The Bovine Calpastatin Gene Promoter and a New N-terminal Region of the Protein Are Targets for cAMP-dependent Protein Kinase Activity

To investigate the regulation of calpastatin gene expression, we isolated bovine heart calpastatin cDNAs and 5′-regions of the calpastatin gene. Analysis of 5′-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 PKA phosphorylation sites but shares no homology with other regions of calpastatin or with any known protein. Immunoblot studies demonstrated that heart and liver contain a calpastatin protein of 145 kDa on SDS-polyacrylamide gel electrophoresis that comigrates with full-length bacterially expressed calpastatin and 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 in vivo. 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 to inhibit or stimulate transcriptional activity. Exposure of transfected cells to dibutylryl cAMP resulted in a 7–20-fold increase in promoter activity for constructs containing at least 272 nucleotides of upstream promoter sequence. Deletion analysis indicates that at least one cAMP-responsive element resides within 102 nucleotides of transcription initiation.

Calpastatin is a proteinase inhibitor that is specific for calpains, a family of calcium-activated neutral proteases that regulate a wide range of Ca2+-dependent cellular processes (1–6). Calpastatin binds to and inhibits the calpains in the presence of Ca2+, although how this interaction is regulated remains largely unknown.

Calpastatin is encoded by a single gene in birds and mammals that produce a number of closely related protein isoforms via alternative splicing (7, 8). Partial calpastatin cDNAs have been cloned from several species, including cattle, pig, rat, rabbit, and human (9–12). Conceptual translation using an initiation codon first identified in the rabbit cDNA yields a protein of 718 amino acids with a predicted molecular mass of 76 kDa (9). The protein contains four repeating domains, each of which is capable of binding to calpain and inhibiting proteolytic activity such that a single calpastatin molecule can inhibit several calpain molecules in vitro (13–15). An additional N-terminal L domain shares no homology with the inhibitory domains and is of unknown function.

Although mechanisms controlling calpastatin inhibitory activity are not yet understood, studies in vitro and in vivo suggest that calpastatin is regulated both at the level of protein abundance and via posttranslational modification. Following administration of β-agonists to steers, calpastatin mRNA and protein levels and calpain inhibitory activity increase 2-fold, and this increased calpastatin activity has been linked to decreased protein degradation during muscle hypertrophy (16–18). β-Agonists bind to the β2-adrenergic receptor and activate signaling cascades that involve cAMP-dependent protein kinase (PKA), raising the possibility that calpastatin gene transcription is responsive to a PKA-dependent signaling pathway. Calpastatin is also phosphorylated by PKA (3, 19), and some evidence suggests that phosphorylation can alter calpastatin function. A phosphorylated form of calpastatin isolated from rat skeletal muscle has been reported to have a lower Ki for inhibition of m-calpain than for inhibition of μ-calpain, whereas an unphosphorylated form preferentially inhibited μ-calpain (3, 19). This relative specificity for m- versus μ-calpain can be shifted in vitro by PKA-dependent phosphorylation or alkaline phosphatase treatment.

These findings indicate that PKA may be involved in regulating both calpastatin gene transcription and the extent of calpastatin protein phosphorylation. Relatively little is known about the regulation of calpastatin gene expression, however, since no calpastatin gene promoter has been characterized in any species. Furthermore, analysis of the known calpastatin amino acid sequence has identified only a single potential PKA phosphorylation site within domain I. As each of four domains within calpastatin shows potent calpain inhibitory activity, it has not been understood how phosphorylation at a single site might modulate calpastatin activity.

Here we report the cloning of 5′-regions of calpastatin cDNAs and transcriptional regulatory regions of the bovine calpastatin gene. Analysis of the cDNA sequence identified a previously unreported upstream translation initiation site that yields a calpastatin protein with 68 additional N-terminal amino acids. This “XL” region is present on calpastatin protein in heart and liver, and is phosphorylated by PKA. Transfection experiments show that the calpastatin gene promoter is up-regulated by PKA-dependent phosphorylation.

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dibutyryl cAMP, indicating that both the calpastatin promoter and protein are targets for PKA activity.

**EXPERIMENTAL PROCEDURES**

5’-Rapid Amplification of Complementary DNA 5’-Ends (RACE)—5’-RACE was performed as described (20) using total RNA from adult bovine heart and primers derived from a published partial calpastatin cDNA sequence (12). Three nested calpastatin-specific primers, CPBS (5’-CCGTATCTGGAAGCTGGTGG-3’), CPBT (5’-GGTAGCTTTGGGCTCTGTGTG-3’), and CPB1 (5’-GTCCTGGTCTCTAAAAGG-3’) were used. Single-stranded cDNA template was generated using 5 μg of bovine heart total RNA and the CPB8 primer according to standard protocols. Following removal of excess primers using Centri-con 100 spin filters (Amicon Corp.), polyA was added to 5’-cDNA ends by incubation in a solution containing 10 units of terminal deoxynucleotidyl transferase (Promega), 0.2 mM dATP, 100 μM ccdexylool (pH 6.8), 1 mM CoCl2, and 0.1 mM dithiothreitol 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 QP (5’-CCCACTGACGAGATGGGACTCCGAGCTCTGTTTGGAAGGTTTTCCTGGG-3’) and two rounds of PCR were then performed using the primers QP (5’-CCCTAGGAGCAGATGGGACTCCGAGCTCTGTTTGGAAGGTTTTCCTGGG-3’) and the calpastatin-specific primer CPBS, followed by the primer QP (5’-CCTATCTGGAAGCTGGTGG-3’) and CPB1. PCR reaction products were separated on a 1% agarose gel, excised, and sequenced.

Isolation of Bovine Calpastatin cDNA and Genomic Sequences—Approximately 1.7 × 109 recombinant phage from a bovine heart UniZAP XR cDNA library (Strategene) were plated and screened at moderate stringency (final wash: 0.1% SDS, 0.1 × SSC at room temperature for 30 min) using a 152-bp 5’-RACE PCR product. Clones giving positive signals were plaque-purified and rescreened two additional times to eliminate false positives. The plaque-purified cDNA 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.

To isolate bovine genomic sequence in the 5’-region of the calpastatin gene, 1.1 × 109 recombinant phage from a bovine heart genomic library (Strategene) were screened using a 152-bp PCR product from 3′-end of the calpastatin cDNA as probe. Clones giving positive signals were plaque-purified, and genomic inserts were subjected to restriction enzyme analysis. Fragments hybridizing to an oligonucleotide from the extreme 3′-end of the calpastatin cDNA were subcloned into pBluescript for additional characterization.

**Northern Blot and Primer Extension**—Total RNA was isolated from adult bovine heart according to the method of Chomczynski and Sacchi (21). Northern analysis was performed essentially as described (22) using a 413-nt PCR product from the middle of the cDNA. For primer extension, an oligonucleotide (CPB522; 5’-GACG-3’) was used to isolate bovine genomic sequence in the 5′-region of the calpastatin gene. 1.1 × 109 recombinant phage from a bovine heart genomic library (Strategene) were screened using a 152-bp PCR product from 3′-end of the calpastatin cDNA as probe. Clones giving positive signals were plaque-purified, and genomic inserts were subjected to restriction enzyme analysis. Fragments hybridizing to an oligonucleotide from the extreme 3′-end of the calpastatin cDNA were subcloned into pBluescript for additional characterization.

**Production of Recombinant Calpastatin Proteins and PKA Phosphorylation Assay**—Calpastatin cDNAs representing full-length calpastatin, domain L plus region XL, or domains 1–4 were cloned into expression vectors containing an N-terminal 26-amino acid calmodulin binding unit. Recombinant plasmids expressing full-length, domain L, domain L plus region XL, or domains 1–4, or domain L and region XL were designated pcCalp786, pcCalp569, and pcCalp217, respectively. Recombinant PKA was used to phosphorylate calpastatin, or a chicken cDNA sequence (12). Three nested calpastatin-specific primers, CPBS (5’-CCGTATCTGGAAGCTGGTGG-3’), CPBT (5’-GGTAGCTTTGGGCTCTGTGTG-3’), and CPB1 (5’-GTCCTGGTCTCTAAAAGG-3’) were used. Single-stranded cDNA template was generated using 5 μg of bovine heart total RNA and the CPB8 primer according to standard protocols. Following removal of excess primers using Centri-con 100 spin filters (Amicon Corp.), polyA was added to 5’-cDNA ends by incubation in a solution containing 10 units of terminal deoxynucleotidyl transferase (Promega), 0.2 mM dATP, 100 μM ccdexylool (pH 6.8), 1 mM CoCl2, and 0.1 mM dithiothreitol 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 QP (5’-CCCACTGACGAGATGGGACTCCGAGCTCTGTTTGGAAGGTTTTCCTGGG-3’) and two rounds of PCR were then performed using the primers QP (5’-CCCTAGGAGCAGATGGGACTCCGAGCTCTGTTTGGAAGGTTTTCCTGGG-3’) and the calpastatin-specific primer CPBS, followed by the primer QP (5’-CCTATCTGGAAGCTGGTGG-3’) and CPB1. PCR reaction products were separated on a 1% agarose gel, excised, and sequenced.

Isolation of Full-length Bovine Calpastatin cDNAs—To identify 5′-bovine calpastatin mRNA sequence, 5′-RACE (20) was performed using mRNA from adult bovine heart and primers derived from a published partial calpastatin cDNA (12). A 152-bp cDNA fragment containing additional upstream sequence identified by 5′-RACE was used to screen a bovine heart cDNA library. Three independent cDNAs were obtained that differed in the amount of 3′-untranslated region and in their 3′-termination point (Fig. 1A). Overlapping regions of all clones were identical except for a 66-nt deletion within cDNA 3 that probably represents an alternatively spliced exon (8). The size of these cDNAs (4296, 4340, and 3544 nt) corresponded approximately to the size of three mature mRNAs identified by Northern blot using heart muscle RNA (Fig. 1B; see also Ref. 9).

Published characterization of full-length calpastatin cDNAs from human, rabbit, pig, and cattle have proposed a translation initiation site that yields a predicted protein of 639–718 amino acids, depending on species (9–12, 16). Designation of this translation start site derived from the rabbit cDNA (9), 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. Additionally, cDNAs 2 and 3 extend 5' beyond an AUG that is in frame and 204 nt upstream of the previously designated initiation of translation. B, Northern blot of bovine heart RNA showing three bands obtained using calpastatin cDNA as probe. The size of these three bands corresponds to the size of cDNAs 1–3.

Fig. 1. A, 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'-terminus point. cDNA 2 and 3 extend 5' beyond an AUG that is in frame and 204 nucleotides upstream of the previously designated initiation of translation. B, Northern blot of bovine heart RNA showing three bands obtained using calpastatin cDNA as probe. The size of these three bands corresponds to the size of cDNAs 1–3.

Fig. 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 boldface type. The dashed underlined region denotes the sequence absent from cDNA 3. The single underlined sequence denotes regions that differ from a previously published bovine skeletal muscle calpastatin cDNA (12). Asterisks indicate polyadenylation sites. (GCCAUGG) is a poor translation initiation site (37). Conceptual translation from the upstream AUG produces a protein containing 68 additional N-terminal amino acids (region XL). The XL region shares no homology with other regions of calpastatin or with any known protein. Full-length calpastatin cDNA sequence and conceptual translation are shown in Fig. 2.
Calpastatin Regulation by PKA

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. 3A). The largest translation product arising from the upstream ATG of cDNA 2 runs on SDS-PAGE at an apparent molecular mass of 145 kDa (Fig. 3B), almost twice the predicted molecular mass of 84 kDa. cDNA 1 produced a protein of 135 kDa, reflecting initiation at the downstream AUG. The anomalous migration of calpastatin on SDS-PAGE has been previously reported (10, 14, 25).

To determine whether the upstream AUG is utilized in vivo, Western analyses were performed using whole cell extracts, an ammonium sulfate fraction of crude heart homogenate (Fig. 3C), showing pCalp786 (lane 1), pCalp569 (lane 2), and pCalp217 (lane 3). All three protein products were phosphorylated by PKA.

FIG. 3. 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 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 at 135 kDa, cDNA 2 produced a predominant product of 145 kDa. C, immunoblot analysis of bacterially expressed calpastatin or whole heart or liver lysates. mAb 1F7, produced against a C-terminal calpastatin epitope, recognized a protein of approximately 150 kDa, representing full-length calpastatin, plus two smaller bands of approximately 120 and 110 kDa. In contrast, an antisera raised against a peptide from the N-terminal XL region recognized only the 145-kDa band in the ammonium sulfate fraction (lane 5).

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 4-kDa calmodulin binding 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.

FIG. 4. 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 4-kDa calmodulin binding 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.

To confirm that calpastatin contains the XL region in vivo, a polyclonal antisera was prepared against a 17-amino acid peptide from the XL region (amino acids 17–33). This antisera recognized only the 145-kDa band in the crude ammonium sulfate fraction (Fig. 3C, lane 5). Taken together, these results demonstrate that the upstream AUG is utilized and that the XL region is present within calpastatin protein in vivo.

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 and a fourth site within domain 1 (Fig. 4). To determine whether the XL domain is a target of cAMP-dependent phosphorylation, full-length and truncated calpastatin proteins were expressed in E. coli, partially purified, and then challenged in a kinase assay using purified PKA. As shown in Fig. 4, both an N-terminal partial calpastatin protein containing regions XL and L, and a C-terminal partial protein consisting of domains 1–4, are phosphorylated by PKA.

Structure of the 5′-Region of the Bovine Calpastatin Gene—Using 5′-sequence from the calpastatin cDNA as probe, four clones were isolated from a bovine genomic library. Extensive subcloning and sequencing has defined the structure of the 5′-region of the calpastatin gene (Fig. 5). Comparison between the genomic and heart cDNA sequences revealed 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.

To define the transcription initiation site, primer extension was performed using heart muscle RNA as template and a 22-nt primer located just 5′ to the upstream AUG (Fig. 6).

Three major extension products were obtained of 118, 124, and 130 nt. Each band may reflect a distinct transcription initiation site, since multiple start sites are common for genes that lack TATA boxes and that are GC-rich in their proximal promoter regions (see below). The region between the upstream AUG and the 5′ terminus of the 130-nt primer extension product is contiguous within the genomic sequence, indicating that it is encoded by a single exon.

**PKA Responsiveness of the Calpastatin Gene Promoter**—Sequence immediately upstream of exon 1 is GC-rich and contains four potential SP-1 binding sites between nt −162 and −41 (relative to the most 5′ initiation of transcription identified by primer extension) and a putative CAT box between nt −125 and −122 (Fig. 7). To determine whether this upstream genomic region possesses promoter activity, DNA fragments extending from nt −1667, −1242, −944, −671, −272, −102, or −31 to nt +130 were cloned immediately upstream of the CAT reporter gene and transfected into NIH3T3 cells. Promoter activity was assessed 48 h later by enzymatic CAT assay.

Transient transfection studies show that the 5′-flanking region of the calpastatin gene possesses moderate transcriptional activity (Fig. 8, Table I). CAT expression from pCS-1667CAT was approximately 0.8% of pCATControl, which contains the CAT gene under control of the strong SV40 viral promoter. Progressive deletion of promoter sequence identified regions that both stimulate and suppress transcriptional activity. Deletion of sequence between nt −1667 and −444, for example, increased promoter activity more than 2-fold, while deletion to nt −671 reduced CAT activity more than 7-fold. Truncation at nt −102, which deleted a potential CAT box element at nt

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**Fig. 6.** Primer extension analysis of calpastatin mRNA. Bovine heart poly(A)^+ 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.

**Fig. 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. Sp1 and GC boxes are located in two clusters within the proximal promoter region and upstream of nt −750. Double underlines indicate sequences that are potentially important for cAMP responsiveness. The single underline denotes a putative CAT box.
was induced to moderate levels (approximately equivalent to pCS-102, which showed extremely low basal promoter activity, not increased by dibutyryl cAMP treatment. Interestingly, promoter activity for constructs containing at least 272 nt of promoter sequence were transfected into NIH3T3 cells. 24 h following transfection, dibutyryl cAMP (1 mM) was added to some cultures. All cultures were sacrificed 48 h following transfection. The graph shows CAT activity relative to pCATControl, which contains the SV40 promoter driving CAT. Data represent the average of at least three independent experiments. Representative CAT assays are shown.

**Fig. 8.** cAMP responsiveness of the bovine calpastatin promoter. Calpastatin-CAT reporter constructs containing varying amounts of promoter sequence were transfected into NIH3T3 cells. 24 h following transfection, dibutyryl cAMP (1 mM) was added to some cultures. All cultures were sacrificed 48 h following transfection. The graph shows CAT activity relative to pCATControl, which contains the SV40 promoter driving CAT. Data represent the average of at least three independent experiments. Representative CAT assays are shown.

### TABLE I

| Plasmid     | Relative CAT activity | Induction ratio |
|-------------|-----------------------|----------------|
|             | Bt2cAMP               | +Bt2cAMP       |
| pCATControl | 100.0                  | 90.0           | 0.9          |
| pCATBasic   | 0.0                   | 0.0            |             |
| pCS-1667    | 0.8                   | 5.4            | 6.8          |
| pCS-1242    | 1.2                   | 5.8            | 4.8          |
| pCS-944     | 1.8                   | 11.0           | 6.1          |
| pCS-671     | 0.2                   | 3.4            | 17.0         |
| pCS-272     | 0.6                   | 4.5            | 7.5          |
| pCS-102     | 0.03                  | 0.5            | 16.7         |
| pCS-91      | 0.0                   | 0.0            |             |

−125, reduced CAT expression to barely detectable levels. Further deletion to nt −31 abolished promoter activity. These results demonstrate that DNA elements located within nt −1667 to initiation of transcription 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.

β-Agonist-induced muscle hypertrophy is accompanied by increased steady-state levels of calpastatin mRNA (18). 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 dibutyryl cAMP, a constitutive activator of PKA. As shown in Fig. 8 and Table I, the addition of dibutyryl cAMP to culture medium resulted in a 5–17-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 dibutyryl 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 dibutyryl cAMP. At least one cAMP-responsive element is therefore likely to reside within 102 nt of transcription initiation.

### DISCUSSION

A New N-Terminal Region of Bovine Calpastatin—We have cloned calpastatin cDNAs from bovine heart and identified a new upstream translation initiation site that yields 68 previously unidentified amino acids on the N terminus of the protein. This XL region is present in calpastatin in vivo and is a substrate for phosphorylation by PKA. We have also cloned the 5′-region of the calpastatin gene and defined an active promoter region, the transcriptional activity of which is enhanced by dibutyryl cAMP, a constitutive activator of PKA. Thus, both the calpastatin gene promoter and the XL region of the protein are targets of PKA activity.

A wide range of molecular weights have been reported for calpastatin isolated from cells and tissues, with estimates ranging from 17 to 172 kDa (1). Some of this variability may be due to protein degradation, while some is likely to result from alternative splicing or different translation initiation sites (7–9, 11, 30). Autolysis may represent an additional mechanism for regulating calpain activity (31–33), and proteolytic processing may also be involved in regulating calpastatin activity (34). Further confusion has arisen from the widespread observation that calpastatin runs anomalously on SDS-PAGE (10, 14, 25). The largest reported calpastatin protein, isolated from bovine heart muscle, has an apparent molecular mass of 145 kDa (35). A calpastatin protein of similar apparent molecular mass has also been reported in skeletal muscle (36).

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 (9). Bovine calpastatin cDNAs do not contain a stop codon at this site. 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 (9, 10, 27). Third, the downstream AUG is a poor potential start site, whereas the upstream AUG shows excellent consensus with other translation initiation sites (37). Fourth, 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 molecular mass 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 108 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. In bovine, Western blots of whole liver extracts detected calpastatin bands of 145 and 135 kDa (Fig. 3C). 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 phosphorylates sites within the XL region, which are present in full-length (145-kDa) calpastatin but are 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.

**PKA Responsiveness of the Calpastatin Gene Promoter**—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 to both stimulate and inhibit gene expression. Deletion to nt −102, which eliminates a putative CAAT box, almost completely abolishes promoter activity.

The finding that dibutyryl cAMP increases calpastatin promoter activity between 5- and 20-fold demonstrates that transcription of the calpastatin gene can be up-regulated 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 (18). 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) (38). Variations of the canonical CRE have also been reported, including CRE half-sites that can bind proteins such as CREB, ATF-2, and Jun, albeit at reduced affinity compared with the intact palindromic CRE (for a review see Ref. 39). pCIS-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, one (GGTCA) at nt −76 just downstream of an Sp1 site, and a second (TGAC) located at nt −20. Detailed deletion and mutation studies are presently under way to determine whether these or other non-CRE cis elements confer cAMP responsiveness to the calpastatin promoter.

Taken together, our results indicate that activation of a cAMP-dependent signaling pathway increases both calpastatin gene transcription and calpastatin protein phosphorylation. Modulating protein activity through changes in gene transcription, however, represents a relatively slow regulatory mechanism; one possibility is that newly synthesized calpastatin is phosphorylated in the XL region, rendering it inactive pending a subsequent regulatory step. Calpastatin is phosphorylated in vitro (30, 40) and also appears highly susceptible to proteolysis, which has been shown to cleave the L domain (and therefore also the XL region) from domains 1–4. Since this aminoterminal region is a target of PKA phosphorylation, its removal from the calpain inhibitory domains may serve a regulatory function.

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