 kDa and likely representing full length calpastatin, plus two smaller bands of approximately 120 and 110 kDa. In contrast, an antiserum raised against a peptide from the N-terminal XL region recognized only the 145 kDa band in the ammonium sulfate fraction (lane 5).
IV. cAMP Dependent Phosphorylation of the N-terminal XL Region by Protein Kinase A.

Analysis of the deduced amino acid sequence of the full length calpastatin protein identified three potential PKA phosphorylation sites clustered within the N-terminal XL region: Thr^{16} or Ser^{17}, Ser^{29} and Thr^{35}. A fourth site at Ser^{269} within domain 1 was also present (Fig. 4.12A). To determine whether the XL domain is a target of cAMP-dependent phosphorylation, full length and truncated calpastatin proteins containing domain XL plus L domain or domains 1 to 4 were expressed in *E. coli*, partially purified (fig. 4.7, 4.8, 4.11) and then challenged in a kinase assay using purified PKA. As shown in Fig. 4.12B, both the N-terminal partial calpastatin protein containing regions XL and L and the C-terminal partial protein consisting of domains 1-4 are phosphorylated by PKA.
Figure 4.11. Expression of calpastatin domain XL+L fusion polypeptide in E. Coli. Plasmids containing calpastatin domains XL+L coding sequence were transformed into BL21(DE3) cells and protein expression was induced by IPTG. Lanes 2 and 3 were loaded with cell lysate samples prepared following 0 h and 2.5 h of IPTG induction. Partially purified calpastatin fusion protein was loaded in Lane 4. Standard protein markers were loaded in lane 1.
Figure 4.12. PKA Kinase Assay of Bacterially Expressed Calpastatin Proteins. (A) Constructs used to express full length or truncated calpastatin proteins in *E. coli*. pCalp786 represents full length calpastatin; pCalp569 is truncated just N-terminal to domain 1; pCalp217 contains regions XL and L. Asterisks indicate predicted PKA phosphorylation sites. Gray boxes indicate a 5 kDa calmodulin domain present to facilitate protein purification. (B) SDS PAGE Autoradiograph showing pCalp786 (Lane 1), pCalp569 (Lane 2), and pCalp217 (Lane 3). All three protein products were phosphorylated by PKA.
V. Discussion.

Calpastatin cDNAs from bovine heart were cloned and a new upstream translation initiation site was identified which yields a protein containing 68 previously unidentified N-terminal amino acids. This XL region is present in calpastatin in vivo, and is a substrate for phosphorylation in vitro by PKA.

A wide range of molecular weights has been reported for calpastatin isolated from cells and tissues, with estimates ranging from 17 to 172 kDa (Croall and Demartino, 1991). Some of this variability may be due to protein degradation, while some seems likely to be the result of alternative splicing or different translation initiation sites (Ishida et al., 1991; Lee et al., 1992; Emori et al., 1987; Asada et al., 1989; Otsuka et al., 1987). Autolysis may represent an additional mechanism for regulating calpain activity (Hathaway et al., 1982; Suzuki et al., 1981a and b), and proteolytic processing may also be involved in regulating calpastatin activity (Mellgren et al., 1986). Further confusion has arisen from the widespread observation that calpastatin runs anomalously on SDS-PAGE (Takano et al., 1988; Emori et al., 1988; Maki et al., 1988). The largest reported calpastatin protein, isolated from bovine heart muscle, has an apparent molecular weight of 145 kDa (Mellgren et al., 1983). A calpastatin protein
of similar apparent molecular weight has also been reported in skeletal muscle (Lepley et al., 1985).

Several observations argue strongly that the upstream AUG we have identified in bovine heart calpastatin mRNA is the predominant translation initiation site. First, prior designation of a downstream initiation of translation was based upon a partial rabbit calpastatin cDNA containing a stop codon 135 nt upstream of the proposed AUG (Emori et al., 1987). Bovine calpastatin cDNAs do not contain a stop codon at this site, and neither do the published cDNA sequences from other species. Second, the upstream AUG located at nt +129 is the first potential start site downstream of transcription initiation. An open reading frame extends from this AUG through to the C-terminus of the protein that is in-frame with calpastatin amino acid sequence derived from peptide sequencing (Emori et al., 1987; Takano et al., 1988; Takano et al., 1991). Third, RT-PCR analyses demonstrate that the upstream AUG is present in calpastatin mRNA. Fourth, the downstream AUG is a poor potential start site, whereas the upstream AUG shows excellent consensus with other translation initiation sites (Kozak, 1986). Fifth, calpastatin produced from the upstream AUG by coupled in vitro transcription-translation runs at an apparent molecular mass of 145 kDa, comigrating with calpastatin
identified by western blot in whole cell extracts from heart and liver. Finally, antibodies prepared against a peptide from the predicted amino acid sequence of the XL region recognizes this 145 kDa protein. The protein running at 145 kDa therefore represents full length calpastatin, contains the XL region and has a predicted true molecular weight of 84 kDa.

Since our results clearly show that the upstream AUG is utilized in vivo, the question arises as to whether the downstream AUG is functional. For rabbit, the presence of a stop codon 135 nt 5’ of the downstream AUG precludes the use of any potential upstream start site. Calpastatin cDNA sequence presently available from other species does not extend far enough 5’ to determine whether upstream start sites are present. However, published partial cDNA sequences do not contain stop codons upstream of the second ATG.

Western blots of bovine whole liver extracts detected calpastatin bands of 145 kDa and 135 kDa (Fig. 4.10). The lower band comigrates with an in vitro translation product initiated at the downstream AUG, raising the possibility that both start sites can be utilized. Alternatively, the 135 kDa calpastatin protein might arise through proteolysis. We have provided evidence indicating that PKA phosphorylation sites exist within
the XL region, which is present in full length (145 kDa) calpastatin but absent from smaller calpastatin proteins. Regardless of whether the 135 kDa calpastatin arises through alternative start site selection or through proteolysis, removal of the XL region might play a regulatory role by altering phosphorylation patterns on the protein.
Chapter 5

The Bovine Calpastatin Gene Promoter is a Target for cAMP Dependent Protein Kinase Activity

This chapter will be presented in seven sections in the following order: (1) isolation of bovine calpastatin genomic DNA clones and determination of the bovine calpastatin gene 5' structure; (2) determination of the transcription initiation site; (3) sequence analysis of the calpastatin gene promoter; (4) transient transfection analysis of the calpastatin gene promoter; (5) effect of dibutyryl cAMP on calpastatin promoter activity; (6) site directed mutagenesis mapping of cAMP responsive elements; (7) discussion. Results given are representative of all experiments unless otherwise noted.

β-adrenergic agonists are potent stimulators of muscle growth (reviewed in Yang and McElligott, 1989). Muscle growth in response to β-agonists represents true hypertrophy in that fibers increase in size without
the incorporation of new satellite cells. Stimulation of muscle growth is rapid, with changes detectable within a few days in vivo (Emery et al., 1984; McElligott et al., 1989). β-agonist effects are selective in that not all muscles of treated animals undergo hypertrophy.

β-agonists bind to the type II β-adrenergic receptor and activate intracellular signaling cascades that are mediated by the cAMP dependent protein kinase A pathway. The β2-adrenergic receptor contains seven transmembrane loops, which is characteristic of G-linked receptors. Binding of ligand (epinephrine or β-agonists) to the β2 receptor activates the G protein complex which stimulates adenylate cyclase to produce cyclic AMP (cAMP). cAMP in turn binds to the regulatory subunit of cAMP-dependent protein kinase A, causing release of the catalytic subunit which then phosphorylates target proteins. Effects include a short term stimulation of glucose metabolism and longer term increases in muscle growth. Although the mechanism by which β-agonists induce muscle growth is not known, evidence suggests that β-agonists act directly on muscle fibers, as denervated muscles and neonatal rat muscle cells in culture respond to β-agonist treatment (Maltin et al., 1987; McMillan et al., 1992).
β-agonists induce changes in the calpain system in both ovine and bovine muscle (Killefer and Koohmaraie, 1994; Higgins et al., 1988; Kretchmar, 1990; Parr et al., 1992) that include alterations in levels of calpains and calpastatins. In steers, a 37% increase in muscle mass following β-agonist treatment was coincident with a 96% increase in calpastatin mRNA levels and a 76% increase in calpastatin-specific activity (Parr et al., 1992). m-Calpain-specific activity increased 27%.

It has been proposed that growth-promoting effects of β-agonist treatment is due to suppression of protein breakdown rather than stimulating protein synthesis (Parr et al., 1992). An important unanswered question is how the increases of calpastatin mRNA levels as well as protein activities following β-agonist treatment relate to muscle hypertrophy. One possible mechanism is that β-agonists could increase the rate of calpastatin gene transcription, consequently increasing the amount of calpastatin protein. Experiments in this chapter will seek to determine whether calpastatin gene is the target for β-agonists.
I. Isolation of Bovine Calpastatin genomic DNA Clones and Determination of the Bovine Calpastatin Gene 5' Structure.

Lee et al. (1992) cloned portions of the human calpastatin gene and identified an exon containing the second ATG that was proposed as the translation initiation codon by Emori et al (1987). cDNA sequence diverged from the genomic sequence 39 nucleotides upstream of this site, identifying an intron-exon boundary. Lee et al. (1992) therefore designated the exon containing the apparent translation initiation site as exon 2, presuming that the upstream region was encoded by a single exon. Cloning of the calpastatin gene in other species has not been reported, and a calpastatin gene promoter has not been identified in any species.

To identify the calpastatin promoter, a 238bp PCR product was generated containing 25 bp of 5'-untranslated region and 213 bp of 5' protein coding sequence. The PCR product was isolated and labeled as a probe to screen a bovine heart genomic library. Four genomic clones were isolated and extra long PCR showed that each clone has an average size of 20 kb (data not shown). Restriction mapping and southern hybridization analyses of each clone identified the exon-containing region, and nucleotide sequencing revealed that each phage clone contained a single exon. The
structure of the 5' region of the calpastatin gene was defined and is shown in Figs. 5.1 and 5.2. Comparison between the genomic and heart cDNA sequences showed that the previously proposed (downstream) translation initiation site is located in exon 4. mRNA sequence further 5' is encoded by three exons separated by large introns spanning at least 60 kb.
Figure 5.1: Genomic organization and lambda phage clones spanning the 5' region of the bovine calpastatin gene. Intron and exon structure is shown along with the location of some restriction enzyme recognition sites (H) *Hind III*; (X) *Xba I*; (S) *Sma I*; (N) *Nde I*. The upstream and putative downstream translation sites in exons 1 and 4, respectively, are shown.
Figure 5.2: Nucleotide sequences of exons 1-4 of the bovine calpastatin gene. 68 Additional N-terminal amino acids arising from translation at the upstream AUG are bolded.
II. Determination of the Transcription Initiation Site.

To define the transcription initiation site, primer extension was performed using heart muscle total RNA as template and a 22 nt primer located just 5' to the upstream AUG (Fig. 5.3). An extension product of 130 nt was obtained. Two smaller extension products of 118, 124 nt were also obtained, which may represent calpastatin mRNA initiating from two downstream sites, as multiple start sites are common for genes lacking TATA boxes and that are GC rich in their proximal promoter regions (see below). Alternatively, the secondary structure at the 5' end of calpastatin mRNA may cause pausing and premature release of some reverse transcriptase molecules.

The 5' terminus of the 130 nt primer extension product contains 36 nt beyond the 5' end of calpastatin cDNA clone #2. To determine whether this extra 36 nt is transcribed and whether it is contained within the same exon as the most 5' sequence in cDNA #2, or is encoded by an additional upstream exon, RT-PCR was performed. Genomic sequence was used to design a 5' primer immediately upstream of the 5' terminus of cDNA #2. When RT-PCR was performed using this primer and a downstream primer within the calpastatin mRNA, a PCR product of 503 nt was obtained (Fig.
5.4), indicating that the region between the upstream AUG and the 5’
terminus of the 130 nt primer extension product is transcribed and encoded
by a single exon.

In summary, a comparison of the 5’ bovine calpastatin cDNA
sequence with the corresponding genomic sequence led to the identification
of 4 exons ranging in length from 60 bp (exon 4) to 158 bp (exon 1).
Primer extension identified the location of the transcription initiation site
and indicated that the downstream initiations may also occur at residues +7
and +13.
Figure 5.3: Primer extension analysis of calpastatin mRNA. Bovine heart polyA+ mRNA was annealed to a primer located just 5' of the upstream AUG. Lane 1 is a G sequencing ladder used as a size marker. Radiolabeled primer extension products are shown in Lane 2. Three major bands were obtained of 118, 124 and 130 nt.
A.  

PCAT1.8F18: 5' - TCCCAGTCAGGTCTGCGG - 3'
CPB1: 5' - GTTCCTTTGGCTTTACTTCTTGGG - 3'

B.  

Figure 5.4: RT-PCR and product sequencing confirmed the 5' boundary of exon 1. (A) Schematic diagram of the primers used for RT-PCR. (B) RT-PCR product (lane 2). 100bp DNA marker is shown in lane 1.
III. Sequence Analysis of the Calpastatin Gene Promoter.

A genomic Hind III fragment containing 1667 bp of 5' flanking region, 158 bp of exon 1 and approximately 2400 bp of intron 1 was cloned into pBluescript KS and served as template for constructing pCS-1667. Nucleotide sequencing was performed using the primers indicated in Fig. 5. 5 as well as in Table 5.1.

Table 5.1

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Location</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4139F1</td>
<td>-1430 - -1448</td>
<td>5'-AAGCCGCACAAAAACACACC-3'</td>
</tr>
<tr>
<td>P4139F2</td>
<td>-1114 - -1130</td>
<td>5'-TCCAGCCCCCTCCTCTCTG-3'</td>
</tr>
<tr>
<td>P4139F3</td>
<td>-944 - -927</td>
<td>5'-CGGAAAAGCTGCCTCAC-3'</td>
</tr>
<tr>
<td>CPB522</td>
<td>+109 -+130</td>
<td>5'-ATGGCGACGATGGATGTGTCC-3'</td>
</tr>
<tr>
<td>CPF422</td>
<td>+104 -+125</td>
<td>5'-CTCTCGGAACACATCCATCGTC-3'</td>
</tr>
</tbody>
</table>

In eukaryotic cells, transcription is initiated by binding of RNA polymerase II to cis elements within the proximal promoter region. After the holoenzyme binds at a specific promoter site, the polymerase causes the double-stranded DNA structure to melt or unwind, so that transcription can begin. Typically a defined region between 20-30 bases upstream is
Figure 5.5: Schematic diagram of plasmid construct p4139. Calpastatin exon 1 together with 1667 bp of 5' flanking sequences and 2400 bp of intron 1 was cloned upstream of chloramphenicol acetyl transferase (CAT) coding sequence. Exon 1 region is indicated by the solid box. Intron and exon boundary is shown along with the location of some restriction enzyme recognition sites (H) Hind III; (P) PstI; (S) Sma I; (A) Apal; (N) Nde I; (X) Xba I. Primers used for sequencing are indicated by arrows.
required for normal initiation at the start site. In most eukaryotic genes, this region contains the consensus sequence TATA(A/T)A(A/T), called the TATA box (Cordon et al., 1980). More distal elements in the promoter region, usually extending 100 or more bases 5' to the transcription initiation site, play a dominant role in controlling the frequency of initiation, and a second consensus sequence, the CAAT box, is often located in this more distal region (Benoist et al., 1980).

Sequence immediately upstream of exon 1 in the calpastatin gene is extremely rich in GC residues (70% GC content within the first 100 nucleotides, Fig 5.6) and contains no TATA box. These structural features may explain the presence of multiple transcription initiation sites, because the role of the TATA box is to determine the precise point of initiation.

So far, a large number of genes, including hypoxanthine phosphoribosyltransferase (Melton et al., 1984, 1986), adenosine deaminase (Valerio et al., 1985), and hydroxy-3-methylglutaryl coenzyme A reductase (Reynolds et al., 1984), have been studied in which no TATA box is evident by sequence analysis. Most of these genes contain a stretch of 20-50 nt rich in GC residues residing within the first 100-200 bases upstream of the start site. A DNA-binding factor called SP1 recognizes GC
Figure 5.6: Calpastatin promoter region GC content.
rich sequences, and genes lacking TATA boxes may rely on these GC-rich sites and the transcription factors bound to them to initiate transcription. It is also notable that most of these genes are “housekeeping” genes and expressed at low to moderate levels in a great variety of tissues. Therefore, our data support the idea that calpastatin belongs to the family of housekeeping genes in which the absence of a TATA box is a common feature.

5’ Flanking sequence of the calpastatin gene was analyzed using MatInspector. Results showed that it contains four potential SP-1 binding sites between nt-162 and -41 (relative to the most 5’ initiation of transcription); a putative CAAT box between nt-125 and -122; two AP2 binding sites between nt-28 and -68; four NFκB binding sites clustering between nt-480 and nt-200, a fifth NFκB site locates far more upstream at nt-971; and MyoD and E47 binding sites at nt-563 to nt-558, (Fig. 5.7). These putative responsive elements located within the proximal promoter region suggest that transcription regulation of this gene may involve a complex array of regulatory factors.

The rabbit calpastatin gene has an in-frame TGA stop codon 135 nt upstream from the second AUG start site, which is located in exon 4. cDNA sequence comparison did not show any stop codons in other species,
raising the possibility that this TGA is a sequencing error. Alternatively, sequence immediately upstream of exon 4 can act as a second promoter to drive calpastatin gene transcription from exon 4. To test this possibility, sequence immediately upstream of exon 4 was analyzed using MatInspector (Fig. 5.8). Results show that although this sequence contains some potential AP1 and NFκB cis elements, a TATA box or SP1 binding sites are absent, making it unlikely that this intron sequence can act as a second calpastatin promoter.
Figure 5.7: Sequence of the Bovine Calpastatin Gene 5' Flanking Region. The sequence extends 1667 nt upstream of the initiation of transcription, which is designated as nt +1. Truncation points for promoter-CAT constructs are indicated by numbers. SP1 and GC boxes are located in two clusters within the proximal promoter region and upstream of nt -700. Double underlines indicate sequences that are potentially important for cAMP responsiveness. Single underline denotes a putative CAAT box. Potential NFκB, IκB and AP2 sites are also indicated.
Figure 5.8: Intron 3 sequence immediately upstream of exon 4. Putative AP1 and NFκB sites are boxed. Arrow shows intron 3 and exon 4 boundary.
IV. Transient Transfection Analysis of the Bovine Calpastatin Gene Promoter.

To determine whether the 5' flanking region of the bovine calpastatin gene possesses promoter activity, a genomic Hind III fragment containing 1667 bp of 5' flanking region, 158 bp of exon 1 and approximately 2400 bp of intron 1 was cloned into pBluescript KS vector. Using this clone as a template, by PCR a series of 5' deletions were generated and cloned upstream of the chloramphenical acetyl transferase (CAT) reporter gene in the vector pCAT-Basic. The structures of the resulting constructs are shown in Fig. 5.9 A. Clones pCS-1667, pCS-1242, pCS-944, pCS-671, pCS-272, pCS-102, or pCS-31, represent promoter fragments extending from nt-1667, -1242, -944, -671, -272, -102 or -31 to nt +130, relative to the transcription initiation site. Calpastatin-CAT constructs were tested for promoter activity by transient transfection into the mouse cell line NIH3T3. 48 h following transfection, cells were harvested and assayed for CAT activity. CAT activities were normalized to protein content within extracts, and each construct was assayed in at least three independent transfection experiments. A pCAT-control plasmid containing the SV40 promoter driving CAT was used as a transfection control.

Transient transfection studies showed that the 5' flanking region of the calpastatin gene possessed moderate transcriptional activity (Fig.5.9B). CAT expression from the longest construct, pCS-1667, was relatively low,
approximately 0.8% of pCAT-Control. Progressive deletion of promoter sequence both stimulated and suppressed transcriptional activity. Deletion of sequence from nt-1667 to -944, for example, resulted in an increase in promoter activity of more than two-fold, while deletion to nt-671 reduced CAT activity more than seven fold. This indicates that the region between -944 to -671 positively regulates the promoter activity of the downstream region (-671 to 0). Deletion to nt-102 (pCS-102), which maintained potential three SP1 binding sites but deleted a potential CAAT box element at nt -125, reduced CAT expression to barely detectable levels. Further deletion to nt-31 totally abolished promoter activity. These results demonstrate that DNA elements located within 1667 nt of transcription initiation of the calpastatin gene can direct moderate level expression of a heterologous cDNA, and that cis elements within this promoter region can both stimulate and inhibit transcriptional activity.
Figure 5.9: Effect of positive and negative elements on calpastatin promoter activity. Constructions of various CAT plasmids are illustrated in A. Gray boxes indicate exon 1 sequence (nt+1 - nt+130). B) Calpastatin-CAT reporter constructs containing varying amounts of promoter sequence were transfected into NIH3T3 cells. Cells were harvested 48 h after transfection. Promoter activity of each construct was determined by CAT expression in NIH3T3 cells. pCAT-Basic was used as a negative control.
V. Effects of Dibutryl cAMP on Calpastatin Promoter Activity.

β-agonist-induced muscle hypertrophy is accompanied by increased steady-state levels of calpastatin mRNA (Parr et al., 1992). Since β-agonists act through the cAMP-dependent protein kinase signaling pathway, we asked whether transcription of the calpastatin promoter is enhanced following administration of dibutryl cAMP, a constitutive activator of PKA.

As shown in Fig. 5.10 and Table 5.2, addition of dibutryl cAMP to culture medium resulted in a five to seventeen-fold increase in calpastatin promoter activity for constructs containing at least 272 nt of promoter sequence. Transcriptional activity of pCAT-Control was not increased by dibutryl cAMP treatment. Interestingly, pCS-102, which showed extremely low basal promoter activity, was induced to moderate levels (approximately equivalent to basal levels of pCS-1667) following administration of dibutryl cAMP. At least one cAMP responsive element is therefore likely to reside within 102 nt of transcription initiation.

A consensus CRE site constitutes an 8 bp palindromic sequence (TGACGTCA). Several genes which are regulated by a variety of endocrinological stimuli contain similar sequences in their promoter regions, although at different positions (Table 5.3). Binding site specificity
appears to require 18-20 bp, since the five or so bases flanking the core consensus have been shown to dictate, in some cases the permissivity of transcriptional activation (Deutsch et al., 1988). In many genes, the CRE sequence is located in the first 200 bp upstream from the cap site. However, variations of the canonical CRE have also been reported, including CRE half sites which can bind proteins such as CREB, ATF-2 and Jun, albeit at reduced affinity compared with the intact palindromic CRE (for review see Habener et al., 1995).

Table 5.2
Effect of Dibutryl cAMP on Calpastatin Promoter Activity

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Rel. CAT Activity$^a$</th>
<th>Induction Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Bt$_2$cAMP</td>
<td>+Bt$_2$cAMP</td>
</tr>
<tr>
<td>pCAT-Control</td>
<td>100.0</td>
<td>90.0</td>
</tr>
<tr>
<td>pCAT-Basic</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pCS-1667</td>
<td>0.8</td>
<td>5.4</td>
</tr>
<tr>
<td>pCS-1242</td>
<td>1.2</td>
<td>5.8</td>
</tr>
<tr>
<td>pCS-944</td>
<td>1.8</td>
<td>11.0</td>
</tr>
<tr>
<td>pCS-671</td>
<td>0.2</td>
<td>3.4</td>
</tr>
<tr>
<td>pCS-272</td>
<td>0.6</td>
<td>4.5</td>
</tr>
<tr>
<td>pCS-102</td>
<td>0.03</td>
<td>0.5</td>
</tr>
<tr>
<td>pCS-31</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

$^a$ CAT activities are relative to pCAT-Control, which contains the CAT gene driven by the SV40 viral promoter.
Figure 5.10 cAMP Responsiveness the Bovine Calpastatin Promoter. Calpastatin-CAT reporter constructs containing varying amounts of promoter sequence were transfected into NIH3T3 cells. 24 h following transfection, dibutryl cAMP (1 mM) was added to some cultures. All cultures were sacrificed 48 h following transfection. Graph shows CAT activity relative to pCAT-Control, which contains the SV40 promoter driving CAT. Data represents the average of at least three independent experiments. Representative CAT assays are shown.
Sequence analysis of the calpastatin proximal promoter region identified two potential CRE-like half sites, CRE1 (GTCA) at nt-76 just downstream of an SP1 site, and CRE2 (GTCA) located within exon 1 at nt+13.

It has been reported that mouse ornithine decarboxylase (ODC) promoter contains three functional CREs, one of which is located at +95 bp relative to the transcription start site (Abrahamsen et al., 1992). To test whether calpastatin CRE2 is involved in activation of promoter activity by cAMP, the 130nt exon 1 sequence was removed and a calpastatin promoter fragment containing nt-944 to nt-1 was cloned into the pCAT-Basic expression vector (pCAT-944). pCAT-944 or pCS-944, which contains the 130 nt exon 1 sequences in addition to the 944 bp promoter sequence, were transfected into NIH3T3 cells. 24 h following transfection, some cultures were treated with dibutyryl cAMP. Results showed that both constructs displayed approximately 4-fold increases in CAT activity after treatment with dibutyryl cAMP (Fig. 5.11), indicating that the putative CRE2 located within exon 1 is not functional. It is worth noting, however, that deletion of nt+1 to nt+130 reduced CAT activity approximately three fold. This difference in basal expression between pCS-944 and pCAT-944 may arise from the fact that they utilize different transcription initiation sites, because potential downstream initiation sites have been deleted.
Table 5.3

cAMP Responsive Elements in Mammalian Gene Promoters

<table>
<thead>
<tr>
<th>cAMP-Responsive Element</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGACGTCAaggtaaaaATGACGTCA</td>
<td>Human glycoprotein hormone, α-subunit (Delegeane et al., 1987)</td>
</tr>
<tr>
<td>-114 gcgcggggcTGCCGTAgggccTGCGTCAgtca-80</td>
<td>Prokephalin (Comb et al., 1986)</td>
</tr>
<tr>
<td>-332cccccattacagaggtgtcataatagacatcTGCGTCAgaggttccacctggtc-278</td>
<td>cFOS (Treisman, 1986)</td>
</tr>
<tr>
<td>-94 cccatggcGTCAactcgTGACGTCA -70</td>
<td>Human vasoactive intestinal polypeptide gene (Tsukada et al., 1987)</td>
</tr>
<tr>
<td>-71 gtagatcgggggcctctggcTGACGTCAagagagagtt -27</td>
<td>Somatostatin (Montminy et al., 1986)</td>
</tr>
<tr>
<td>-71 agaggtggggggccagaggtgGTACGTCAgttggtttctgctggttc -27</td>
<td>TH (Lee et al., 1987)</td>
</tr>
<tr>
<td>-207 AGGCGGTGAGGTACGTCAAGGGGTGGGGGA -183</td>
<td>Major Histocompatibility Complex (MHC) (Hamada et al., 1989)</td>
</tr>
<tr>
<td>-200 TGACGTCAatataa_cacttcGTACGTCA -150</td>
<td>pituitary-specific homeobox gene GHFI (McCormick et al., 1990)</td>
</tr>
<tr>
<td>-243 TTGATGGACAGTGACGCTAGCAAG -225</td>
<td>Bovine P-45017-α (Zanger et al., 1991)</td>
</tr>
<tr>
<td>-129 GGCCACTCTGTGGGCGGGTCGCCGGGAGGCTACC -96</td>
<td>Human Steroid 21-Hydroxylase (Kagawa et al., 1990)</td>
</tr>
<tr>
<td>-131 TA-TGT-T---------------A-A--------CAAG--G-------- -98 Bovine Steroid 21-Hydroxylase (Kagawa et al., 1990)</td>
<td></td>
</tr>
<tr>
<td>-109 TA-TGT---------------TT*-----------AACA*----------A--------*---------- -79 Mouse Steroid 21-Hydroxylase (Kagawa et al., 1990)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.11: Effect of deletion of CRE2 in exon 1 on induction of CAT expression by dibutyryl cAMP. (A) Schematic diagram of constructs pCS-944 and pCAT-944. Filled box represents exon 1 sequences (nt+1 to nt+130, relative to the calpastatin transcription initiation site). Single lines represent 5'-flanking sequences of the calpastatin gene. Two putative CRE half sites are indicated as dark circles. (B) NIH3T3 cells transfected with 2 μg of the two plasmids were incubated in the absence or presence of dibutyryl cAMP. After 48 h cells were harvested and CAT activity was measured as described under "Materials and Methods". Fold increases are indicated above the bars.
VI. Site Directed Mutagenesis Mapping of cAMP Responsive Elements.

Deletion analysis of calpastatin promoter region demonstrated that at least one cAMP responsive element resides within 102 nt of transcription initiation (Table 5.2). Nucleotide sequence analysis showed that a potential CRE half site is located at nt-76. To test whether this is the functional CRE, site directed mutagenesis was used to change (GTCA) at nt-76 to (ATCT) using PCR based method of mutagenesis. This mutation generated pCS-102mutAT, which is identical to pCS-102 except for the two point mutations (Fig. 5.12A). As shown in Fig. 5.12B, this mutation totally abolished the cAMP responsiveness, indicating that this is the core sequence for calpastatin gene up-regulation by β-agonists.

To determine whether additional CRE sites are located further upstream, fragments of the calpastatin 5′-flanking region were generated by PCR (Table 5.4) and cloned upstream of a minimal Herpes simplex virus thymidine kinase promoter (-105 - +51) driving CAT (pTK-CAT). *SalI* and *XbaI* cloning sites located 179 bp upstream of the promoter region were used to insert DNA fragments from the calpastatin 5′-flanking region (Fig. 5.13A). Plasmids containing calpastatin promoter fragments were then transfected into NIH3T3 cells and CAT activities were compared with the parental plasmid. Results showed that a 3.2-fold increase was observed
upon dibutyryl-cAMP treatment in pCS5-TKCAT transfected cells, which contains the proximal calpastatin promoter region from nt-346 to nt-16 sequences, (Fig. 5.13 B). No cAMP induction was observed when cells were transfected with the plasmids containing calpastatin 5’ flanking sequences further upstream, suggesting that functional CREs are not present within the more distal regions of the calpastatin promoter. When cells were transfected with pCS3-TKCAT, containing calpastatin 5’-flanking region nt-947 to nt-577, basal CAT activity was about twice as high as the parental plasmid, pTKCAT, indicating that some positive elements located within this region can upregulate gene transcription. This result is consistent with the promoter deletion assays in which deletion from -944nt to nt-671 reduced CAT activity more than seven fold.
Figure 5.12: Effect of CRE element mutation on basal and induced expression of CAT fusion constructs. (A) Schematic diagram of pCS-102 and pCS-102mutAT. CRE-like sequences are indicated in the boxes. Filled box represents exon 1 sequences (nt+1 to nt+130, relative to the calpastatin transcription initiation site). Single lines represent 5'-flanking sequences of the calpastatin gene. Mutations were made from GTCA to ATCT. (B) cAMP responsiveness of the mutated bovine calpastatin promoter. NIH3T3 cells transfected with 2 μg of the two plasmids were incubated in the absence or presence of dibutyryl cAMP. After 48 h cells were harvested and CAT activity was measured as described under "Materials and Methods".
Table 5.4

PCR primers for constructing calpastatin-TKCAT

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>Position relative to the transcription initiation site</th>
<th>PCR Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCSF1</td>
<td>cggtcgacCACAGCCCCAAAACCAGGAAG</td>
<td>-1653–1634</td>
<td></td>
</tr>
<tr>
<td>PCSB1</td>
<td>ctagtctagacTTTTCCCCCGACTTGCC</td>
<td>-1143–1126</td>
<td>528 bp</td>
</tr>
<tr>
<td>pCSF2</td>
<td>cggtcgaccGGAGTGGGGCTTGAGATGTAGAC</td>
<td>-1248–1226</td>
<td></td>
</tr>
<tr>
<td>pCSB2</td>
<td>ctagtctagacGTGTAGGCGAGCTTTTCCGAC</td>
<td>-946–926</td>
<td>323 bp</td>
</tr>
<tr>
<td>pCSF3</td>
<td>cggtcgaccGGTGGAAAAGCGCCCTCAC</td>
<td>-947–928</td>
<td></td>
</tr>
<tr>
<td>pCSB3</td>
<td>ctagtctagacGGTCTGACTCCACACCCTTTC</td>
<td>-599–577</td>
<td>371 bp</td>
</tr>
<tr>
<td>pCSF4</td>
<td>cggtcgaccGGTCACCTCTAGAGGAGGGTTAG</td>
<td>-679–656</td>
<td></td>
</tr>
<tr>
<td>pCSB4</td>
<td>ctagtctagacCTGTCTCTCATCCACAACTTTTCC</td>
<td>-283–259</td>
<td>421 bp</td>
</tr>
<tr>
<td>pCSF5</td>
<td>cggtcgacCATGGTGGCAGTCAGGAC</td>
<td>-346–325</td>
<td></td>
</tr>
<tr>
<td>pCSB5</td>
<td>ctagtctagacCTGTCAGGGGGTGAGCAAG</td>
<td>-33–16</td>
<td>331 bp</td>
</tr>
</tbody>
</table>

Note: sequences in small letters indicate SalI or XbaI linkers added at the 5’ end of the primers.
Figure 5.13: Effect of dibutyryl cAMP on promoter activities. (A) Schematic diagram of constructs used for transfection. The 5'-flanking DNA fragments were inserted upstream of the herpes simplex virus thymidine kinase promoter driving transcription of CAT. (B) Calpastatin-TKCAT reporter constructs containing varying amounts of promoter sequence were transfected into NIH3T3 cells. Cells were harvested 48 h after transfection. Promoter activity of each construct was determined by CAT expression in NIH3T3 cells. pTKCAT was used as a negative control. Relative CAT activities of the various constructions are shown taking the expression of pTKCAT without dibutyryl cAMP treatment as 1.0. Fold increases after treatment with dibutyryl cAMP are indicated.
VII. Discussion.

The proximal promoter region of the bovine calpastatin gene is GC rich and lacks a TATA box, characteristics of genes that are widely expressed in different cell types. Transfection analyses show that the 5' flanking region of the calpastatin gene can direct moderate level transcription of a heterologous cDNA, and that sequence elements between nt-1667 and -272 can act both to stimulate and to inhibit gene expression. Deletion to nt-102, which eliminates a putative CAAT box, almost completely abolishes promoter activity.

The finding that dibutryl cAMP increases calpastatin promoter activity between five and twenty fold demonstrates that transcription of the calpastatin gene can be upregulated by PKA. Binding of β-agonists to the β2 adrenergic receptor activates a PKA-dependent signaling pathway and leads to an increase in calpastatin protein levels (Parr et al., 1992). Our results suggest that this occurs through an increase in gene transcription. Some cAMP responsive genes contain a consensus cAMP responsive cis element (CRE) consisting of the palindromic sequence (TGACGTCA) (Delmas et al., 1994). Variations of the canonical CRE have also been reported, including CRE half sites which can bind proteins such as CREB, ATF-2 and Jun, albeit at reduced affinity compared with the intact
palindromic CRE (for review see Habener et al., 1995). pCS-102, which shows no basal promoter function, exhibits moderate transcriptional activity following exposure of transfected cells to dibutyryl cAMP, indicating that at least one cAMP responsive element is located within 102 nt of transcription initiation. This proximal promoter region contains two potential CRE half sites, CRE1 (GGTCA) at nt-76 just downstream of an SP-1 site, CRE2 (GTCA) located at nt+13 within exon 1. Deletion of CRE2 failed to decrease dibutyryl cAMP dependent induction, indicating that CRE2 is not necessary for calpastatin gene upregulation by cAMP. Cells transfected with plasmids lacking exon 1 sequences showed much lower CAT activity compared to cells transfected with the plasmid containing calpastatin exon 1, suggesting calpastatin exon 1 sequence is necessary for complete promoter activity. PCR-based site directed mutagenesis showed that CRE1 (GGTCA) at nt-76 is necessary for cAMP induction.
Chapter 6

Conclusions and Discussion

I. The Bovine Heart Calpastatin Gene and Protein are Both Targets for cAMP-Dependent Protein Kinase Activity.

a. Discovery of A New N-terminal Region of The Bovine Calpastatin Protein.

Cloning and sequence analysis of bovine calpastatin cDNAs identified a previously unrecognized translation initiation site that gives rise to a protein with additional 68 N-terminal amino acids, called the XL region. This XL region shows no homology to other regions of calpastatin or to any known protein. Amino acid analysis indicates that it contains three potential protein kinase A phosphorylation sites. Coupled in vitro transcription/translation experiments demonstrated that this upstream ATG is functional in vitro and generates a protein with an apparent molecular mass of 145 kDa on SDS-PAGE. Western blot analysis using bacterially-expressed full length calpastatin and tissue homogenates from bovine heart and liver shows that a protein with the size of 145 kDa is recognized by a
monoclonal antibody to an epitope at the calpastatin C-terminus, suggesting that the upstream ATG is functional \textit{in vivo}. To prove that this XL region exists within the native calpastatin protein, polyclonal antibodies were raised against a polypeptide within this XL region. Western blot confirmed that the full length calpastatin protein migrating at an apparent size of 145 kDa, but not smaller calpastatin proteins, was recognized by this polyclonal antibody, demonstrating that this XL region exists within native calpastatin protein \textit{in vivo}. PKA phosphorylation assays using bacterially-expressed full length calpastatin, domain 1-4 or domain XL plus L showed that both XL region and domain 1 are targets for PKA.

b. Identification of the Calpastatin Gene Promoter.

A calpastatin gene promoter had not been previously identified in any species. To investigate the regulation of bovine calpastatin gene transcription during muscle growth and hypertrophy, the 5' region of the bovine calpastatin gene was cloned by screening a bovine heart genomic DNA library. The genomic structure of calpastatin gene 5' region shows that the newly discovered translation initiation site is in exon 1, while the previously reported translation start site is located in exon 4; intervening large introns span at least 60 kb. Primer extension defined the 5'-most
transcription start site, although two other minor bands have also been
found just a few bases downstream. Multiple transcription initiation sites
are common for house-keeping genes which lack TATA boxes and that are
GC rich in their proximal promoter regions.

Nucleotide sequence analysis within the promoter region shows that
the calpastatin promoter is GC rich and contains one putative CAAT box,
four SP-1 binding sites and several NFkB and AP2 binding sites, reflecting
the complexity of transcriptional regulation by different transcription
factors. Promoter activity analysis shows that the calpastatin promoter
contains moderate promoter activity. Serial deletion of the calpastatin
promoter identified positive and negative regulatory regions which can
activate and inhibit basal calpastatin promoter activity.

c. *In vitro* Analysis of Regulation of the Calpastatin Gene Promoter by
PKA.

The effects of β-agonists on calpastatin gene transcription has also
been investigated. Since β-agonists can stimulate a cAMP-dependent
signaling pathway, a cAMP analogy dibutyryl cAMP was used to mimic the
β-agonist effect. Addition of dibutyryl cAMP increased calpastatin
promoter activity 5 to 20 fold, demonstrating that the calpastatin
transcription is upregulated by PKA. Promoter deletion studies showed that at least one cAMP responsive element (CRE) resides within nt-102 of the transcription initiation site. Additional CREs were not detected further upstream. Deletion of exon 1 dramatically decreased basal promoter activity but not cAMP induction, indicating that while exon 1 sequence does not contain a functional CRE, unidentified cis elements are present that influence promoter activity. Sequence analysis of the proximal promoter region shows a CRE half site located at nt-76 (GTCA). Mutation of (GTCA) to (ATCT) by PCR-based mutagenesis abolished dibutyryl cAMP mediated promoter induction, indicating that this is the functional CRE in the calpastatin gene promoter.

II. Effects of Calpastatin Phosphorylation by PKA on the Regulation of Calpain Activity.

a. Possible Roles of PKA Phosphorylation in Domain 1.

Calpastatin protein has been studied for several decades and the cDNAs cloned from many species. The previously proposed translation start site was designated because nucleotide sequence analysis of the rabbit
cDNA showed a stop codon 135 bp upstream. Since then, this ATG has been widely accepted as the translation initiation site in many species. Difficulties in obtaining amino acid sequence of the N-terminus, anomalous migration of calpastatin protein on SDS-PAGE, and rapid proteolysis of calpastatin during isolation obscured the possibility that calpastatin protein might contain additional previously unidentified regions. The calpastatin protein studied extensively in many laboratories has a reported size of 110-120 kDa, which is approximately the size of a bacterially expressed calpastatin polypeptide representing domain 1-4. Our findings demonstrate that the 110-120 kDa protein lacks the L domain and XL region, and is therefore a partial calpastatin protein. The potential roles of these regions on calpastatin function have therefore not been studied.

Salamino et al. (1994) isolated an unphosphorylated and phosphorylated form of the 110 kDa partial calpastatin protein from rabbit heart and kidney (designated calpastatin I and II) that displayed preferential inhibitory efficiencies towards μ- and m-calpain, respectively. Although sites of phosphorylation remain unknown, our results show that a calpastatin protein truncated to remove domain L and region XL can be phosphorylated by PKA. A predicted PKA phosphorylation site resides within subdomain B of domain 1, the domain exhibiting the highest calpain
inhibitory activity. It is therefore reasonable to hypothesize that PKA phosphorylation in domain 1 maybe responsible for the observed shift in calpastatin specificity from \( \mu \)- to \( m \)-calpain. Using bacterially expressed calpastatin domain 1-4, it should be straightforward to precisely define the PKA dependent phosphorylation sites by tryptic peptide mapping and site directed mutagenesis. Phosphorylation assays could then be used to test the role of specific PKA phosphorylation sites on calpastatin protein function \textit{in vitro}.

The ability of the 110 kDa calpastatin to be phosphorylated \textit{in vivo} could also be assayed. cDNAs encoding wild type calpastatin or calpastatin containing mutations in PKA phosphorylation sites could be epitope tagged and transfected into cells growing in the present of \(^{32}\text{P}\). Transfected cells would then be treated with dibutyryl cAMP and expressed calpastatin immunoprecipitated from cell lysates using antibody against the affinity tag. Bound calpastatin could then be analyzed for the effect of dibutyryl cAMP on phosphorylation.

b. Possible Roles of PKA Phosphorylation in XL region.

Since our \textit{in vitro} phosphorylation data suggest that the XL region of bovine heart calpastatin is also a target of PKA, the question arises as to
what the function of XL region is and what the role of this PKA phosphorylation might be for regulating calpastatin inhibitory function. Amino acid sequence analysis shows that the XL region is extremely basic with a predicted pI of 10.07. This is consistent with the possibility that the XL region is an extension of domain L, which is also extremely basic and can non-specifically associate with the negatively charged phospholipids.

As discussed above, the XL region contains three PKA phosphorylation sites that can be phosphorylated by PKA in vitro. Since almost all published studies have utilized the 110 kDa partial calpastatin that lacks this XL region, the function of this region and its phosphorylation by PKA has yet to be investigated.

In the literature review it was noted that in some tissues, notably heart and liver, there is sufficient calpastatin to fully inhibit endogenous calpain activity. Since calpains are clearly active within these cells, one possibility is that regulatory mechanisms might inactivate calpastatin and/or sequester calpastatin from calpain. Whether calpains can be fully inhibited in vivo by calpastatin depends on their degree of association. PKA activation may contribute to muscle growth by phosphorylating sites within the XL region, which might then bring calpastatin and calpain molecules together so that calpastatin can inhibit calpain’s activity. Further work is
needed to determine the intracellular distribution of calpastatin following
β-agonist treatment, especially the correlation between the phosphorylation
by PKA within the XL region and protein localization.

III. Model for β-adrenergic agonist effect on muscle growth.

Administration of β-agonist to mammals can stimulate muscle
hypertrophy to a remarkable degree. The calpain system is believed to play
an important role in regulating protein degradation and muscle growth.
We provide the first evidence at the molecular level that β-agonist
treatment can both stimulate calpastatin gene transcription and protein
phosphorylation, which is consistent with the previously reported data that
β-agonist treatment can increase both m-calpain and calpastatin mRNA
levels and protein activities (Parr et al., 1992). Based on the data presented
above, the following model is provided to explain the effect of β-agonist
on muscle growth (Fig 6.1):

Treatment of animals with β-agonists leads to cAMP-dependent
protein kinase stimulation via the GTP-binding protein (G protein )-
adenylate cyclase-cAMP signal transduction pathway. Stimulation of this
pathway leads to increased calpastatin gene transcription and
phosphorylation of the calpastatin protein in two regions: phosphorylation
within region XL might be involved in the localization of calpastatin molecules to the same microenvironment as calpains, while phosphorylation at domain 1 in the central inhibitory region might switch its inhibitory efficiency from \( \mu \)-calpain to \( m \)-calpain. The physiological significance of this switch remains unknown. Increases in the level of calpastatin and its ability to inhibit calpains would nevertheless lead to decreased levels of protein degradation, resulting in muscle growth.
Figure 6.1: Model for β-agonist effects on muscle growth.
LIST OF REFERENCES


Dependent Proteolysis., R. L. Mellgren, and Murachi, T., ed. (Boca

Gorman, C. M. (1983). Recombinant genomes which express
chloramphenicol acetyltransferase in mammalian cells. Mol. Cell.
Biol. 2, 1044-51.

regulation of gene transcription by cAMP response element-binding
protein and cAMP response element modulator. Vit. and Horm. 51,
1-57.

Hamada, K., Gleason, S., Levi, B., Hirschfeld, S., Appella, E., and Ozato,
K. (1989). H-2RIIBP, a member of the nuclear hormone receptor
superfamily that binds to both the regulatory element of major
histocompatibility class I genes and the estrogen response element.

Hanrahan, J., Quirke, J., Bomann, W., Allen, P., McEwan, J., Fitzsimons,
J., J., K., and Roche, J. (1986). In In Recent Anvances in Animal

of transcription factors, c-Jun and c-Fos, by calpain. FEBS Let. 287,
57-61.

free calcium in frog skeletal muscle fibers estimated with fluo-3.
Biophysical Journal 65, 865-81.

autolysis reduces the Ca^{2+} requirement of a smooth muscle Ca^{2+}-

between dietary restriction or clenbuterol (a selective beta 2 agonist)
treatment on muscle growth and calpain proteinase (EC3.4.22.17)
and calpastatin activities in lambs. British Journal of Nutrition 60,
645-52.


Reynolds, G., Basu, S., Osborne, T., Chin, D., Gil, G., Brown, M.,
negatively regulated gene with unusual promoter and 5' untranslated

Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai,
N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut,
C., and et al., (1995). Mutations in the proteolytic enzyme calpain 3

perspectives in molecular diversity and physiological-pathological
involvement. FASEB J. 8, 814-22.

Salamino, F., De Tullio, R., Michetti, M., Mengotti, P., Melloni, E., and
tissues by reversible phosphorylation and dephosphorylation.

New muscle fiber production during compensatory hypertrophy.
Medicine & Science in Sports & Exercise 12, 268-73.

purified from bovine skeletal muscle that inhibits the Ca2+-
dependent proteinase. In Intracellular Protein Catabolism., E. A.
Khairallah, J. W. C. Bird and J. S. Bond, eds. (New York: Alan R.

Smith, T., Dana, R., Krichevsky, A., Bilezikian, J., and Schonberg, M.
(1981). Inhibition of beta-adrenergic responsiveness in muscle cell
cultures by dexamethasone. Endocrinology 109, 2110-6.

Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S.,
mammalian calcium-dependent protease distinct from both m- and
mu-types. Specific expression of the mRNA in skeletal muscle. J.
Biol. Chem. 264, 20106-11.


