THE INVOLVEMENT OF CALPASTATIN IN MYOBLAST FUSION

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Myoblast fusion is a dynamic event that can be recreated *in vitro* using tissue culture. Portions of cDNA encoding inhibitory and non-inhibitory domains of ovine calpastatin were expressed in murine C2C12 myoblasts as fusion proteins with enhanced green fluorescent protein (EGFP). The ability of these myoblasts to fuse into myotubes, the dynamics of particular indicator proteins, and the subcellular localisation of fluorescence were assessed.

Formation of skeletal muscle is a multistage process characterised by the biochemical and morphological maturation of myoblasts (mononucleate) into myotubes (multinucleate). A key event in the transition from myoblast to myotube is fusion. Fusion is a dynamic event which can be induced and monitored by culturing muscle cells *in vitro*. An expanding body of evidence indicates that proteolytic activity from the ubiquitous calpains (μ- and m-) is obligatory for fusion and myotube development.

Calpastatin is the specific endogenous inhibitor of calpain. Domains 1, 2, 3 and 4 are homologous and possess inhibitory potential. The N-terminal domain L may play a role in regulating calpastatin action and / or directing the localisation of calpastatin to the plasma membrane. Proteolytic activity is prevalent around the plasma membrane during fusion. It was decided to investigate domain L's potential role in targeting calpastatin to this region.

The polymerase chain reaction (PCR) was used to amplify regions of ovine calpastatin cDNA corresponding to domains L, 1 and L1. The PCR reaction enabled the introduction of restriction enzyme sites to permit in-frame ligation of the

amplimers into a prokaryotic expression vector. Using *E.coli*, domains L and 1 of ovine calpastatin were expressed separately and together, in combination with glutathione *S*-transferase (GST). The GST tag enabled purification of the expressed product which was then characterised in terms of heat stability, molecular weight and activity. The proteins behaved normally in respect to their ability to inhibit calpain, and were used as antigens for the successful production of polyclonal anti-calpastatin antibodies.

C2C12 myoblasts were cultured under proliferation conditions before being induced to fuse. The rate and extent of fusion in control cells was assessed by microscopic examination and by fluorescent automated cell sorting (FACS). FACS analysis was unable to accurately distinguish myotubes and significantly underestimated the percentage fusion at all times. Three proteins (myosin, desmin and troponin T) were reported to be indicators of biochemical differentiation while desmin and troponin T are also potential substrates for calpain. Levels of these proteins and patterns of migration were determined using Western analysis. While the levels of all three proteins reflected biochemical differentiation, only troponin T Westerns exhibited truncation products. It could not be concluded if this truncation was associated with calpain activity. Levels and fragmentation patterns of μ-calpain, m-calpain and calpastatin were also assessed. Both proteases were detected in C2C12 extract in an active form. Despite one of the polyclonal anti-calpastatin antibodies demonstrating immunoreactivity against native murine calpastatin, no endogenous calpastatin was detected in C2C12 extract.

The polymerase chain reaction (PCR) approach used to produce prokaryotic expression vectors was also used to produce eukaryotic expression vectors. Amplimers of domains L, 1 and L1 were ligated into a mammalian expression vector containing EGFP cDNA. Conditions for transfection of C2C12 myoblasts were optimised using a control EGFP expressing plasmid. Cells were transfected with one of four expression plasmids (three experimental plasmids and one control), encouraged to proliferate and induced to fuse. There were no differences in terms of fusion rate or extent attributable to the expression of calpastatin. FACS analysis did not reveal any qualitative differences in terms of fluorescence between treatments.

Western blot analysis of myosin, desmin, troponin T, μ-calpain and m-calpain did not detect any qualitative or quantitative differences. Western analysis of the expressed calpastatin products revealed complex banding patterns which may reflect structural instability and / or proteolytic susceptibility. A schematic model is proposed which seeks to depict the truncation points.

Finally, fluorescent microscopy and confocal microscopy were used to localise the fluorescent component of the recombinant calpastatin – EGFP hybrids. Examination of transfected cells indicated that the active component of the expressed calpastatin was localised, together with EGPF, within the nucleus. In contrast, domain L did not influence the subcellular localisation of EGFP, although this may be explained by the probable separation of domain L from EGFP suggested in the schematic model.

KEYWORDS: myoblast fusion, C2C12, proteolysis, calpain, calpastatin domains, recombinant protein, glutathione *S*-transferase (GST), enhanced green fluorescent protein (EGFP), subcellular localisation, fluorescence, fluorescent automated cell sorting (FACS).

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Chapter 1

Review of Literature

1.1 Muscle and myoblast fusion

Processes associated with muscle development have attracted considerable interest in recent years. One reason for this is that muscle represents a good example of specialised tissue differentiation and growth. Mature skeletal muscle cells are permanently multinuclear and non-mitotic, and form the foundation of the muscle fibre. These fibres are composed of many multinucleate myotubes which are formed during embryogenesis by the fusion of mononucleate myoblasts (see Figure 1.1).

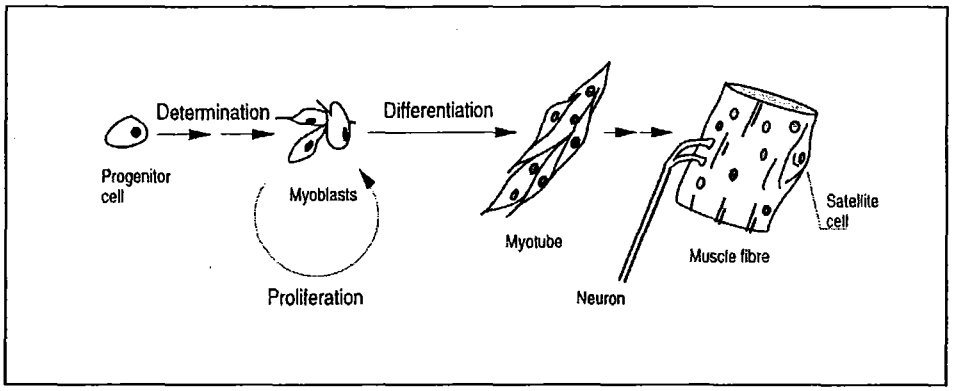

Figure 1.1 Schematic diagram showing the general development of skeletal muscle. Precursor cells (myoblasts) arise from pluripotent stem cells (progenitor cells). Myoblasts proliferate until they reach a stage at which cell division ceases. Fusion is initiated and concurrently muscle specific proteins are produced (differentiation). Following the formation of multinucleate myotubes, further maturation occurs in the form of innervation, and relocation of nuclei to the tissue periphery (adapted from Huynen 1994).

The process of myoblast fusion within a primary cell culture was first witnessed in the late 1950's (Holtzer *et al.*, 1958). Since this time several myoblast cell lines have become available exhibiting continuous multiplication while retaining the capacity to differentiate and fuse (Yaffe, 1968). These cultures are invaluable tools

for examining myogenesis, and have given rise to several excellent reviews describing aspects of this process (Wakelam 1985; Olsen 1992; Stockdale 1994).

1.1.1 Embryonic development of muscle

Muscle is derived from the lateral plate and paraxial somitic mesoderm during embryonic development (Goldspink 1974). The first discernible step in myogenesis is the accumulation of mitotically active cells at sites destined to later become muscle. These myogenic progenitor cells enter a phase of determination, become spindle shaped and proliferate. Mammalian muscles are variable with respect to their position, size, shape and contractile properties. Consequently it was recognised that myoblasts do not represent an homogeneous population, but rather one that is composed of distinct myogenic sub-lineages (Donoghue & Sanes 1994). Proliferation continues during embryogenesis up until a point at which myoblasts withdraw from the cell cycle and differentiate both biochemically and morphologically. The trigger for this transition is unknown.

1.1.2 Biochemical differentiation

The biochemical differentiation of a myogenic progenitor into a mature myoblast is characterised by the cell exiting the cell cycle, the expression of lineage determination genes, and the appearance of typical muscle proteins such as myosin and actin (Okazaki & Holtzer 1966). The differential expression of lineage determination genes is crucial for committing cells to a particular myogenic path (Olsen 1992). Members of the myogenic transcription factor gene family are important agents in this transmutation. This family includes MyoD1, myogenin, MRF4 and *myf-5* genes, all of which produce basic helix-loop-helix master regulatory nuclear proteins (Buckingham 1992). These genes appear to be pivotal in myogenic determination and their expression can even force cells of a different parentage into becoming myoblasts (Filvaroff & Derynck 1996). The complex interaction between genes and gene products makes the muscle development cascade an auto- and cross-regulatory network where the activity of one member may influence its own activity

or the activity of others (Li *et al.*, 1992; Rudnicki *et al.*, 1992; Weintraub 1993; Kaushal *et al.*, 1994).

1.1.3 Morphological differentiation

A series of events characterise morphological differentiation. After withdrawing from the cell cycle, recognition takes place, cell-cell contact is established and adhesion occurs. The initial recognition step assists fusion between myoblasts, and precludes fusion between dissimilar cell types (Wakelam 1985). After cell-cell contact myoblasts elongate and align with each other, leading to parallel alignment along the long axis of the myoblast. Both recognition and alignment are likely to involve muscle specific proteins, which may only appear at a specific stage of biochemical differentiation. Adhesion was recognised in experiments where myoblasts were cultured under fusion permissive conditions and progressively became undissociable before they were recognisably multinucleate (Wakelam 1985).

1.1.3.1 Myoblast Fusion

Fusion between two myoblasts or a myoblast and a myotube takes place after alignment. It is now generally accepted that fusion results from a series of distinct events. Doberstein *et al.* (1997) produced an excellent article which describes the morphological changes together with an analysis of gene expression. Although their work utilises the fruit fly (*Drosophila melanogaster*) as a model system, the process of myogenesis in this species shares many characteristics with myoblast fusion in vertebrates. Initially, in prefusion myoblasts, an intense concentration of vesicles appear touching the cytoplasmic face of the juxtaposed plasma membranes. The vesicles pair up across the membranes and an electron-dense material, associated with the plasma membrane and accumulating in the extracellular space, forms between paired vesicles. This morphology may cover $1\mu m^2$ of the cell surface and is named a "prefusion complex". Other characteristic features include stretches of electron-dense material ≈500nm long which form along regions of membrane breakdown. These have also been reported in vertebrate myoblasts where they are called membrane plaques. Although they are rare relative to prefusion complexes, the

authors suggest they result from vesicle pairing. Cytoplasmic continuity is established through multiple pores of local fusion between opposed plasma membranes. Membrane vesiculates along the zone of contact and forms sacs enclosing what was formerly extracellular space. These vesicles become progressively rounder as the membrane breaks up and are later recycled in a manner not clearly understood. These observations provide the foundation for the model of myoblast fusion shown in Figure 1.2 (Doberstein *et al.*, 1997).

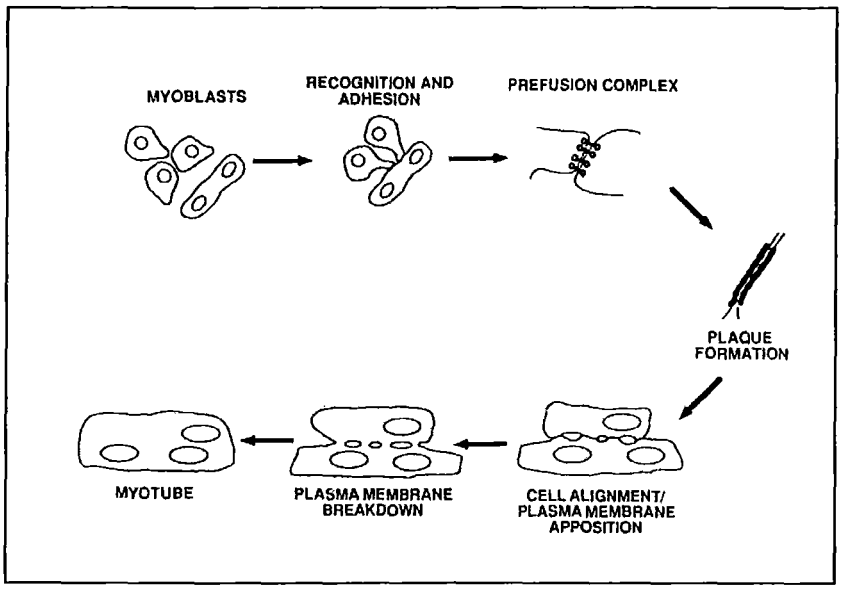

Figure 1.2 Diagram showing significant morphological changes occurring at the ultrastructural level during the fusion process (Doberstein *et al*,.1997).

Myoblast fusion is specifically calcium dependent and other divalent cations cannot substitute for calcium, although they may play a supporting role (Petrie *et al.*, 1996). The *in vitro* optimal calcium concentration for fusion is 1.4mM. Reducing this to 0.3mM inhibits fusion. Proliferation and alignment seem independent of calcium concentration.

Wakelam (1985) has reviewed the research characterising myoblast surface biochemistry during fusion. Generally, there are no obvious changes in lipid composition of the plasma membrane correlating with transitions and phases in myoblast fusion. However, membrane fluidity appeared to be a critical factor in

fusion competence which stimulated interest in surface protein changes. Research has focused upon aspects of protein synthesis, accumulation, modification and degradation, and there is considerable information describing the fluctuations in specific surface proteins. Electrophoresis of surface proteins shows a general trend towards a decline in high molecular weight proteins and a greater amount of low molecular weight proteins, possibly indicating proteolytic involvement.

1.2 The Calpain Protease

Thirty-five years ago, Gurhoff (1964) reported the existence of a calcium-dependent soluble protease that would later be named "calpain" (Murachi 1989). Calcium-dependent proteolytic activity has since been detected in many different species and tissues, and several distinct members have been added to the family. Despite their ubiquitous nature, a clear physiological function for these enzymes remains elusive. However, substantial research has been carried out and a number of review papers have highlighted significant advances (Suzuki *et al.*, 1987; Croall & Demartino 1991; Melloni & Pontremoli 1991; Saido *et al.*, 1994b; Johnson & Guttmann 1997; Sorimachi *et al.*, 1997).

1.2.1 Calpain Structure and Function

Two ubiquitous calpains were the first members of this family to be described in detail. These are known as μ-calpain (or calpain I, calcium-dependent neutral protease (CANP-1)) and m-calpain (or calpain II, (CANP-2)). The distinction between them is based upon either a μM or mM calcium requirement for half-maximal activity *in vitro* (Mellgren 1980; Dayton *et al.*, 1981). Both proteins have been purified, and characterised as cysteine proteases.

The native enzymes are found as heterodimers with subunit molecular weights of 80 kDa and 30 kDa (Suzuki 1990). Peptide mapping, cDNA sequence analysis and immunological techniques demonstrate that the small subunit originates from a single gene, while μ- and m-calpain large subunits arise from two separate

genes. Considerable cDNA sequence homology exists between μ- and m-calpain, with particularly high homology found in the proteolytic region. Comparing the peptide sequences from different species also reveals significant homology, indicating evolutionary conservation. The large subunit of μ- and m-calpain comprises four domains, while the smaller subunit is partitioned into two domains (see Figure 1.3).

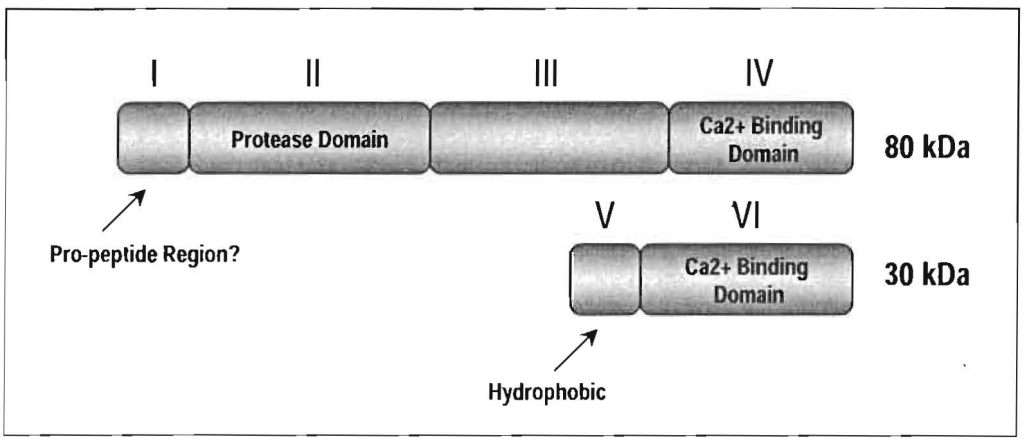

Figure 1.3 Schematic diagram showing the domain structure of the ubiquitous μ- and m-calpain.

The role of each domain may be suggested by comparing it to other defined proteins. Domain II includes the active site, while domain IV contains EF - hand structures defining it as a calcium-binding domain. Domains I and III remain functionally nondescript, although autoproteolytic modification of the N-terminus of domain I during protease activation may hint at a regulatory role. With respect to the small subunit, two domains (V and VI) can be structurally defined. Domain V contains a predominance of hydrophobic residues, which may relate to the protease associating with the plasma membrane. Similarity between domains IV and VI, establish that the latter also functions as a calcium-binding domain.

It is unclear if the two subunits remain associated during *in vivo* proteolysis. Co-immunoprecipitation studies have shown the subunits remain associated during substrate proteolysis (Zhang & Mellgren 1996), and reports of bacterially expressed large and small subunits show that formation of a heterodimer is obligatory for activity (Graham-Siegenthaler *et al.*, 1994; Elce *et al.,* 1995; Simon *et al.*, 1995).

However, Yoshizawa *et al.,* (1995a) reported that the calpain subunits dissociate in the presence of calcium, and that this event is essential for enzymatic activity. Moreover, Meyer *et al.* (1996) expressed the large subunit of human μ-calpain in insect cells and were able to detect high levels of proteolytic activity. It is possible that the subunit could associate with naturally expressed small subunit of μ-calpain. This protein subunit has been detected in Drosophila (Pinter *et al.*, 1992), but not in bacteria. Furthermore, Yoshizawa *et al.* (1995b) demonstrated that under optimal refolding conditions the re-natured large subunit had full enzyme activity *in vitro*, even in the absence of the small subunit. With respect to Meyer's work (Meyer *et al.*, 1996) it should be noted that expression of active large subunit calpain has not been reproduced in other laboratories despite concerted efforts (Goll; personal communication 2001).

1.2.2 Catalytic properties of calpain

The calpain active site contains cysteine and histidine residues characteristic of cysteine proteases, and a conserved asparagine residue appears crucial in stabilising this pairing (Simon *et al.*, 1995). Calpains are inhibited by agents such leupeptin and E-64, which also inhibit other sulfhydryl proteases, and chelating agents such as EDTA and EGTA. The most obvious catalytic difference between the two ubiquitous enzymes is their dissimilar demand for calcium. At micromolar calcium concentrations (5 – 50 μM) μ-calpain reaches half-maximal activity, which contrasts with the millimolar calcium demands of m-calpain to attain the same degree of activity. The wide range of reported calcium requirements may be due to variations in experimental conditions, especially the ionic strength of *in vitro* preparations (Elce *et al.*, 1997). These requirements are intriguing because under normal conditions *in vivo* calcium concentrations range between 100 and 200 nM with transient increases up to 500 - 800 nM occurring at certain stages of cell division (Poenie *et al.*, 1985; 1986; Bitar *et al.*, 1986), and with certain localised concentrations of calcium reaching 30 μM (Davies & Hallett 1998). The discrepancy between prevailing calcium concentrations and calcium concentrations required for calpain activity maybe associated with autolysis of both subunits. Specifically, while it is not uncommon for proteolytic enzymes to autolyse, autolysis of calpain reduces the calcium

concentration required for half-maximal activity (Suzuki *et al.*, 1981). This is one mechanism through which calpains may reduce prohibitive calcium demands.

1.2.3 Autoproteolytic modification of calpain

Autolytic conversion of calpain entails the removal of N-terminal portions from both subunits. It is unclear, however, whether it is autolysis of the large or the small subunit that is responsible for increased calcium sensitivity and subsequent proteolysis, or indeed whether autolysis is obligatory for proteolysis. Kinetic studies show that autolysis of μ- and m-calpain subunits occur at different rates (Saido *et al.*, 1994a). *In vitro*, the large subunit of μ-calpain degrades rapidly and is swiftly followed by small subunit degradation (Saido *et al.*, 1992a), whereas autolysis of m-calpains large subunit takes place more slowly than that of the small.

Autolysis of μ-calpain large subunit is a two stage process producing 78 kDa and 76 kDa products which result from the initial cleavage of 14 amino acids followed by a further 12 amino acids (Inomata *et al.*, 1988; Zimmerman & Schlaepfer 1991). Autolysis of m-calpains large subunit is also a two-stage process involving an initial loss of 9 amino acid residues, followed by the loss of a further 11 (Brown & Crawford 1993). The slightly smaller truncation of m-calpain produces isoforms that are almost indiscernible by SDS-PAGE. Extensive autolysis of large subunits from both μ- and m-calpain generates fragments ranging between 21 and 42 kDa (Nishimura & Goll 1991; Crawford *et al.*, 1993). Autolysis of the small subunit involves the complete removal of domain V and proceeds through 27 kDa and 23 kDa intermediates before resulting in an 18 kDa product (McClelland *et al.*, 1989; Hathaway & McClelland 1990).

Brown & Crawford (1993) observed that increased calcium sensitivity of m-calpain correlated with the initial autolysis (9 residues) of the large subunit but not with truncation of the small subunit. Results from Saido *et al.*, (1994a) contradict this. By employing antibodies specific to either 8 or 18 N-terminal residues of m-calpains large subunit, these authors concluded that removal of these regions is not obligatory

for increased calcium sensitivity. In fact, truncation of the small subunit was the only detectable structural change associated with proteolytic activity. Recently, Elce *et al.* (1997) mutated recombinant rat m-calpain and established that removal of amino-terminal fragments from the large subunit (and not small subunit autolysis) was responsible for enzyme sensitisation. These workers also observed significant effects of ionic strength and pH on *in vitro* enzyme activity and suggest that inconsistencies in the literature result from variations in experimental conditions. It has been suggested that the increased sensitivity displayed by autolysed calpain is a consequence of removal of a region blocking the active site of the proteins. However recent work by Hosfield *et al.*, (1999) has determined from crystallization studies that the first 20 – 28 amino acids are located some distance from the active site and their removal is not critical for "unblocking" the active site. Moreover, Crawford *et al.* (1993) reported that E64 could bind to the active site of unautolysed calpain, indicating this site is functional prior to autolysis. While not dependent upon autolysis, this binding does require calcium (Thompson *et al.*, 1990).

While there is little doubt that autolysis increases the sensitivity to calcium, does calpain exist as an inactive pro-enzyme requiring autolysis for activation, or is it active in its native form and simply becomes more sensitive to calcium following truncation? The almost synchronous occurrence of autolysis and proteolysis has led to the hypothesis that autolytic conversion of a calpain pro-enzyme is the likely mechanism of activation. Saido *et al.* (1994a) reported that autolysis of both μ-calpain subunits preceded casein proteolysis, as did autolysis of m-calpains small subunit. Significantly autolysis of the large subunit of m-calpain lagged behind casein hydrolysis suggesting that truncation of the catalytic subunit is not required for activity. As noted, however, large subunit truncation of m-calpain can be difficult to detect by SDS-PAGE. In order to detect μ-calpain activity, microtubule-associated protein 2 (MAP2), was used as a highly sensitive calpain substrate (Baki *et al.*, 1996). The 76-kDa isoform of μ-calpain was generated in a linear manner in concert with the hydrolysis of MAP2, passing through origin. The hydrolytic profile of MAP2 exhibited a lag phase when incubated with native μ-calpain, but not when incubated with the autolysed enzyme. Thus, the autolytic removal of an N-terminal fragment, possibly including an auto-inhibitory region, appears obligatory for proteolysis.

This suggestion is challenged by other work which showed significant proteolytic activity of μ-calpain at calcium concentrations (40 – 50 μM) which were insufficient to simulate autolysis (Cong *et al.*, 1989). Moreover, a monoclonal antibody specific for the calcium binding domain of the small subunit was found to increase the calcium demand for m-calpain autolysis without affecting the requirements for proteolysis (Cong *et al.*, 1993). The observation that the calcium concentrations required for μ- and m-calpain autolysis are, or can be made, greater then those required for proteolytic action, strongly indicates that the protein does not exist as an inactive proenzyme. The lag phase which precedes substrate proteolysis by μ-calpain has been thought to indicate an autolytic "activation" step. However, there is an alternative interpretation (Crawford *et al.*, 1993). Unautolysed μ-calpain has a lower affinity for calcium and maybe the predominant form of the protease at the start of experiments exploring the temporal relationship between substrate proteolysis and autolysis. As a consequence, the rate of proteolysis will appear low initially compared to later stages when autolysis has generated truncated calpain with its greater affinity for calcium. More recently, Elce *et al.* (1997) expressed full length recombinant rat m-calpain large subunit containing a mutation which prohibited N-terminal autolysis but found the mutated calpain was still capable of normal activity. One likely effect of μ- and m-calpain truncation is a decrease in enzyme stability (Thompson *et al.*, 1990; Elce *et al.*, 1997), which may represent a self-regulation stratagem.

Prolonged autolysis of purified bovine μ- and m-calpain generates several fragments (Nishimura & Goll 1991; Crawford *et al.*, 1993). The largest contains all domain II (catalytic domain) and a portion of domain I, but fails to exhibit proteolytic activity. Two intermediate fragments, between 20 kDa and 23 kDa, embrace domain IV and a portion of domain III. The smallest fragment (18-kDa) contains the calmodulin-like domain of the small subunit (domain VI). Each intermediate fragment can non-covalently associate with the 18 kDa fragment (Crawford *et al.*, 1993). A similar profile of fragments is seen when chicken calpain is allowed to autolyse to completion. Chicken calpain produces a large fragment (54-

kDa) containing all of domain II, most of domain III and a portion of domain I, and degrades to a smaller fragment (37-kDa) with the loss of a portion of domain III. Finally, two smaller fragments are produced, a 32-kDa fragment containing domain IV and some of domain III, and an 18-kDa fragment identical to that from bovine calpains. Again, the fragments containing domains IV and VI associate non-covalently. This probably reflects the manner in which the large and small subunits bind in the native enzyme, and may or may not require the full domain sequences.

Studies investigating calpain autolysis usually employ mM calcium concentrations to stimulate an autolytic response. Because supraphysiological concentrations of calcium appear to be required for activation of the calpain system, scientists are investigating other *in vivo* calpain activation theories.

1.2.4 Calpain activation theories

Some interest has focused on the fact that μ- and m-calpain undergo autolysis, producing isoforms with diminished calcium requirements. In 1987, this observation was interpreted by two groups to represent a possible pro-enzyme activation mechanism (Mellgren 1987; Suzuki *et al.,* 1987). More recently, Tompa *et al.* (1996) has suggested a mechanism whereby μ-calpain, in the presence of μM calcium, catalyses limited proteolysis of m-calpain alleviating its extreme calcium demands. He proposes that this heterolytic, intermolecular reaction represents a "calpain cascade" causing activation of μ- and m-calpain. However, a recent paper by Thompson *et al.* (2000) refutes this hypothesis by demonstrating that after incubation of μ-calpain with m-calpain at a 1 : 50 ratio (in the presence of sufficient calcium to activate μ-calpain) m-calpain remained intact and its calcium requirements for activity remained unchanged. The ratio of m-calpain to μ-calpain is probably a significant contributor to this effect, since Tompa *et al.* (1996) successfully demonstrated proteolysis of m-calpain by μ-calpain by employing a higher ratio of 1 : 5 (μ-calpain : m-calpain) in their experiments. While autolysed μ-calpain demonstrates half-maximal activity at a physiological calcium concentrations (Edmunds *et al.*, 1991), autolysed m-calpain still demands calcium concentrations in excess of physiological

levels despite a 20-50 fold increase in its sensitivity. Moreover, autolytic fragments of the calpains are not observed in cell extracts (Edmunds *et al.,* 1991; Saido *et al.,* 1992a) and both μ- and m-calpain have demonstrated relatively long half lives *in vivo* (Zhang *et al.,* 1996). In strict molecular terms calpain must be active for proteolysis to take place and, coupled with the fact that not all available evidence supports the pro-enzyme theory, autolysis remains an unsubstantiated mechanism of activation.

In vitro, calpains show weak calcium-independent hydrophobic associations, but strongly hydrophobic regions are exposed following calcium binding (Gopalakrishna & Barskey 1985). The hydrophobic nature of the small subunits N-terminus has given rise to the membrane activation theory (Suzuki *et al.,* 1987; Mellgren 1987). This proposes that calpains activate after associating with plasma membrane phospholipids in a calcium dependent manner (Thompson *et al.,* 1990). Specifically, polyphosphoinositides have been shown to promote μ-calpain activity (Saido *et al.,* 1992b). After association take place, calpain undergoes autolysis (Pontremoli *et al.,* 1985a; 1985b) and is released into the cytosol. This membrane activation theory remains incompletely tested *in vivo,* although in crude tissue preparations containing calcium, calpain has been shown to associate with subcellular organelles in a hydrophobic manner (Gopalakrishna & Barskey 1986), and calcium dependent binding to erythrocyte plasma membrane (Pontremoli *et al.,* 1985a; Molinari *et al.,* 1994) and to insoluble fraction of rat lenses (Lampi *et al.,* 1992) has also been observed. Additional support for the theory is provided by Michetti *et al.* (1996) who found that erythrocyte calpain released from the membrane following autolysis. Interestingly, association appeared to be mediated through the large subunit and it may be that membrane affinity requires regions in both subunits (Melloni *et al.,* 1992).

There is, however, significant doubt concerning the validity of the membrane activation theory. Several findings fail to support this theory (Goll *et al.,* 1989; Goll *et al.,* 1992). Goll suggests that the simplest mechanism for activation is the involvement of an "activator". This may be a kinase or phosphatase, whose activity is dependent on calcium fluxes, which phosphorylate or dephosphorlyate calpain. This modification to calpain increases the calpain binding affinity for calcium.

Alternatively, a calmodulin-like protein may bind calcium, deliver it to calpain, and have the additional effect of reducing the calcium demands of calpain. Researchers in Italy have also proposed the existence of an activator protein which binds calpain with a high specificity (Pontremoli *et al.,* 1990b; Melloni *et al.,* 1992; Melloni *et al.,* 2000b). In erythrocytes, the protein (40 kDa) is associated with the plasma membrane, possibly through another membrane bound protein, where it promotes autolysis of calpain (Salamino *et al.*, 1993). An analogous activator has been purified from rat skeletal muscle and shows specificity for m-calpain (Melloni *et al.*, 1992). This 35 – 45 kDa protein is associated with the cytoskeleton and after autoproteolytic conversion of the bound m-calpain, the active calpain enzyme is freed into the cytoplasm. In a recent paper, the same researchers isolated a protein from rat skeletal muscle called acyl-CoA-binding protein (Melloni *et al.*, 2000a). This 20 kDa protein reduces the calcium concentration required for optimum m-calpain activity from 300 μM to 10 μM. This result, coupled to the discovery of a 30 kDa protein in bovine brain tissue which specifically decreases the calcium requirement for μ-calpain and accelerates autolysis (Melloni *et al.*, 1998b; Melloni *et al.,* 1998c), may represent an enzyme specific regulatory system.

All these observations underscore the elusive nature of the activation mechanism(s) of calpains. It is not surprising to find that attempts to reconcile the evidence have produced different models.

1.2.5 Calpain Substrates

As the ubiquitous nature of the calpains became apparent, the nature of the endogenous substrates was actively investigated. Many cellular proteins have been reported as being susceptible to calpain hydrolysis (Wang *et al.*, 1989; Carillo *et al.*, 1994). They can be grouped into three broad categories; enzymes, cytoskeletal / membrane proteins, and receptor proteins. Interestingly, the relatively large proportion of membrane associated substrates is consistent with the calpain activation proposals. There are many synthetic peptides which are susceptible to calpains but will not be discussed here.

Calpains display strict substrate specificity with few differences existing between the specificity of the two calpain isoforms and the fragment patterns they generate (Sasaki *et al.*, 1984; Croall *et al.*, 1996). It has been reasoned that if calpain recognises a specific residue pattern or peptide bond then this would be present at the region removed through autoproteolysis. This was investigated and a general calpain cleavage sequence was suggested (McClelland *et al.* 1989). The P_2 position is typically hydrophobic (eg. leucine or isoleucine) the P_1 is a non-specific residue, P_1' is a neutral residue and P_2' residue is either hydrophobic or neutral. These conclusions are comparable to those of Sasaki *et al.* (1984) who suggested a preference for residues such as tyrosine, methionine and arginine at the P_1 position, preceded by leucine or valine at P_2. These guidelines are often transcended although inconsistencies may be due to calpain recognising higher order 2° and 3° structures such as hydrophobic-hydrophilic boundaries rather than linear structures (Wang *et al.*, 1989; Stabach *et al.*, 1997). If proteolysis is dependent upon the gross structure of substrates then this could explain why proteolysis often results in relatively large protein fragments which contain sequences that are not subject to cleavage.

One potential higher order candidate structure is the calmodulin-binding domain which is manifest in several calpain substrates. Calmodulin is a calcium-binding protein that interacts with other proteins regulating their physiological processes. It has been reported that the calmodulin-like domains within calpain interacts with calmodulin-binding proteins (Molinari *et al.*, 1995b) which then become susceptible to calpain proteolysis. However, calmodulin-binding proteins lacking the binding domain are still susceptible to proteolysis (Croall *et al.*, 1996). Thus an alternative mechanism for substrate recognition must exist. Rogers *et al.* (1986) were the first to recognise that proteins containing sequences rich in proline (P), glutamine (E), serine (S) and threonine (T) residues were relatively short lived. PEST sequences have the potential to sequester Ca^{2+} ions and may consequently enable calpain to overcome its calcium restrictions. An examination of calmodulin-binding proteins has revealed several strong PEST sequences (Wang *et al.*, 1989). It has been suggested that calpain is primarily driven to proteolysis by recognising PEST sequences and that the association of the calmodulin - binding and calmodulin - like domains enhance the substrate/protease coalition which gives the appearance

of a functional requirement for these regions. While some substrates display a positive correlation between PEST content and calpain susceptibility (Smith *et al.*, 1993; Noguchi *et al.*, 1997) several substrates do not obey the rule (Molinari *et al.*, 1995a; Carillo *et al.*, 1996). This prompted some investigators to suggest PEST sequences are not necessary for calpain proteolysis (Johnson & Guttmann 1997) and that the propensity for a substrate to be susceptible to calpain action is multifactorial.

Post-translational modification of substrates may influence the proteolytic potential of calpains. For example the phosphorylation of some connexin molecules protects them from calpain degradation and presumably, dephosphorylation has the opposite effect (Elvira *et al.*, 1994). The relationship between this modification and proteolysis is possibly more complex than this study implies. The phosphorylated state of the insulin receptor substrate-1 does not, for example, influence its susceptibility to calpain (Smith *et al.*, 1993). In contrast, phosphorylation of troponin I with protein kinase A confers protection whilst protein kinase C (PKC) phosphorylation appears to increase substrate susceptibility (Di Lisa *et al.*, 1995). With respect to the latter, these effects are only observed with μ-calpain and not m-calpains (Di Lisa *et al.*, 1995). In addition, kinases are themselves calpain substrates, and degradation of PKC to co-factor independent PKM is catalysed by both μ- and m-calpain. While m-calpain continues to degrade PKM making its appearance transitory, the kinase is unaffected by μ-calpain and is theoretically able to act on substrates inaccessible to its parent enzyme (Cressman *et al.*, 1995). These observations indicate the complex interrelationships which influence the functionality of calpain.

1.2.6 Other members of the calpain family

The large subunit of calpain was cloned for the first time in 1984 from chicken calpain (Ohno *et al.*, 1984). It is now recognised that this calpain isoform represents an intermediate type known as μ/m-calpain (Sorimachi *et al.*, 1995b). Since then, various calpain isoforms have been identified and their structures determined through cDNA cloning (Sorimachi *et al.*, 1997). The large subunit of m-calpain has been sequenced in humans (Imajoh *et al.*, 1988), and a novel large subunit

(nCL) isoform has been cloned from *C. elegans* (Barnes & Hodgkin 1996). In mammals the ubiquitous calpains (μ- and m-) have been joined by tissue specific isoforms. This distinction now forms the basis of calpain classification (Sorimachi *et al.*, 1997).

The tissue specific division includes the skeletal muscle specific p94, also known as calpain 3 and nCL-1 (Kinbara *et al.*, 1998b), the stomach specific isoforms known as nCL-2 and nCL-2' (Sorimachi *et al.*, 1993a), and the new rodent lens specific isoforms Lp82 (Ma *et al.*, 1998; Ma *et al.*, 1999) and Lp85 (Ma *et al.*, 2000). p94 is a monomeric protein whose structure is similar to that of the large subunit of μ- and m-calpain except for the inclusion of 3 short sequences (Sorimachi *et al.*, 1993b). The mRNA levels of p94 exceed those of the ubiquitous calpains, and while the protein is relatively stable in skeletal muscle (Spencer *et al.*, 1997) it undergoes rapid autolysis following extraction (Sorimachi *et al.*, 1993c; Kinbara *et al.*, 1998a). nCL-2 is homologous to other large subunits containing a cysteine protease domain and calcium binding domain, while nCL-2' is composed of the protease domain only and lacks the calcium binding domain (Sorimachi *et al.*, 1993a). Lens protein 82 (Lp82) represents a splice variant of p94 and is the most abundant calpain isoform in young mouse and rat lens (Ma *et al.*, 1998; Ma *et al*, 1999). Lp82 demonstrates a reduced sensitivity to calpastatin inhibition (Nakamura *et al.*, 1999) and, in alliance with Lp85, may form a system involved in lens specific proteolysis. Other atypical homologues of calpain have been reported in mammals, insects, nematodes, fungi and yeast (Sorimachi *et al.*, 1997).

1.2.7 Functions of the calpains

The physiological function(s) of the members of the calpain family remains unclear although several suggestions have been forwarded. The ubiquitous nature of μ- and m-calpain has suggested they are involved in cell signalling. As already mentioned, protein kinase C maybe converted by calpain proteolysis to an active, cofactor-independent protein kinase M (Nishizuka 1985). In addition, however, hormonal polypeptides can induce PKC down-regulation by stimulating calpain to proteolyse this membrane associated signalling molecule (Eto *et al.*, 1995). A model

showing the possible role of calpains in signal transduction has been presented by Suzuki *et al.* (1987). Alternatively, Emori & Saigo (1994) reported that calpain localises in a manner suggesting an involvement with cytoskeletal remodelling, especially of actin structures, during Drosophila embryogenesis. A similar report was presented by Schollmeyer (1988) who suggested that m-calpain is involved in critical spindle disassembly during mitosis. The possibility that calpain is involved in cell cycle progression has led to an investigation of the proteolysis of nuclear proteins (Gilchrist *et al.*, 1994). Several nuclear proteins are substrates for calpain, and nuclear kinase activity can be solubilized by calpain (Mellgren 1991). In particular m-calpain has been shown to proteolyse nuclear proteins at concentrations of calcium normally required by μ-calpain, suggesting that m-calpain is sensitised to calcium in the presence of DNA (Mellgren *et al.*, 1993). These observations may be significant in light of a report that calpain regulates adipocyte differentiation, possibly through the degradation of a transcriptional activator gene product (Patel & Lane 1999). Differentiation of neurites also appears to be regulated by calpain (Pinter *et al.*, 1994) although this is probably mediated through structural protein turnover rather than changes in gene expression. A well-established function of calpains is their involvement in post-mortem myofibrillar protein degradation (Koohmaraie 1988) which has been expanded to include regulation of the turnover of the myofibrillar proteins *in vivo* (Huang & Forsberg 1998). Finally, calpain may be involved in apoptosis. Evidence suggests calpains participate in an escalation of proteolytic activity during the final stages of a cell's life cycle (Martin & Green 1995; Chan & Mattson 1999).

Calpains have been associated with several pathological conditions (Wang & Yuen 1994; Johnson & Guttmann 1997). An elevated level of m-calpain is a feature of cataracts, a condition that can be alleviated by administering the calpain inhibitor, E64 (Shearer *et al.*, 1991). Similarly, neurodegenerative symptoms during ischaemic myocardial infarction and cerebral ischaemia can be reduced through application of calpain inhibitors (Wang *et al.*, 1996). Dystrophic muscle from hamsters are characterised by elevated m-calpain levels and heightened activity compared to normal muscle (Johnson & Hammer 1988). The gene responsible for Limb-Girdle-Muscular-Dystrophy-type-2A (LGMD2A) has been mapped and found to be the

gene encoding for p94 (Sorimachi *et al.,* 1995a). Evidence also suggests an involvement of the calpains in Alzheimers Disease (Nixon *et al.,* 1995). Finally, damage to the bone joint extracellular matrix by externalised calpain may contribute to the inflammation seen in arthritis (Menard & El-Amine 1996).

In summary it is apparent that over-action of calpains is probably an important component of several metabolic disorders, but the cause of the perturbation is not always clear. It may be from elevated calpain levels or from a reduction in calpastatin, the naturally occurring endogenous inhibitor of calpains. While calpain inhibition by synthetic inhibitors is a promising pharmacological avenue for disease prevention (Wang & Yuen 1994), much of the understanding of calpain inhibition to date is based upon an understanding of the natural inhibitor.

1.3 Calpastatin

Calpastatin was first described and partially purified by Drummond & Duncan (1966) who noted it was an inhibitor of cardiac phosphorylase *b* kinase. Twelve years later two papers related its potential to inhibit calpain (Nishiura *et al.*, 1978; Waxman & Krebs 1978). Since then various papers have described primary and tertiary structures of calpastatin, its functional relationships, the mechanism of calpain inhibition, and its regulation. Initial reports of chromatographically isolated erythrocyte calpain suggested a molecular weight of 300 kDa (Murakami *et al.*, 1981). However, subsequent SDS-PAGE analysis revealed a variety of sizes (Parkes 1986). Several theories have explained the size discrepancies including the formation of calpastatin tetramers (Takano & Murachi 1982) and undefined post-translational modifications (Emori *et al.*, 1987; Imajoh *et al.*, 1987). Amino acid sequencing and cDNA analysis have revealed that calpastatin contains an unusually high proportion of charged amino acids (Takano *et al.*, 1988) which may result in anomalous electrophoretic migration. Western blotting has revealed two predominant isoforms by employing antibodies specific to the native monomeric proteins; a 110 - 120 kDa protein found in all tissues examined, and a 70 kDa form in erythrocytes. These two forms show a strong amino acid similarity and immunological cross reactivity. The 40 kDa difference is due to the absence of domains L and 1 in the erythrocyte isoform,

and are probably a result of specific proteolytic processing after translation (Lee *et al.*, 1992). Recently, Cong *et al.* (1998) reported that bovine heart calpastatin cDNA possesses a translation initiation codon (AUG) 204 nucleotides upstream of the previously designated start point. Translation from this point results in the addition of 68 amino acids to calpastatin creating a region termed "XL". An antibody raised against "XL" recognises a 145 kDa band, which is evidence for the upstream AUG being utilised, and the 145 kDa band representing full-length calpastatin *in vivo*. Geesink *et al.* (1998) purified heart and skeletal muscle calpastatin from several species. Western blotting demonstrated the presence of a higher molecular weight calpastatin isoform (145 kDa) in heart tissue only. In contrast, skeletal muscle appeared to contain the 125 kDa calpastatin isoform only. Some heart extracts contained the 125 kDa isoform. It remains to be established if the difference in size is caused by the inclusion of domain XL in the 145 kDa isoform.

Analysis of complementary DNA (cDNA) sequences has demonstrated that numerous calpastatin variants exist in tissues from different sources (Lee *et al.*, 1992; Servadio & Casari 1993; De Tullio *et al.*, 1998; Takano *et al.*, 2000). Many differences are attributable to exon skipping during transcription and may lead to proteins with different susceptibilities to post-translational modifications. Examination of cDNA's revealed the presence of four homologous domains of approximately 120 - 140 amino acids sharing roughly 30 % sequence homology (Takano *et al.*, 1986b, Emori *et al.*, 1987). These repetitive sequences were called domains 1-4 and in full size calpastatin are joined by an N-terminal leader region, of similar size, referred to as domain L. Domains 1 - 4 represent inhibitory domains. Each contain three conserved sequences named regions A, B and C.

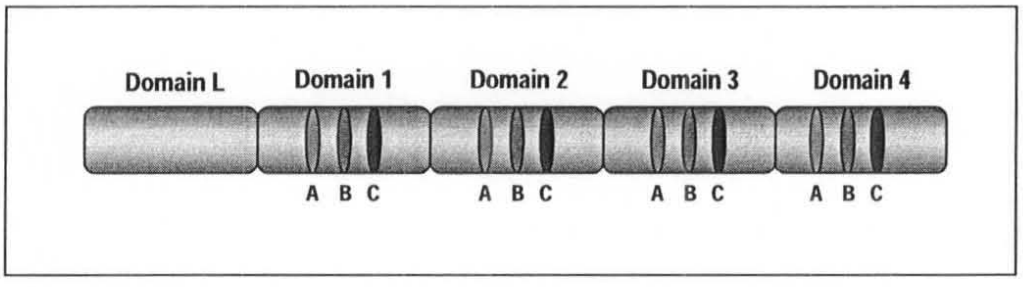

Figure 1.4 Schematic diagram of calpastatin. The typical 110 - 120 kDa calpastatin isoform is composed of four homologous inhibitory domains (1 - 4), and an N-terminal domain L. Within each inhibitory domain three regions (A,B,C) are conserved.

1.3.1 Structure and function relationships

In 1987, a 130 amino acid fragment of calpastatin was isolated and found to have inhibitory activity (Maki *et al.*, 1987a). Later studies on cell extracts from *E. coli* containing recombinant plasmids showed that each of the four domains possessed inhibitory activity against both μ- and m-calpain (Maki *et al.*, 1987b; Emori *et al.*, 1988). No inhibitory potential was observed when incubating recombinant domain L with calpain. These observations led Maki *et al.* (1987a) to conclude that the repetitive region is the functional unit of the protease inhibitor. The comparison of amino acid sequence of calpastatin with other known sequences revealed there was homology with the calcium-binding domain of calmodulin. However the characteristic EF hand structure was not found (Takano *et al.*, 1986b).

The physical arrangement of calpastatin's amino acid residues was determined and revealed that conserved regions A and C, at the N and C terminal ends of each domain respectively, were likely to be internalised and possessed a hydrophobic profile. Region B displayed a predominantly hydrophilic profile and probably presents itself on the surface of the folded protein where it is able to interact with calpain (Maki *et al.*, 1988). The A and C conserved regions form α-helical structures which may be important in stabilising the tertiary structure of the whole domain. The removal of regions A and C through restriction digestion or site-directed mutagenesis of cDNA generated pruned peptides with significantly lower inhibitory potential than whole inhibitory domains (Emori *et al.*, 1988; Maki *et al.*,1988). Further deletions towards the central region B caused inhibitory activities to fall below detectable levels. A detailed structure of the region B was presented by Maki *et al.* (1989). They synthesised a 27 amino acid peptide based on this region which displayed strong inhibitory potential. The oligopeptide embraced a 12 residue region known as the central consensus sequence (CCS). This region is common to all four inhibitory domains and is exposed on the surface of the calpastatin molecule where it can interact with calpain. More recently, several low molecular weight inhibitors have been shown to effectively inhibit calpain (Li *et al.*, 1996). While no function is clearly ascribed to the remaining bulk of the inhibitor there is evidence to suggest that some regions associate with calpain but not for the purpose of

inhibition. Kawasaki *et al.* (1993) investigated effects of calpastatin on the ability of calpain to bind to the plasma membrane and found that a region which was not associated with calpain inhibition, interacted with a non-catalytic region of calpain. The effect of this association was to prevent calpain from binding phospholipid but it did not reduce calpains activity.

1.3.2 Mechanism of calpain inhibition by calpastatin

The mechanism by which calpastatin inhibits calpain has attracted a lot of attention. It appears that calpastatin does not inhibit calpain by sequestering free calcium since increasing calcium concentrations do not reverse the inhibition (Nishiura *et al.*, 1978). However, calpastatin has an absolute requirement for calcium to bind calpain. The binding and inhibition can be reversed by calcium chelators (Melloni *et al.*, 1982). Calcium has the effect of changing the conformation of the calpain (Tsuji *et al.*, 1981) possibly by altering its structure into a form that allows calpastatin to bind. Following removal of calcium, the physical association between calpain and calpastatin is reversed. The central consensus sequence does not contain the preferred proteolytic site of calpain, which indicates that calpastatin does not operate as a suicide substrate despite the fact that it is substrate both *in vivo* and *in vitro* (Doumit & Koohmaraie 1999).

Kapprell & Goll (1989) reported that autolysed μ-, autolysed m- and unautolysed m-calpain require more calcium for half-maximal activity than was required for their half-maximal binding to calpastatin. These enzymes could not activate *in vivo* unless; 1) calpastatin is relocated and removed from calpain, 2) calpains are localised at a region where calcium concentrations are high enough to permit activity, 3) some factor regulating the interaction between calpain and calpastatin increases the calcium requirement for inhibition over activation, 4) the calcium requirement for calpain activation is lowered to enable activation. Unautolysed μ-calpain, however, requires less calcium for activation than is required for calpastatin binding. This suggests that under certain circumstances μ-calpain could be active in the presence of calpastatin.

Nishimura & Goll (1991) found that autolytic fragments of calpain, which contain the calmodulin-like domains but not the proteolytic domain, bind to a calpastatin affinity column in a calcium dependent manner. The fact that the proteolytic domain failed to bind to the column is difficult to reconcile since calpastatin is the competitive inhibitor of calpain. Experiments by Ma *et al.* (1993) established, however, that the binding of calpain to calpastatin is not correlated to its inhibition. This was determined by exploring the associations between various recombinant calpastatins and an immobilised recombinant calmodulin-like domain (CaMLD) engineered from μ-calpain. Ma showed that only calpastatin domains 1 and 4 could bind to immobilised CaMLD. After removal of region B (the inhibitory region), regions A and C of domain 1 associated with CaMLD, displayed no inhibitory potential and yet were susceptible to hydrolysis by calpain. In contrast, recombinant domain B failed to bind to the column but inhibited calpain. Interestingly, substituting region A from domain 1 into domain 3 enabled this domain to associate with CaMLD. Thus, it would appear that region A is critical for CaMLD association but not for inhibition. The latter function is fulfilled by region B. Furthermore, calpastatin binding to CaMLD's is not directly involved in calpain inhibition. Further work revealed that mutating conserved residues in regions A and C reduced the binding of calpastatin to the calpain calmodulin-like domain as well as the ability of calpastatin to inhibit calpain. This observation, coupled with the fact that region B is solely responsible for inhibition of calpain, lead Ma *et al.* (1994) to conclude that regions A and C potentiate the inhibitory activity of calpastatin by interacting with calpains calmodulin-like domain which allows region B to form a tighter complex and, as a consequence, exert its inhibitory influence. It would appear that the interaction of region A with the CaMLD of calpain is based upon the hydrophobic properties of the CaMLD which appear after exposure to calcium (Yang *et al.*, 1994). Calcium has been shown to lead to calmodulin developing a hydrophobic surface (Tanaka & Hidaka 1980). This may be the mechanism of interaction between calmodulin-binding peptides and calmodulin.

Calpastatin can be degraded by calpain after the two have associated in the presence of calcium (Mellgren *et al.*, 1986; Doumit & Koohmaraie 1999). This degradation requires a relatively high ratio of calpain to calpastatin. The degree of

calpastatin degradation increases with the addition of more calpain (Mellgren & Lane 1990). Furthermore, the degradation of calpastatin by calpain is a relatively slow process compared to the degradation of casein (Mellgren *et al.*, 1986). It is important to recognise that the degradation of calpastatin by calpain does not lead to a loss of inhibitory function and calpastatin fragments resulting from extensive proteolysis still retain inhibitory potential (DeMartino *et al.*, 1988; Nakamura *et al.*, 1989). This observation indicates that the inhibitory region is resistant to cleavage and/or that the structural arrangement of calpain and calpastatin precludes its degradation. However, a considerable body of evidence has described a gradual decline in detectable calpastatin activity during post-mortem storage of skeletal muscle (Koohmaraie *et al.*, 1987; Morton *et al.*, 1999).

1.3.3 Regulation of calpastatin

The mechanisms regulating calpastatin inhibitory activity are not completely understood. Protein and mRNA abundance and post-translational modifications are all points at which regulation may occur.

The existence of multiple calpastatin isoforms has been reported in rat skeletal muscle and brain tissue (Pontremoli *et al.*, 1991; Averna *et al.*, 1999) and human haematopoietic cell lines (Adachi *et al.*, 1991). In some instances the isoforms are different with respect to their domain composition. For example, the classical calpastatin is composed of one N-terminal domain (domain L) and four similar inhibitory domains (domains 1-4). However, in the instance of rat brain tissue, isoforms were discovered which comprised of only domain L and another of domains L and 1 only (Averna *et al.*,1999). In contrast, other isoforms are less easily distinguished. Pontremoli separated two forms of calpastatin on a DE32 chromatography column which displayed similar molecular weights. However calpastatin I, which eluted first, exhibited greater inhibitory potential over μ-calpain than m-calpain, whereas the less prevalent calpastatin II was more effective against m-calpain. The same laboratory later discovered that calpastatin I and II are reversibly dephosphorylated and phosphorylated (respectively) forms of the same calpastatin isoform (Pontremoli *et al.*, 1992). More recently, these researchers

identified calpastatins I and II in rat brain tissue and found that calpastatin I had inhibitory potential against μ- and m-calpain, while calpastatin II displayed an overall poor inhibitory potential (Salamino *et al.*, 1997). In this instance it is speculated that the calpastatin II isoform comprises a reservoir of low activity inhibitor whose behaviour is utilised under specific cell conditions. For example, conversion of the less active calpastatin II to calpastatin I followed by the elevation of intracellular calcium levels (Salamino *et al.*, 1994a). This possible mechanism of differential calpain regulation represents post-translational control.

It is not clear which cellular enzyme system is responsible for calpastatin phosphorylation. Both cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) have the ability to phosphorylate calpastatin (Mellgren & Carr 1983; Adachi *et al.*, 1991; Salamino *et al.*, 1994a; Averna *et al.*, 1999) although details about their efficiencies vary. Phosphorylation occurs at the serine and threonine residues (Adachi *et al.*, 1991) and has been specifically seen at a single domain L serine residue in some rat brain isoforms (Averna *et al.*, 1999). Serine and threonine residues are strongly represented in the recently revealed domain XL making it a potential substrate for phosphorylation. Cong *et al.* (1998) suggest that the absence of region XL, by proteolysis or alternative start site selection, may play a regulatory role by altering the phosphorylation patterns of the protein. These authors also revealed the existence of a calpastatin promoter susceptible to PKA phosphorylation, and demonstrated that a constitutive activator of PKA could cause a 7-20 fold increase in promoter activity. Thus calpastatin may be regulated at the transcriptional level.

1.3.4 Calpastatin membrane associations

Using immunostaining, calpastatin has been detected at the sarcoplasmic reticulum and sarcolemma, as well as displaying a granular/fibrillar localisation in thyroid epithelial cells (Karlsson & Nilsson 1997). Bovine sarcolemmal vesicles were extracted and calpastatin was found associated with the cytoplasmic face (Mellgren *et al.*, 1987a; Mellgren *et al.*, 1987b). Therefore, in an intact myofibril the inhibitor would exist on the intracellular side where it would be able to interact with calpains. Calpastatin is a hydrophilic protein containing relatively few hydrophobic residues.

Hydrophobic association with membranes is, therefore, unlikely. The high proportion of charged residues, the fact that soluble calpastatin can associate with biological membranes in low ionic strength buffers, and the effect of pH and ionic strength all suggest that ionic interactions are important in this process of membrane binding (Mellgren 1987). All phospholipid species possess a polar nature and the affinity of various phospholipids to calpastatin was examined. Purified myocardial calpastatin bound to two acidic phospholipids; phosphatidylserine and phosphotidylinositol. There was no binding between the neutral phospholipids and calpastatin (Mellgren *et al.*, 1989). In contrast, purified erythrocyte calpastatin did not bind to phosphatidylserine or phosphotidylinositol under the same experimental conditions (Mellgren 1987). As noted previously, erythrocyte calpastatin lacks domains L and 1. Digestion of purified myocardial calpastatin with cyanogen bromide produced a 20 kDa fragment which binds to phosphotidylinositol but does not exhibit any inhibitory activity (Mellgren *et al.*, 1989). Sequence analysis revealed there was significant homology with porcine calpastatin domain L. The amino acid composition of domain L is strongly basic and has a high isoelectric point relative to domains 1 - 4 (Lee *et al.*, 1992). The net charge of this region could, therefore, facilitate an association with acidic charged groups as in phospholipids. Finally, Adachi *et al.* (1991) showed using ^{32}P - labelled calpastatin that 30 % of the calpastatin was membrane bound, compared to 6 % using ^{35}S-methionine labelled calpastatin. The authors suggest that membrane associated calpastatin is either preferentially phosphorylated or phosphorylated calpastatin migrates to membranes. These alternatives represent possible mechanisms of regulating calpastatin.

1.4 Calpains and calpastatin as regulators of myoblast fusion

Studies indicate that the plasma membrane region is an important site of calpain activity where there are many potential calpain substrates. The disruption of structural proteins can increase membrane fluidity and may represent a precursor step in membrane fusion. More specifically, the degradation of membrane cytoskeletal proteins from membrane patches and / or the removal of intrinsic proteins from membranes, may increase the local lipid concentration and prepare the

region for fusion. Several laboratories have investigated the role of calpain proteolysis in myoblast fusion.

It has been established that myoblast fusion is strictly calcium dependent and is accompanied by significant changes in the surface protein composition (Wakelam 1985). This prompted researchers to search for an association between protease activity and fusion. One of the earliest reports observed that myoblast extracts contain calcium-dependent protease activity whose activity correlated to fusion but was undetectable in undifferentiated myoblasts (Kaur & Sanwal 1981). However, an increase in activity was also observed in non-fusing myoblast variants suggesting that the level of activity was not dependent upon, or sufficient to ensure fusion. However, by investigating the fusion of L6 myoblasts Schollmeyer (1986a) was able to determine that EGTA prevented fusion while treatment with A23178 promoted precocious fusion. This evidence has been repeated (Hayashi *et al.*, 1996; Barnoy *et al.*, 1997) and underscores the importance of calcium and its effectors in fusion.

Schollmeyer's research into myoblast fusion continued and using immunocytochemistry she was able to report that the distribution of m-calpain changed from a predominantly diffuse distribution to a more peripheral distribution in pre-fusion myoblasts (Schollmeyer 1986b). In non-fusing variants, m-calpain distribution did not change following treatment to induce fusion. The localisation of μ-calpain didn't change in either normal or non-fusing variants, leading the authors to suggest that it was the extracellular distribution of m-calpain that is responsible for myoblast fusion. More recently, Kwak *et al.* (1993a) determined that an increase in m-calpain expression and activity, and not μ-calpain, correlated with myoblast fusion. These results are reinforced by quantification of μ- and m-calpain mRNA (Poussard *et al.*, 1993). In this study the mRNA level of m-calpain was seen to increase over the fusion period whilst μ-calpain mRNA only increased after fusion. The authors suggest that this supports the involvement of m-calpain in fusion and that the delayed elevation of μ-calpain may hint at its involvement in the control of other biological activities. The measurement of mRNA levels was repeated in 1994 by the same laboratory (Cottin *et al.*, 1994) together with the quantification of calpain

activity. They found that m-calpain activity was detectable at 2 and 4 days, and maximal at 6 days, which is concurrent with maximum fusion. At 8 days, the rate of fusion declined in accord with m-calpain activity, however, μ-calpain was maximal at 8 days after becoming measurable at 6 days. These results differ from those reported by Ebisui *et al.* (1994) who found that both μ- and m-calpain activity increased very slightly up to day 6 beyond which, m-calpain activity stabilised and μ-calpain escalated rapidly. While cell fusion was most rapid between 2 and 4 days the "percentage cells fused" value was still increasing at 10 days.

In support of m-calpains involvement, Brustis *et al.* (1994) observed a drastic increase in fusion when m-calpain was added to the growth media surrounding cells. Furthermore, these workers used monoclonal antibodies specific to m-calpain to reduce fusion by 76 %. The cells had not been made permeable so the binding effects of the antibodies are thought to have been occurring at the exterior surface. The inhibitory effect of anti-m-calpain antibody appears to be reversible and to diminish over time suggesting the degradation or internalisation of the antibodies (Dourdin *et al.*, 1997). No similar impediment to fusion was apparent when dosing the media with monoclonal anti-μ-calpain antibodies. Balcerzak *et al.* (1995) employed an 18-mer antisense oligodeoxyribonucleotide targeting a region of m-calpain mRNA to reduce calpain synthesis by 48% and myoblast fusion by 70%. No effect was observed on fusion when antisense oligonucleotides against μ-calpain or p94 were used.

Numerous experiments have sought to modify fusion using inhibitors or promoters of proteases. Kumar *et al.* (1992) used the cysteine protease inhibitors, leupeptin and E64, to block the fusion of C_2C_{12} myoblasts as has Dourdin *et al.* (1997). While Ebisui *et al.* (1994) and Barnoy *et al.* (1998) have used calpeptin to disrupt this process. Poussard *et al.* (1993) reports that insulin ,which elevates calpain mRNA levels, stimulates fusion, while transforming growth factor-β (TGF-β) suppressed fusion (Barnoy *et al.*, 1997). Cottin *et al.* (1994) expanded this list to include IGF-1 and corticosterone as stimulators of fusion. Both were also found to increase calpain mRNA levels. However, Hayashi *et al.* (1996) cautioned that

myoblast fusion is a complex, multifactorial process and those agents which influence fusion could be operating though a mechanism other than the up- or down-regulation of calpain.

More compelling evidence for the involvement of calpains in fusion may be provided by discovering its location during fusion. Initial work revealed that m-calpains distribution changed from being diffuse throughout the cytoplasm to being predominantly associated with the plasma membrane (Schollmeyer 1986b). Brustis *et al.* (1994) also witnessed a surface distribution of m-calpain, although these authors considered that its association was more with the extracellular matrix surrounding myoblasts. While these experiments used monoclonal antibodies to localise calpain, less precise methodology has also indicated an involvement of exteriorised calpain. Leupeptin is able to inhibit calpain but is not cell permeable. Myoblasts cultured in media containing leupeptin fail to fuse, which suggests that the leupeptin's anti-fusion effects are manifesting at the exterior of the cells (Brustis *et al.*, 1994). Cell penetrating calpain inhibitors such as calpeptin also inhibited fusion. However, since these inhibitors are usually included in the culture media there is no evidence to suggest that their effects are occurring intracellularly (Kwak *et al.,* 1993b; Ebisui *et al.*, 1994; Barnoy *et al.*, 1998).

The peripheral relocation of m-calpain during myoblast fusion was challenged by Moraczewski *et al.* (1996) who observed the distribution of the ubiquitous calpains during *in vitro* fusion of human satellite cells. These workers reported that μ-calpain is predominantly localised in the nuclei, but as myoblasts fuse to become young myotubes (2-3 nuclei) and then older myotubes (12+ nuclei) it moves towards a more cytoplasmic distribution. Irrespective of the differentiation stage, m-calpain was detected in punctuated peri-nuclear regions, which possibly hints at its localisation in the endoplasmic reticulum.

As a regulator of calpain activity, it is logical to expect that calpastatin may play a role in regulating the fusion process. Recent work by Temm-Grove *et al.* (1999) demonstrated that microinjection of calpastatin into myoblasts completely abolished fusion, in contrast to microinjection of m-calpain which accelerated the

process. Moreover, Kosower & Barnoy (2000) reported that calcium dependent protease activity is only detectable once calpain is separated from calpastatin. The authors concluded that calpains and calpastatin are associated during proliferation and at the beginning of fusion. Accordingly, the relationship between the enzyme and its inhibitor must change for calpain to be active during fusion. Kaur and Sanwal (1981) found that in rat myoblasts, the levels of an inhibitor of calcium-dependent protease activity declined during fusion. More recently Barnoy *et al.* (1996) reported that calpastatin levels (protein and activity) decrease by a factor of 10 during differentiation which may allow calpain activity to become apparent. Other research indicates the decrease in calpastatin is due to diminished mRNA levels and that artificially reproducing this with antisense oligodeoxyribonucleotides will stimulate fusion (Balcerzak *et al.*, 1998). The authors concluded that acute "hyperactivity" of m-calpain from reduced calpastatin is directly correlated to the onset of the fusion process. Not surprisingly, the addition of calpastatin to cultures containing myoblasts, reduced fusion by 70% (Brustis *et al.*, 1994). Some reports do not support the idea of a calpastatin decline (Kwak *et al.*, 1993a). While it is difficult to ascribe a reason, Balcerzak *et al.* (1998) has attributed dissimilar results to differences in the experimental cell lines.

1.5 Summary and experimental proposal

Mature muscle tissue arises from embryonic stem cells via a distinct series of steps. This involves the proliferation of mononucleate myoblasts followed by their biochemical and morphological differentiation into multinucleate myotubes which form the basis of higher order muscle structures. Fundamentally, biochemical and morphological differentiation are represented by the expression of muscle specific proteins and the fusion of myoblasts respectively. Myoblast fusion is accompanied by significant cell surface restructuring and controlled proteolysis.

The calpains (μ- and m-) are members of an expanding family of calcium dependent proteases and have been the subject of intensive investigation over the last three decades. In structural and functional terms, the proteases and their endogenous inhibitor calpastatin have been well defined. Despite this, however,

many details concerning *in vivo* activation and the specific functions of the ubiquitous proteases remain unknown. Several aspects of myoblast fusion, notably, the reliance upon calcium and proteolysis have inspired several researchers to search for an involvement of calpains in the fusion process.

A variety of approaches have been used to investigate this involvement although the majority of work employs myoblasts grown in tissue culture as a model system. Unfortunately, differences between culture systems (eg. cell type and media composition) have sometimes resulted in diverging results. Nevertheless, a compelling body of evidence has demonstrated that calpain, especially the m-calpain isoform, is of crucial importance in the normal fusion process.

This work seeks to investigate the role calpastatin plays in regulating fusion. Calpastatin is the endogenous inhibitor of the calpains and its levels can be manipulated to produce dramatic impacts upon fusion. Moreover, aspects of the structure of calpastatin suggest possible mechanisms by which the protein may be able to perturb events at specific cellular locations. For example, the N-terminal "domain L" of calpastatin may be responsible for directing the protein to the plasma membrane; a location where calpain activity is thought to occur. Consequently this thesis aims to:

(i) Manipulate the ovine calpastatin cDNA to create vectors containing specific regions of calpastatin. The regions will be selected on their inhibitory activity and putative subcellular localisation signals.

(ii) Express the proteins encoded by the vectors in bacteria and assess the basic aspects of their structure and function.

(iii) Use the expressed proteins as antigens with which to produce antibodies for subsequent usage.

(iv) Characterise the fusion of myoblasts grown in tissue culture, and assess whether calpain and calpastatin activities are useful indicators of the process.

(v) Generate vectors encoding regions of calpastatin which will be suitable for expression in myoblasts and which will enable detection of the expressed product.

(vi) Optimise the methodology for the introduction of plasmid DNA into myoblasts.

(vii) Introduce expression vectors into myoblasts and monitor the effect of expression of calpastatin regions on the fusion process. Specific attention will be given to the subcellular localisation of the expressed product.

It is expected that this thesis will contribute to the understanding of the involvement of calpastatin in myoblast fusion and to an appreciation of the structure and function of calpastatin.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Solutions

The most commonly used solutions were:

TBE (1 X):

45 mM Tris base, 45 mM boric acid, 1 mM EDTA (pH 8.2)

Tris Buffered Saline (1 X TBS):

20 mM Tris-HCl pH 7.5, 0.5 M NaCl

DNA Loading buffer (5 X):

0.25 % bromophenol blue, 40 % (w/v) sucrose

PBS (1 X):

0.8 % NaCl, 0.02 % KCl, 0.14 % Na_2HPO_4, 0.024 % KH_2PO_4 (pH 7.3)

Protein Denaturing Buffer (6 X PDB):

0.35 M Tris-HCl pH 6.8, 10 % SDS, 30 % glycerol, 5 % β-MCE, 0.012 % bromophenol blue

Running Buffer (10 X):

0.25 M Tris-HCl, 2.0 M glycine and 10 % SDS

Transfer Buffer (1 X):

25 mM Tris-HCl pH 8.3, 192 mM Glycine, 20 % Methanol (v/v), 1 % SDS

$CaCl_2$ Buffer (1 X):

60mM $CaCl_2$, 15 % glycerol, 10mM PIPES (pH 7.0)

2.1.2 Antibiotics

Ampicillin (Amp) interferes with a terminal reaction in bacterial cell wall synthesis. A stock solution was made as a 100 mg/ml in water, filter sterilised, and used at a final concentration of 50 μg/ml for broth cultures and at 100 μg/ml for agar plates.

Kanamycin (Kana) binds to the 70S ribosomes and causes misreading of messenger RNA. A stock solution was prepared by dissolving the solid at 60 mg/ml, and used at a working concentration of 30 μg/ml for broth cultures and 50 μg/ml for agar plates.

Geneticin is also known as Neomycin or G418 and blocks protein synthesis by interfering with ribosomal functions. It was purchased as a stock solution of 50 mg/ml, working concentration for selection was 800 μg/ml and 100 μg/ml for maintenance.

2.1.3 Antibodies

Table 2.1 List of primary (1°) antibodies. Antibody dilutions are noted in individual Figure legends.

Antisera	Host	Antigen	Type	Reference
D3	Mouse	Desmin	Monoclonal	Danto & Fischman (1984)
CT3	Mouse	Troponin T	Monoclonal	Jin *et al.* (1989)
MF20	Mouse	Myosin	Monoclonal	Bader *et al.* (1982)
Anti-GFP	Mouse	GFP	Monoclonal	Roche (Cat # 1814 460)
Anti-GST	Goat	GST	Polyclonal	APB (Cat # 27-4577)
551	Rabbit	Domain L	Polyclonal	This Thesis
552	Rabbit	Domain 1	Polyclonal	This Thesis
566	Rabbit	Domain L	Polyclonal	This Thesis
567	Rabbit	Domain 1	Polyclonal	This Thesis
107-82	Mouse	m-calpain	Monoclonal	ABR Geesink & Goll (1995)
1F7E3D10	Mouse	Calpastatin	Monoclonal	ABR Thompson & Goll (Unpublished)
2H2A7C2	Mouse	μ-calpain	Monoclonal	ABR Thompson & Goll (Unpublished)
3B9	Mouse	Calpastatin	Monoclonal	Doumit & Koohmaraie (1999)
B2F9	Mouse	μ-calpain	Monoclonal	Geesink & Koohmaraie (1999)
Forsberg	Rabbit	m-calpain	Polyclonal	Huang & Forsberg (1998)
Forsberg	Rabbit	μ-calpain	Polyclonal	Forsberg (Unpublished)

Table 2.2 List of secondary (2°) antibodies and the appropriate dilutions.

Antisera	Host	Antigen	Type	Conjugate	Dilution	Source
A-3688	Goat	Mouse IgG	Polyclonal	Alkaline Phosphatase	1:2000	Sigma
A-0418	Goat	Rabbit IgG	Polyclonal	Alkaline Phosphatase	1:2000	Sigma
A-2168	Rabbit	Goat IgG	Polyclonal	Alkaline Phosphatase	1:2000	Sigma

2.1.4 Oligonucleotides

Oligonucleotides used in this work were all "guaranteed oligos" synthesised by GENSET. They were used without further purification.

Table 2.3 Oligonucleotides used in this work.

Name	Oligonucleotide Sequence	Reference
PGEX^{5}	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	Chapter 4
PGEX^{3}	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	Chapter 4
DLGFP^{5}	5'-TGCCGGAATTCGCCACCATGAATCCCACAGAAGCCAAGGCT-3'	Chapter 5
DLGFP^{3}	5'-CAGCCGGTCGACTGCAGAAGGTTTACTTGGCTTGG-3'	Chapter 5
D1GFP^{5}	5'CAGCCGGAATTCGCCACCATGGCAAAGTCAGACATGGATACTGCT-3'	Chapter 5
D1GFP^{3}	5'-CTGCCGGTCGACCTCTTCCACTTTTCTTCTTTTC-3'	Chapter 5
DLGST^{5}	5'-CTGCCGGAATTCAATCCCACAGAAGCCAAGGCT-3'	Chapter 4
DLGST^{3}	5'-CAGCCGGTCGACTTATGCAGAAGGTTTACTTGGCTTG G-3'	Chapter 4
D1GST^{5}	5-'CTGCCGGAATTCAAGTCAGACATGGATACTGCT-3'	Chapter 4
D1GST^{3}	5-'CTGCCGGTCGACTTACTCTTCCACTTTTCTTCTTTTC-3'	Chapter 4

2.1.5 Bacterial culture media

Bacterial cells were grown at 37°C with aeration using the following media:

Luria-Bertani Medium (LB)

(1 % Bacto-tryptone, 0.5 % yeast extract, 170 mM NaCl)

pH 7.5 with 4M NaOH, make to 1L with ddH_2O, autoclave to sterilize.

2 x Tryptone and Yeast Broth (2 x TY)

(1.6 % Bacto-tryptone, 1 % yeast extract, 280 mM NaCl)

Make to 1L with ddH_2O, autoclave to sterilize

Terrific Broth (TB)

1.2 % Bacto-tryptone, 2.4 % yeast extract, 0.4 % v/v glycerol

supplemented with 17 mM KH_2PO_4 and 72 mM K_2HPO_4

Agar plates for supporting solid bacterial growth consisted of 2 x TY broth or LB broth supplemented with 1.6 % agar. After sterilisation, broth and broth containing agar are cooled to 50°C before the addition of antibiotic.

2.1.6 Plasmids, bacterial strains and mammalian cell lines

2.1.6.1 Plasmids

Table 2.4 Plasmids used in this thesis. Diagrams of vector maps are provided in Appendices A.1, A.2, A.3 and A.4.

Plasmids	Description	Supplier / Reference
pBSCalstn	Bluescript plasmid containing ovine calpastatin cDNA	Collingwood (1994)
pBluescript II KS+	Ampicillin resistant cloning vector	Stratagene
pGEX-4T-1	Prokaryotic GST protein fusion vector	APB (Smith & Johnson 1988)
pEGFP-N3	Eukaryotic GFP protein fusion vector	Clontech

2.1.6.2 Bacterial strains

Table 2.5 Bacterial strains. Both *E.coli* strains are gram negative, non-conjugative and non-pathogenic. DH5α is a K12 *E. coli* derivative, while BL21 originate from B strain *E. coli.*

Strain	Genotype	Supplier	Reference
BL21	F- *ompT gal [dcm] [lon] hsdSB* (r_B-m_B-)	APB	Wood (1966); Studier & Moffatt (1986)
DH5α	F- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K-,m_K+) *phoA supE44* λ- *thi-1 gyrA96 relA1*	NEB	Grant *et al.* (1990)

2.1.6.3 Mammalian cell lines

Table 2.6 Mammalian cells used in this work.

Strain	Description	Supplier	Reference
C2C12	Mouse (strain C3H) skeletal muscle myoblast	ATCC	Yaffe and Saxel (1977); Blau *et al.* (1985)

2.2 Methods

2.2.1 Preparation of competent *E.coli* cells and transformation

Fresh competent *E.coli* cells were prepared according to a modified protocol based upon that described by Sambrook *et al.* (1989). A loop-full of frozen *E.coli* stock was streaked onto pre-warmed LB plates and grown overnight at 37°C. The following day a single colony was used to inoculate 10 ml LB broth which was incubated overnight at 37°C with shaking. The following day 1 ml of the overnight culture was added to 100 ml pre-warmed 2 x TY broth and incubated at 37°C with shaking. Growth was monitored by measuring A_{600} of the culture against an uninoculated 2 x TY reference. When the culture A_{600} was 0.40 (approximately 2 - 4 hours) the culture was cooled on ice to inhibit any further growth.

Bacteria were transferred to 2 chilled sterile 50 ml centrifuge tubes (Falcon), stored on ice for 10 minutes and pelleted by centrifugation at 500 *g* for 10 minutes at 4°C (varifuge). The supernatant was decanted and replaced with 10 ml $CaCl_2$ buffer. The pellet was resuspended by gently swirling the centrifuge tubes until cell clumps could no longer be seen. Bacteria were recovered by repeating the centrifugation step, then resuspended in 10 ml $CaCl_2$ buffer, swirled and held on ice for 30 minutes. Finally, the cells were collected by centrifugation and resuspended in 2 ml $CaCl_2$ buffer. Aliquots of 100 µl were pipetted into sterile 1.5 ml eppendorf tubes and stored upright at -80°C.

For each transformation, a 100 µl aliquot of cells was thawed on ice and a 14 ml polypropylene tube was similarly chilled. The competent cells were gently pipetted into the chilled tubes, and plasmid DNA (10 - 100 ng) was added by moving the pipette through the cells while dispensing. The cells were flick-mixed and incubated on ice for 30 minutes. In all cases a negative control, which received no plasmid DNA, was included. Bacteria were given a heat shock by rapid transfer from ice into a 42°C water bath for 45 seconds, before being returned to the ice for a further 2 minutes. After adding 900 µl room temperature SOC broth (2 % casein hydrolysate, 0.5 % yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose; pH 7.0) to each tube, the cells were incubated with shaking at 37°C for 1 hour to allow expression of antibiotic resistance. Agar plates made from LB broth and including selective antibiotic were prepared. Aliquots of the bacterial cultures (between 50 and 200 µl) were pipetted onto the plates and spread with a glass rod. The control competent cells were plated onto agar plates in the presence and absence of antibiotic. The plates were incubated upside-down at 37°C overnight and the number of transformants counted the next day.

2.2.1.1 α-complementation detection

Plasmids used in this work all contain the N-terminal 146 amino acids of the β-galactosidase gene containing a non-disruptive multiple cloning site. When transformed into host cells that code for the C-terminal portion of the protein only (Lac- bacteria), the two gene products combine through α-complementation to produce an active protein. Lac+ bacteria are easily distinguished because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chromo-3-indolyl-β-D-galactosidase (X-gal).

When DNA is inserted into the plasmid's multiple cloning site, the gene is disrupted and expression of active β-galactosidase is prevented. Agar plates, containing the appropriate antibiotic, were prepared for testing transfection success by spreading with 40 µl stock X-gal solution (20 mg/ml in dimethylformamide) and

4 µl IPTG (200 mg/ml). The plates were incubated at 37°C until all the fluid had evaporated (approximately 3 - 4 hours), and an aliquot (100 µl) of the transformed bacteria was evenly spread with a glass rod. The plates were incubated upside-down at 37°C overnight and the number of successful transformants (white colonies) counted the next day. The percentage of successfully transfected bacteria was calculated according to the following calculation:

$$\frac{\textit{Number White Colonies}}{\textit{Number White + Blue Colonies}} \times \frac{100}{1}$$

2.2.1.2 Preservation of bacteria

Bacterial stock solutions and transformed bacteria may be frozen and stored for indefinite periods at -80°C. LB broth (5 ml) containing the appropriate antibiotic (where required) was aseptically inoculated with a single colony of bacteria, and incubated at 37°C overnight with constant shaking. The following day bacteria were harvested by centrifugation (microfuge; 12 000 rpm, 2 minutes) and supernatant removed by aspiration. The pellet was resuspended in 2 ml LB containing 15 % (v/v) sterile glycerol. Volumes of 100 µl were aseptically transferred into sterile 1.5 ml cryovials and incubated at RT for 1 hour to allow the glycerol to penetrate the cells. Aliquots were stored frozen in an upright position at -80°C.

2.2.2 Plasmid DNA extraction

For the rapid and small scale isolation of plasmid DNA a modification of the alkaline lysis method of Sambrook *et al.* (1989) was used. Plasmid DNA obtained in this way was used for the purposes of checking ligation experiments, and confirming the identity of plasmids by restriction digestion.

2.2.2.1 Small scale preparation of plasmid DNA

Single transformant colonies were aseptically transferred to separate 14 ml polypropylene tubes containing 5 ml LB broth and the appropriate antibiotic. The cultures were incubated at 37°C overnight with constant shaking (180 rpm). Three ml of stationary phase bacteria were harvested and resuspended in 100 µl of solution I (50 mM glucose, 10 mM EDTA , 25 mM Tris-HCl; pH 8.0), then incubated at RT for 5 minutes. The cells were lysed and the DNA denatured following the addition of 200 µl of fresh solution II (1 % SDS, 0.2 M NaOH) and then gently mixed by inverting the tube 5 times before incubating on ice for 5 minutes. Protein and chromosomal DNA was precipitated by the addition of 150 µl of solution III (3.0 M sodium acetate; pH 4.8) and gentle mixing before incubating on ice for 5 minutes. Cell debris and chromosomal DNA was pelleted by centrifugation (microfuge; 12 000 rpm, 10 minutes). Plasmid DNA and RNA was recovered from the supernatant by the addition of 2 volumes (v/v) absolute ethanol, incubation at RT for 5 minutes and centrifugation (microfuge; 12 000 rpm, 10 minutes). After removing the supernatant the pellet was vortexed in 1 ml ice-cold 70 % ethanol. DNA was repelleted by centrifugation and the supernatant removed. The pellet was dried under a centrifugal vacuum using a SpeedVac Concentrator, before being resuspended in sterile ddH_2O containing 0.01 volume 0.5 µg/ml Dnase-free Rnase.

2.2.2.2 Mid-scale preparation of high purity plasmid DNA

For extraction of very high purity plasmid DNA, such as that used for transfection, a Plasmid Midi-Kit was employed. This plasmid purification kit uses a modified alkaline lysis procedure, followed by the binding of DNA to anion-exchange resin, from which impurities are eluted by successive wash steps. DNA is eluted with a high-salt buffer, and concentrated and desalted by isopropanol precipitation. The manufacturers instructions were followed, and high purity DNA (1 µg/µl) was obtained.

2.2.2.3 Extraction and ethanol precipitation of plasmid DNA

Following isolation, nucleic acids may be purified from contaminating protein by sequential extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and then by chloroform. Nucleic acids were precipitated with 0.1 volume (v/v) sodium acetate (NaOAc: 3 M; pH 5.2) or 0.25 volume (v/v) ammonium acetate (NH_4OAc: 10 M) followed by the addition of 2.5 (v/v) volumes of absolute ethanol. Tubes were inverted to ensure complete mixing and incubated on ice for 10 minutes. Following centrifugation (microfuge; 12 000 rpm, 10 minutes) the supernatant was removed and the pellet was vortexed in 1 ml 70 % ethanol. The DNA was repelleted by centrifugation and the supernatant removed. The pellet was dried by spinning in a SpeedVac Concentrator and redissolved in sterile ddH_2O.

2.2.3 Protein extraction and quantification

2.2.3.1 Rapid, small scale extraction of bacterial protein

Protein from small samples of bacterial culture could be rapidly extracted using a freeze/thaw protocol. Cells from 1 ml samples were harvested by centrifugation and washed once with ice-cold PBS. The cells were then pelleted and resuspended in Lysis Buffer (100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml lysozyme, 50 mM Tris-HCl; pH 8.0) to an A_{590} of 0.7. After incubation at 4°C for 30 minutes, the cells were ruptured by freeze-thawing, once at -20°C and twice at -80°C, with thawing performed at RT. Soluble protein was obtained as the supernatant after centrifugation of lysed cells (microfuge; 12 000 rpm, 15 minutes). The pellet, containing cell debris and inclusion bodies, was washed once with ice-cold PBS and solubilized by boiling in 1 X PDB using the same volume as for lysis buffer.

2.2.3.2 Large scale soluble bacterial protein extraction

Cells harvested from 1 litre of 2 x TY bacterial broth culture, grown under shake flask conditions, were collected by centrifugation (Beckman JA10; 5000 *g*, 10 minutes, 4°C). The supernatant was decanted and the tubes inverted onto absorbent

towels to remove as much of the supernatant as possible. The pelleted cells were resuspended in 100 ml of ice-cold PBS containing 1 mM PMSF. Cells were lysed by sonication on ice (10 x 30 seconds, 40 % power) and Triton X-100 was added to the crude bacterial lysate to a final concentration of 1 %. Clarification of the lysate was performed by centrifugation (Beckman JA20, 12 000 *g*, 15 minutes, 4°C) and the soluble protein containing supernatant was collected and frozen at -20°C for no longer than 24 hours.

2.2.3.3 Small scale extraction of soluble protein from skeletal muscle

Soluble protein from small amounts of skeletal muscle were required for SDS-PAGE and Western Blot analysis. Samples (5 gm) from sheep *Longissimus dorsi* and mouse hind limb (1 gm) were trimmed of connective tissue and minced with scissors before being homogenised on ice in 5 volumes extraction buffer (5 mM EDTA, 0.05 % β-MCE, 100 mM Tris-HCl, pH 8.3, 4°C) using a polytron (3000 rpm). The homogenate was centrifuged (Beckman JA20, 27 000 *g*, 30 minutes, 4°C) after which the soluble protein containing supernatant was collected and filtered through glass wool to remove any lipid. An aliquot was removed from the "total soluble protein fraction" and heated to 100°C for 5 minutes. The heated solution was then centrifuged in a microfuge at maximum speed and the supernatant removed and labelled "boiled soluble protein fraction". The protein content from both fractions was determined as described in section 2.2.3.4.

2.2.3.4 Protein quantification

Protein quantification was performed using either the Bio-Rad Protein Assay "Microassay Procedure" (BioRad), or the BCA Protein Assay "Microtiter Plate Assay" (Pierce) according to the manufacturers instructions. Standards covered a range of concentrations from 0.025 mg/ml to 0.5 mg/ml, and were prepared from Bovine Serum Albumin (Pierce). The standard curves typically displayed an r^2 value greater than 0.95, and both standards and samples were analysed in quadruplicate.

2.2.4 Gel electrophoresis

2.2.4.1 DNA

Fractionation of DNA electrophoresis using a Mini-Sub Cell gel tank provided satisfactory resolution for this study. The agarose concentrations selected were based upon the recommendations of Sambrook *et al.* (1989). The gel was prepared by melting the appropriate percentage (w/v) of SeaKem agarose in 1 X TBE, and casting into a "minigel" mold. The DNA samples were coloured and weighted by the addition of 0.2 volumes of 5 X DNA loading buffer before being loaded onto the gel. The electrophoresis buffer used was 1 X TBE containing ethidium bromide (0.5 μg/ml), and gels were typically electrophoresed at 5 V/cm for 1 hour. DNA was visualised by viewing the gel over an UV transilluminator.

2.2.4.2 Protein

Proteins were separated using discontinuous denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The Mini-Protean II apparatus enabled sufficient resolution for this work. Resolving gels were composed of 10 % or 12.5 % acrylamide, 375 mM Tris-HCl (pH 8.8) and 0.1 % SDS. The stacking gels were composed from 4 % acrylamide, 125 mM Tris-HCl (pH 6.8) and 0.1 % SDS. The 30 % stock acrylamide solution had a ratio of 37.5:1 acrylamide :N,N'-methylenebisacrylamide. Polymerisation of the gels was initiated by the addition of 1 % (v/v) freshly prepared 10 % ammonium persulphate and 0.1 % (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED). Samples to be loaded were denatured at 100°C for 3 minutes in 1 X PDB. Gels were pre-run for 10 minutes at 100 V in Running Buffer before loading the samples and electrophoresis was continued at 100 V until the dye front had migrated from the gel. Following electrophoresis, proteins were fixed and stained with a solution containing 50 % methanol, 10 % glacial acetic acid and 0.25 % Coomassie Brilliant Blue R250. Gels were then destained by repeated washes with a solution of 50 % methanol and 10 % acetic acid, and were allowed to reswell in ddH_2O. Stained gels were preserved by drying between two sheets of cellophane using a GelAir Drying System.

2.2.4.3 Gel Documentation and densitometry

A permanent record of agarose and PAGE gels was captured using a Ultra Violet Products (UVP) ImageStore 5000 gel documentation system. Using a dual white-light and UV-C transilluminator, SDS-PAGE and agarose gels were illuminated and images were captured using the incorporated digital camera (resolution set at 752 x 582 pixel's). Images were initially saved as *.BMP files which could be studied using GelBase/GelBlot Pro image analysis software (UPV). Alkaline phosphatase developed Western blots were similarly analysed after being scanned using a MicroTek Scan Maker X6el and stored as computer files.

2.2.5 Purification of DNA fragments with DEAE membrane

Fragments of DNA produced by restriction digestion or PCR were extracted from agarose gels using diethylaminoethyl (DEAE) membrane. Following electrophoresis through an appropriate percentage TBE agarose gel in the presence of ethidium bromide, the DNA fragment of interest was located with a hand-held, long-wavelength ultraviolet lamp and an incision was made directly in front of its leading edge. A piece of DEAE membrane (Schleicher and Schuell NA-45) was cut that was same width but slightly deeper than the gel incision. This was prepared by soaking in 10 mM EDTA (pH 8.0) for 5 minutes, then replacing this solution with 0.5 M NaOH and soaking for a further 5 minutes. The membrane was washed extensively with ddH_2O before being inserted into the gel incision, care was taken to exclude air bubbles. Electrophoresis was resumed at 5 V/cm until the band of interest had migrated onto the membrane. The membrane was removed from the gel and washed with 10 ml low-salt wash buffer (50 mM Tris-HCl, 0.15 M NaCl, 10 mM EDTA; pH 8.0). The membrane was transferred to a microfuge tube, and the DNA eluted by completely immersing the membrane in the least possible volume of high-salt elution buffer (50 mM Tris-HCl, 1 M NaCl, 10 mM EDTA; pH 8.0), and incubating at 65°C for 30 minutes. After removing the fluid to a new microfuge tube, the membrane was incubated for a further 15 minutes in a second aliquot of high-salt elution buffer. The two aliquots of elution buffer were combined and nucleic acid

was extracted using phenol:chloroform and ethanol precipitation as described in section 2.2.2.3.

2.2.6 Assessment of DNA concentration and purity

The yield and quality of plasmid DNA was determined spectrophotometrically. The absorbance at wavelengths 260 and 280 nm were read on a DNA sample which was first diluted (as much as 1000 fold) in ddH_2O. An A_{260} : A_{280} ratio of 1.8 indicates that the DNA is free from RNA and protein contamination. A solution containing 50 µg/ml double-stranded DNA has an absorbance of 1 at 260 nm. Thus the DNA concentration was calculated from the following equation:

$$DNA\ Concentration\ (\mu g/ml) = A_{260} \times 50 \times dilution\ factor$$

Where required, DNA purity and quantity was further assessed by electrophoresis of the sample and standards through an agarose gel and staining with ethidium bromide. Subjective comparisons between the fluorescent intensity of the unknown sample and a known DNA standard were made. Typically, a titration of samples would be compared to a titration of Hind III restriction enzyme digested λ DNA. Knowing the fragment sizes in base pairs and total concentration of the standard DNA enables calculation of the DNA concentration for each fragment (usually encompassing a range from 0.3 – 238.0 ng). By titrating both the sample and the standard it is possible to assess which standard fragments fluorescence most closely resembles that of the sample, and thus estimates the concentration of the sample.

2.2.7 DNA manipulation

2.2.7.1 Restriction enzyme digestion

Restriction enzyme digests of DNA were performed according to the manufacturers recommendations of incubation buffer, temperature, time and

addition of other reagents such as BSA. Where two restriction enzymes with differing optimal salt concentration requirements were used in a single reaction, the buffer conferring the greatest predicted activity to both enzymes was used. Each digest typically contained DNA, endonuclease, buffer and water.

2.2.7.2. Ligation

Ligation was used to introduce purified PCR products into expression vectors. DNAs were ligated in a reaction volume of 10 µl reaction buffer (66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP) containing 1 unit T4 DNA Ligase. The concentrations of vector to insert were chosen based upon the protocol described by Sambrook *et al.* (1989). The mixture was incubated at RT for 3 hours. Ligations were terminated by heating to 65 °C for 10 minutes. The efficacy of the ligation was confirmed by agarose gel electrophoresis of 0.1 volume of the ligation mixture.

2.2.8 Nucleotide sequencing

PCR products were purified from agarose using DEAE paper and ethanol precipitation. The DNA was sequenced by dideoxy-dye chain-termination chemistry using PCR primers described in Table 2.1.4. Termination products were analysed on an Applied Biosystems Automated Sequencer at the Centre for Gene Technology, School of Biological Sciences, University of Auckland (NZ). Sequence analysis was carried out at Lincoln University using DNAMAN (ver 4.0a).

2.2.9 The polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify particular sequences of DNA fragments and to introduce restriction enzyme recognition sequences at critical points of the amplimer. DEAE membrane purified DNA fragments (50 ng) were amplified in a reaction volume of 100 µl consisting of 1.5 mM $MgCl_2$, 1.0 mM dNTP's, 2 µM oligonucleotide primers, and 1 U Taq

polymerase, the reaction volume was overlaid with 50 µl sterile mineral oil. Samples were amplified using a Perkin Elmer GeneAmp PCR System 2400 thermal cycler. The program consisted of 30 cycles of 95°C for 1 minute, 56°C for 2 minutes and 78°C for 2 minutes, cycling was completed with a 10 minute incubation at 78°C before maintenance at 4°C. Nucleic acids were isolated by adding 1 volume of chloroform (150 µl) to the PCR reaction, vortexing and centrifuged (microfuge; 12 000 rpm, 10 minutes). After removing the nucleic acid containing supernatant, PCR products were purified free from amplification primers and reagents with agarose gel electrophoresis and DEAE membrane purification as described in section 2.2.5.

2.2.10 Preparation and purification of GST-fusion proteins

An aliquot of frozen transformed bacteria was thawed on ice before being added to 5 ml LB broth (containing appropriate antibiotic). The inoculated LB culture was grown at 37°C overnight with constant shaking. The following day 1 ml of the stationary phase culture was used to inoculate 1 litre of 2 x TY broth containing appropriate antibiotic which was incubated at 37°C with constant shaking. The absorbance of the culture at 590 nm was monitored by removing 1 ml aliquots at 1 hour intervals and measuring in a spectrophotometer. When the culture reached an absorbance density of 0.8, the bacteria were induced to transcribe the GST plasmid by the addition of 0.5 ml 500 mM IPTG. Samples of bacteria (1 ml) were taken immediately prior to addition of IPTG and at 1 hour intervals. Protein was extracted as described in section 2.2.3.1, and electrophoresis enabled the appearance of the target protein to be monitored.

Once suitable quantities of the desired protein had been expressed, soluble bacterial protein was extracted as described in section 2.2.3.1. The soluble protein containing supernatant was thawed on ice and mixed with 10 ml 50 % (v/v) slurry of glutathione-agarose beads. Binding took place overnight at 4°C with gentle revolution (15 rpm) using a Hybaid oven. GST fusion proteins bound to glutathione-agarose beads were collected by centrifugation (varifuge; 1000 rpm, 15 minutes, 4°C), the supernatant was decanted and replaced with 100 ml of 0.1 % Tween-20. Beads

were mixed by rotation for 20 minutes. Centrifugation and washing steps were repeated twice more with 100 ml of 0.1 % Tween-20, and three times with 100 ml PBS. Finally, the beads (5 ml bed volume) were pelleted by centrifuging (varifuge; 1000 rpm, 10 minute, 4°C) and resuspended in 10 ml of PBS containing 100 cleavage units of thrombin. This mixture was gently rotated at RT for 4 hours. While the GST fragment remained immobilised on the beads, the calpastatin fragments were released and could be recovered from the supernatant. In parallel experiments, the equivalent supernatant fraction from bacteria expressing GST alone from pGEX-4T-1 was also recovered and used as negative controls in calpain inhibition assays. Yields of purified calpastatin fragments were calculated with the BCA protein assay.

2.2.10.1 CBND assay for GST activity

The detection and quantification of GST was accomplished through measuring the appearance of a conjugate which arises from the interaction between GST and 1-chloro-2,4-dinitrobenzene (CBND). A CBND assay solution was freshly prepared (100 mM KH_2PO_4 / K_2HPO_4 pH 6.5, 1 mM CBND, 1mM reduced glutathione) from 100 mM CBND stock dissolved in absolute ethanol. Sufficient volumes were transferred to UV-transparent cuvettes, and sample aliquots of between 1/10 and 1/100 volume were added, volumes in non-sample cuvettes were made to equivalent volume with water. The mixtures were well mixed and placed in a twin-beam spectrophotometer measuring at 340 nm. The reaction is constant within the limits of sensitivity of the spectrophotometer, and quantification of GST is achieved by measuring reaction rate according to the following equation:

$$\Delta A_{340} \,/\, minute \,/\, ml\ sample \quad = \quad \frac{\Delta A_{340}\,(t_2) - \Delta A_{340}\,(t_1)}{(t_2 - t_1)(ml\ sample\ added)}$$

It is necessary to include a control assay solution (lacking sample) to account for any background increases in absorbance. Pure, recombinant GST was used for quantification after determining its protein concentration. A selection of concentrations were assayed and the rate increase plotted, thus allowing the determination of approximate GST concentration in samples.

2.2.11 Recombinant protein activity assay

2.2.11.1 Calpain protease assay

The activity of the calpain proteases was determined by measuring the hydrolysis of a substrate protein (Koohmaraie., 1990). Briefly, aliquots of calpain (made to 1.0 ml with ddH_2O) were incubated at 25°C with 1.0 ml Assay Buffer (100 mM Tris-HCl pH 7.5, 0.7 % casein, 50 mM β-MCE, 1 mM NaN_3) and 100 µl of 0.1 M $CaCl_2$ or 0.2 M EDTA. The reaction was stopped after 30 minutes with 2 ml ice-cold 5 % TCA and the mixture was centrifuged (varifuge; 2 500 rpm, 15 minutes, 20°C). The absorbance of the supernatant was read at 278 nm, after blanking against ddH_2O. One unit of calpain was defined as the amount that gave a calcium dependent increase of 1.0 unit of absorption in 1 hour.

2.2.11.2 Calpastatin assay

Quantification of calpastatin activity is achieved through measuring its ability to reduce the activity of calpain. Aliquots of purified calpastatin were preincubated for 10 minutes at 4°C with a fixed amount of bovine m-calpain (0.2 units) extracted as previously described (Edmunds *et al.,* 1991). The volume was made to 1.0 ml with ddH_2O, and followed with a further 1.0 ml of Assay Buffer. Inclusion of $CaCl_2$, or EDTA, reaction incubation and termination, and the measurement of activity was performed as described for calpain (section 2.2.11.1). The absorbance of samples not containing calpastatin aliquots were calibrated to represent 100 % m-calpain activity, and were used as a comparison for the samples. Activity of calpastatin was expressed either as percentage reduction in calpain activity or Units activity, where 1.0 Unit was defined as the amount of calpastatin which completely eliminated 1.0 Unit calpain.

2.2.12 Amino acid sequencing

For N-terminal amino acid determination, thrombin-treated calpastatin fragments were separated on SDS-PAGE and electroblotted onto polyvinylidine difluoride membrane (PVDF) using 3-[cyclohexylamino]-1-propanesulphonic acid

(CAPS) buffer (10 mM CAPS pH 11, 10 % methanol (v/v)). The positions of the protein bands were ascertained by Coomassie staining and automated Edman degradation was performed using an Applied Biosystems Model 476A sequencer at the Bioprocessing Technology Centre, National University of Singapore, Singapore.

2.2.13 Generation of polyclonal antibodies

Recombinant domain L and domain 1 were purified as described in section 2.2.10. Four 6 week old New Zealand White rabbits were selected for generating antibodies. These were divided into two pairs, one animal (A) within each pair would receive a total of 780 μg protein from 4 immunisations, the other (B) would receive 900 μg protein over 3 immunisations. The two pairs would be exposed to either domain L or domain 1 antigen, resulting in four different immunisation regimes referred to as L(A), L(B), 1(A), and 1(B).

The first immunisation was performed at 6 weeks of age. Antigen (1.2 mg/ml) was emulsified with an equal volume of Freund's Complete Adjuvant, and 100 μl was subcutaneously injected over 4 (A) or 6 (B) sites across the animals back. Boosters were at 4 week intervals, and differed in respect to the volume (75 μl) injected, but not the number of injections or the sites.

Immediately preceding the first immunisation, and 10 days after each booster, 5 ml blood samples were collected by making a small incision in the marginal auricular vein. Prior to the incision being made, the vein region was shaved, bathed in ethanol, rubbed and swabbed with d-limonene. This has a vasodilative effect and ensures swift and simple blood collection. Blood samples taken after the first immunisation were examined for immunogenic response, while the sample taken prior to the first immunisation served as a control.

One month after the second booster, B animals were anaesthetised using Ketamine HCl (100 mg/ml) and Xylazine HCl (22.8 mg/ml) at a 3 : 2 ratio and a final volume of 2.5 ml / 4 kg body weight. A heart puncture was used to exsanguinate the animals, and blood was collected into non-heparinised centrifuge

tubes. The blood was allowed to coagulate and the serum was collected after gentle centrifugation. Animals belonging to the A group were administered a third booster, and 1 month later were sacrificed as described above.

2.2.14 Western analysis

Western blotting uses chromogenic immunological techniques to identify specific proteins within a complex mixture. Protein preparations were first resolved by SDS–PAGE and then electroblotted to Nitrocellulose Membrane using a Transblot Apparatus immersed in Transfer Buffer. Proteins were transferred at RT for 1 hour using a constant supply of 100 V. Upon completion, the membrane was immersed in Blocking Solution I (1 X TTBS containing 5 % (w/v) non-fat dry milk powder) for 1 hour at RT with gentle rocking. The membrane was similarly incubated with Blocking Solution II (1 X TTBS containing 3 % (w/v) non-fat dry milk powder) containing the primary antibody (Table 2.1). After removing the primary antibody solution the membrane was washed 3 times for 5 minutes with TTBS. Secondary antibodies (Table 2.2) were all conjugated to alkaline phosphatase and were added in Blocking Solution II. The membrane was incubated and washed as previously described. Finally, the blot was developed using the Alkaline Phosphatase Conjugate Substrate kit according to manufacture's instructions. Typically blots would be allowed to develop for 5 - 20 minutes before the membrane was washed with copious amounts of ddH_2O and air dried.

2.2.15 Cell culture

2.2.15.1 Maintenance

Skeletal myoblasts (C_2C_{12}, ATCC) were grown on either 100 mm culture plates or T175 Culture Flasks with 10 ml or 40 ml, respectively, "complete growth media" (Dulbecco's Modified Eagle Medium supplemented with 10 % Foetal Calf Serum and 0.1 % (v/v) each of Fungizone and Penicillin-Streptomycin). Plates were incubated at 37°C in a moisture saturated 5 % CO_2 in air atmosphere.

2.2.15.2 Passaging

Immediately prior to cells reaching confluence they were passaged. Media was removed and the plates were washed with 1 X PBS pre-warmed to 37°C, this was then replaced with 2.0 ml pre-warmed 4 % Trypsin/EDTA and the plates were returned to the incubator for 10 minutes or until all cells had released from the plate surface. At this time the solution was removed to a sterile 15 ml centrifuge tube, combined with 8.0 ml complete growth media and centrifuged (varifuge; 400 rpm, 10 minutes, 25 °C). Media was decanted and the cell pellet resuspended in fresh complete growth media. Aliquots of suspension were seeded into new culture vessels to produce a cell density of 20 %.

2.2.15.3 Cryopreservation

For cryopreservation of cells after passaging, the pellet was resuspended in complete growth media supplemented with 5 % (v/v) DMSO. The suspension was then aliquoted as 1.0 ml volumes into cryovials and frozen to -80°C at a rate of -1°C per minute. Any subsequent thawing of cells was performed rapidly to 37°C.

2.2.15.4 Fusion induction

Cells grown in complete growth media could be made to undergo fusion upon reaching confluence. Complete growth media was removed, the plate washed with 1 X PBS pre-warmed to 37°C, and the media was replacing with "differentiation media" (Dulbecco's Modified Eagle Medium supplemented with 2 % Horse Serum and 0.1 % (v/v) each of Fungizone and Penicillin-Streptomycin). For extended growth periods, plates were washed with PBS and supplied with fresh differentiation media every other day.

2.2.15.5 Protein extraction

Total protein was extracted from C2C12s following the release of cells from their support using trypsin/EDTA digestion. After washing with PBS to remove trypsin, cells from a 100 mm plate were pelleted by gentle centrifugation in a 1.5 ml microfuge tube. Excess solution was decanted and 200 µl Lysis Solution (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 0.1 mM AEBSF (4-(2-aminoethyl)-benzenesulphonyl fluoride), 10 µg/ml Aprotonin) was added. The pellet was then disrupted by sonication on ice for 10 seconds at 25 % maximum power. Finally, Triton X-100 was added to a final concentration of 1 %, and the homogenised solution inverted 5 times before being incubated on ice for 20 minutes. Protein concentration was determined as described in section 2.2.3.2 and samples were immediately prepared for SDS-PAGE analysis by dissolving them in an appropriate volume of PDB.

2.2.15.6 Cell staining and microscopy

Where examination using a light microscope was desired, cells were cultured on flame sterilised coverslips in 35 mm plates under whatever conditions were demanded. At appropriate times, the media would be removed and the coverslip washed by bathing the plate three times with pre-warmed 1 X PBS. While still in solution, the coverslip would be gently levered from the plate and placed into 100 % methanol for 5 minutes to fix the sample. After air drying, the cells were stained with Giemsa Solution according to manufacturers instructions. Coverslips with stained cells were mounted cell-side down onto glass slides using a drop of DPX mountant. Routine examination of slides was carried out using an Olympus CH2 light microscope. Photography used Fujichrome Sensia II 400 film in a Pentax Asahi 35 mm reflex camera attached to a Zeiss Standard Microscope with a Zeiss MC63 photomicrographic regulator.

Fluorescent microscopy followed similar lines, cells were grown directly on coverslips and washed with PBS. Fixation was performed by incubating the cells for 30 minutes at RT in 4 % paraformaldehyde dissolved in PBS. Cells were washed

twice with PBS and mounted cell-side down into glass slides with a drop of 50 % glycerol dissolved in ddH_2O. The coverslip was sealed with 10 % molten agarose, dried at RT for 30 minutes, and stored at –20°C until required. Fluorescence was very stable under these conditions and could still be detected after 3 - 4 months. Microscopy and photography was performed as described above with the inclusion of a Zeiss 450~490 – 510 nm block filter, and a Xenon lamp.

For obtaining confocal microscope images, paraformaldehyde fixed samples were examined with a BioRad Microradiance confocal system linked to an Olympus IX-70 inverted microscope and viewed under oil emersion. The blue line of the argon laser was used for excitation and a 510 nm emission filter was used for observing GFP fluorescence. This work was performed at the Plant and Microbial Sciences Department, University of Canterbury, NZ.

2.2.16 Zymographic PAGE analysis

Zymographic gels enable detection of proteolytic activity in protein samples fractionated by non-denaturing PAGE (Raser *et al.*, 1995). Acrylamide resolving gels were prepared from 375 mM Tris-HCl pH 8.8, 12.5 % acrylamide, 0.5 mg/ml casein solution (7 mg/ml casein in 100 mM Tris-HCl pH 8.8), 0.05 % TEMED and 0.5 % APS. The stacking gel was prepared as described in section 2.2.4.2, with the exception that it did not contain SDS. The gel was pre-run at 100 V for 15 minutes. The protein sample was mixed with Non-Denaturing Sample Buffer (4X; 150 mM Tris-HCl pH 6.8, 20 % glycerol (v/v), 0.75 % β-MCE, 0.1 mg/ml bromophenol blue) and loaded. Electrophoresis was performed at 4°C in Non-Denaturing Running Buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 1 mM EDTA, 0.5 % β-MCE) for 8 hours at 100 V. The gel was removed and incubated in $CaCl_2$ solution (50 mM Tris-HCl pH 7.5, 4 mM $CaCl_2$, 0.05% β-MCE) three times for 20 minutes and finally left overnight at RT. The gel was then stained with Coomassie and destained as usual.

2.2.17 FACS analysis

Cells suspended in PBS were analysed using a fluorescence activated cell sorting (FACS) vantage flow cytometer (Becton Dickinson San Jose, CA) equipped with a 488 nm argon ion laser with fluorescence emission detected at 525 nm using a Dichroic Filter (DF) 530/30. This work was performed at the Christchurch Medical School, Christchurch, NZ.

Chapter 3

Expression and purification of ovine calpastatin domains L and 1 in Escherichia coli, and the generation of specific polyclonal antibodies

3.1 Introduction

As outlined in Chapter 1, there is evidence that the primary role of the calpains in myoblast fusion is the proteolytic disassembly of membrane proteins which allows fusion to take place. Microscopic examination and immunocytochemical evidence suggests that this proteolysis predominantly occurs at or around the plasma membrane during fusion. This observation would indicate that calpain is preferentially directed to the plasma membrane. However, the mechanism responsible for this localisation remains unknown.

Previously, calpastatin has been found to localise with membrane preparations from sarcolemma and sarcoplasmic reticulum vesicles (Mellgren *et al.*, 1987a; Mellgren *et al.,* 1987b). A proposed mechanism to account for the facts is that the basic amino acids of calpastatin associate with the acidic membrane phospholipids (Mellgren 1988; Mellgren *et al.*, 1989). It has been noted that domain L is relatively rich in basic amino acid residues which contributes to the high isoelectric point (Lee *et al.*, 1992). Domain L of calpastatin is thought, therefore, to be responsible for targeting the protein to areas of the plasma membrane. The functional *in vivo* significance of domain L is explored in Chapter 4.

Crucially, for calpain to be responsible for proteolysis it must avoid inhibition by calpastatin. Some data suggests that a down regulation of calpastatin mRNA expression and protein levels might accomplish this. At this point in time, information regarding the involvement of calpastatin in the fusion process remains incomplete. This chapter reports on the attempt to express large quantities of recombinant calpastatin variants encoding domain 1, domain L or domains L and 1 combined. The physical characteristics and activities of these variants will then be

compared and contrasted. Purification of the three calpastatin variants will be described, together with their subsequent use in the generation of polyclonal antibodies for use in later experiments.

3.2 Cloning strategy to generate recombinant calpastatin from *E. coli*

The ability to obtain pure protein samples is critical when examining protein structure and function *in vitro*. It is also important in the production of good quality antisera. Traditional chromatographic techniques to separate calpastatin from tissue samples are elaborate (Mellgren & Carr 1983) while more compact methodologies do not generate a pure product (Koohmaraie 1990). Furthermore, despite the improvements in extraction techniques (Geesink *et al.*, 1998), the isolation of pure, native calpastatin with a high specific activity remains laborious.

An alternative strategy is to produce synthetic oligopeptides based upon active calpastatin (Maki *et al.*, 1989). This approach is especially useful when developing therapies demanding extremely pure protein, or investigating specific structure and function relationships, or producing affinity columns (Anagli *et al.*, 1996). This technology, however, is not suited for larger or more complex proteins because of the inability to introduce the appropriate post-translational modifications and the accumulation of errors inherent in the production process.

E. coli and some cell culture systems represent successful vehicles for the over-expression of both prokaryotic and eukaryotic protein Several systems are commercially available which permit the researcher to express a cDNA of interest. Such recombinant DNA techniques, while revolutionary, are not completely free of challenge. Host contamination, product solubility, structural integrity and biological activity are all potential problems.

An early example of expression of calpastatin domains in *E.coli* was provided by Maki *et al.* (1987a; 1987b). In these studies, however, no attempt was made to quantify expression or to purify the recombinant product. Subsequent experiments by the same researchers used boiling, ammonium sulphate precipitation, ion-

exchange and gel filtration chromatography to purify the expressed recombinants (Maki *et al.*, 1988; Ma *et al.*, 1993; Ma *et al,*. 1994) using standard extraction practices applied to tissue samples.

As biotechnology develops, more powerful expression systems have become available. One particularly useful tool is to "tag" distinctive residues to the recombinant product, thus resulting in fusion hybrids. Following expression, the target protein may be purified though relatively simple affinity chromatography. In recent work, recombinant calpastatin was "tagged" with an N-terminal hexahistidine and expressed in *E.coli* and insect cells (Hitomi *et al.*, 1998). The nature of the additional sequence was exploited to facilitate purification via affinity chromatography. However, this system did not permit isolation of calpastatin alone without additional residues. One system which has been used for the purification of many proteins employs the pGEX series of plasmids. These were created by Smith and Johnson (1988) to direct the synthesis of foreign polypeptides in *E.coli* as fusions with a 26 kDa Glutathione S-Transferase (GST) encoded by the helminth *Schistosoma japonicum*. This system has been used successfully to express active regions of calpastatin in bacteria (Doumit *et al.*, 1996; Melloni *et al,* 1998a), and is particularly attractive since it allows the separation of the tag from its fusion partner via specific proteolytic cleavage. This is often important, because tags can be highly immunogenic and hamper immunodetection of target antigens (Youssoufian 1998). The vector encoding GST contains a protein sequence sensitive to thrombin proteolysis at a point between GST and its fusion partner. Cleavage at this point enables isolation of the fusion partner from GST following its immobilisation onto glutathione affinity agarose beads.

Knowledge of the ovine calpastatin cDNA sequence (see Appendix B) permitted the design of PCR primers that would amplify domains L and 1 separately, or in combination. The primer sequences were chosen to contain restriction enzyme sites that matched those of the GST vector's multiple cloning site. After digestion, these permit sticky-ended ligation of the insert into the vector in the correct orientation and reading frame with respect to the N-terminal GST start codon. An examination of the cDNA sequence also confirmed the absence of stop codons

(TAA) that would cause premature cessation of transcription. Figure 3.1 provides a schematic overview of the process.

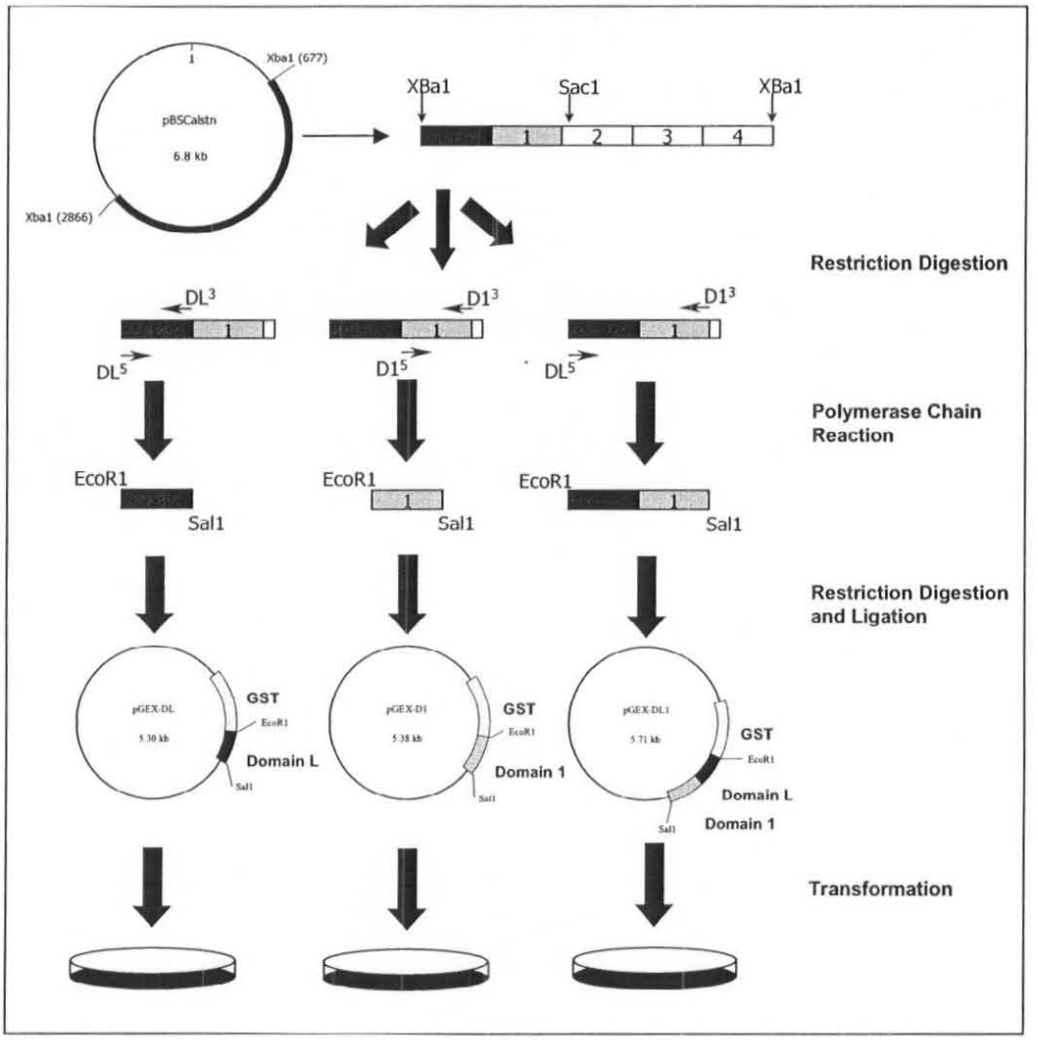

Figure 3.1 Construction of GST-calpastatin plasmids. Ovine calpastatin cDNA contained within pBluescript II KS+ (pBSCalstn) was extracted from transformed bacteria, and purified. Digestion with Xba1 and Sac1 released a fragment containing domains L-1; this was used as template DNA in PCR reactions. Three reactions were performed using primer combinations of $DL^{5'}$ & $DL^{3'}$, $D1^{5'}$ & $D1^{3'}$ and $DL^{5'}$ & $D1^{3'}$, generating amplimers of domains L, 1 and L-1 respectively. The PCR products were gel purified and digested with restriction enzymes to generate sticky-ended products. These could then be ligated into a similarly digested pGEX-4T-1 vector. The resultant construct is then suitable for introduction into E. coli and expression of recombinant fusion protein.

3.2.1 Template preparation

Generation of GST-calpastatin constructs began with a double digestion of pBSCalstn using the restriction enzymes Xba1 and Sac1, as described in section 2.2.7.1. Digestion with Xba1 releases the insert from pBSKS, while Sac1 cleaves at a point within the insert (specifically 71 bp into domain 2, see Appendix B) producing two fragments. The largest of the three fragments produced in this digestion represents the pBSKS vector (see Appendix A.1) while the former two represent the totality of the calpastatin insert. Electrophoresis through an agarose gel separated the products of this digestion (see Figure 3.2). The smaller 862 bp fragment containing domains L and 1 and a short sequence of domain 2 was extracted from the gel using DEAE membrane as described in section 2.2.5. Following purification, band homogeneity and size was confirmed by gel electrophoresis.

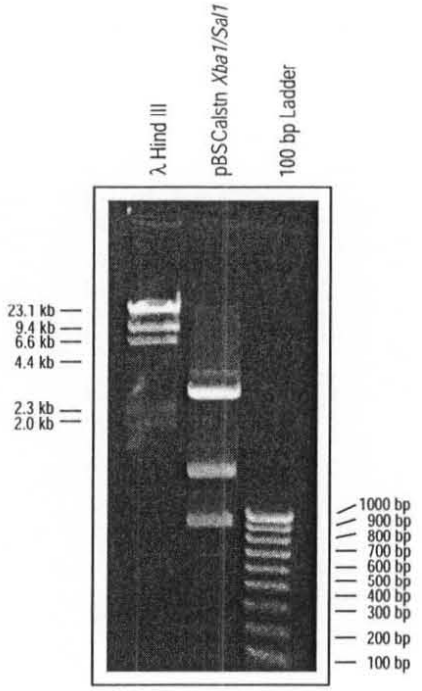

Figure 3.2 Extraction and purification of cDNA encoding domains L and 1. Agarose gel electrophoresis of pBSCalstn digested with Xba1 and Sal1, producing 3 fragments embracing pBSKS vector (2958 bp), calpastatin domains 2 - 4 (1425 bp) and calpastatin domains L - 1 + 71 bp domain 2 (862 bp).

The quantity and purity of the DNA template was determined by measuring its absorbance at 260 and 280 nm.

3.2.2 Amplification of calpastatin domains

Three PCR reactions were performed as described in section 2.2.9 using the 862 bp fragment as template DNA and the primers shown in Figure 3.3. For suitable translation, the product of transcription must contain an initiation codon (usually AUG) and stop codon (UAA, UGA or UAG). The GST cDNA contained in the pGEX-4T-1 plasmid contains an initiation codon upstream of the multiple cloning site, which is suitable for directing translation of the GST fusion proteins. However, there are no stop codons contained within domain L and domain 1 sequences. Thus TTA codons were included in both 3' primers, equating with ATT sequence in the 5' → 3' strand, and a UAA triplet (stop codon) in the consequent mRNA.

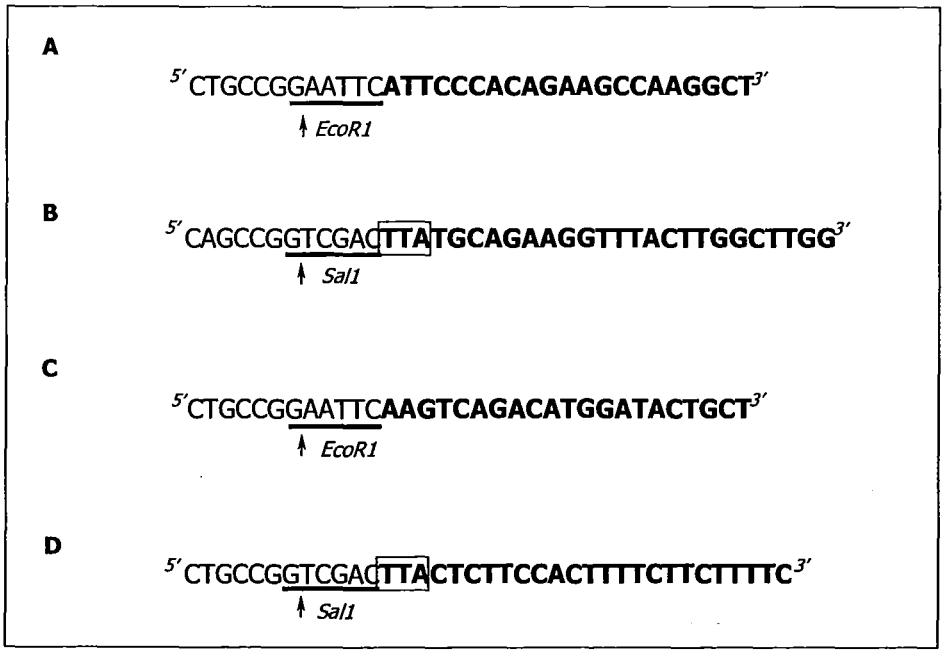

Figure 3.3 PCR primer sequences used for the amplification of domains L and 1. Primer A is the 5' primer for Domain L (DLGST$^{5'}$), primer B is the 3' primer for Domain L (DLGST$^{3'}$), primers C and D are 5' and 3' primers for domain 1 (D1GST$^{5'}$ and D1GST$^{3'}$ respectively). Bold nucleotide sequence is homologous to ovine cDNA, stop codons (boxed) were introduced to downstream primers and restriction enzyme locations are noted together with cut points (arrowed). Remaining nucleotides are non-homologous to ovine cDNA but are included to provide an "overhang" region.

The amplimer products from this PCR include the desired cDNA fragment and an additional 27 base pairs. This extension is composed of the restriction site (6 bp) and a non-homologous overhang (6 bp) contained in both primers, and stop codons (3 bp) in both 3' primers. Accordingly, the resultant amplimer sizes are 357 bp for the leader domain fragment, 432 bp for the domain 1 fragment, and 762 bp fragment combining both leader and 1 (see Figure 3.4).

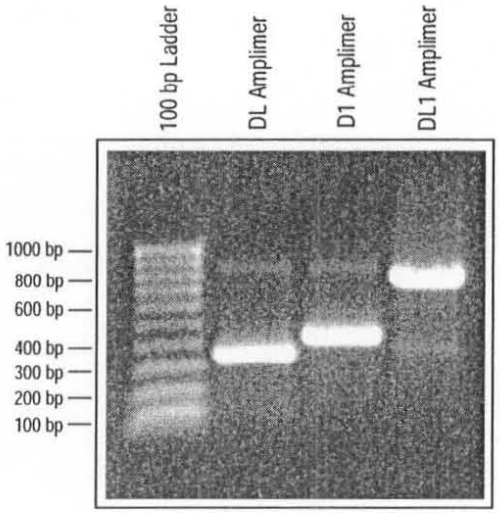

Figure 3.4 Amplimer products resulting from PCR. An 862 bp fragment containing calpastatin domains L and 1 (together with 71 bp of domain 2) was used as template to produce amplimers containing domain L, 1 and L + 1 only.

After PCR, these fragments were gel electrophoresed, extracted using DEAE membrane, ethanol precipitated, and digested with EcoR1 and Sal1 to produce sticky-ends. The pGEX-4T-1 expression vector (see Appendix A.3) was similarly digested before gel fractionation and ethanol precipitated. After precipitation all products (PCR amplimers and digested pGEX-4T-1) were subjected to phenol / chloroform extraction (see section 2.2.2.3) and quantified prior to ligation.

3.2.3 Ligation and transformation

PCR products were ligated separately into digested pGEX-4T-1 and the resultant product used to transform competent BL21 *E. coli* (see section 2.2.1).

Transformed bacteria were selected through their resistance to ampicillin antibiotic. After 24 hours the plates were examined, and based upon "blue-white" selection the procedure appeared to have been 100% successful in producing plasmids containing inserts (see section 2.2.1.1). By digesting the vector to completion with dissimilar restriction enzymes it is possible to produce different sticky-end profiles. With the use of gel fractionation to isolate the desired fragment, the prospect of the digestion products reuniting is eliminated and incorporation of insert is the only way to produce a construct with the potential for replication. The DNA from 10 colonies, peculiar to each transformation treatment, was harvested following the miniprep protocol (section 2.2.2.1) and subjected to a double restriction enzyme digestion designed to remove the insert. The nature of the insert was assessed by examination after gel electrophoresis (see Figure 3.5)

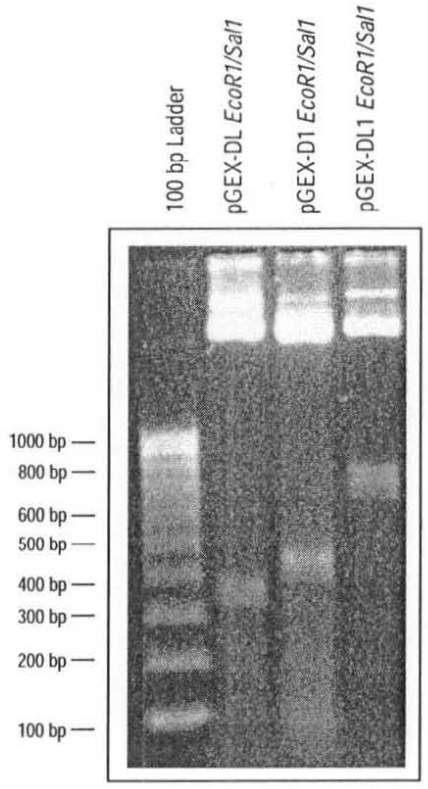

Figure 3.5 EcoR1 and Sal1 digestion of miniprep DNA from successfully transformed bacterial colonies. Gel shows pGEX-4T-1 vector and inserted domains L, 1 and L+1, with predicted sizes of 4959 bp, 343 bp, 418 bp, and 748 bp respectively.

3.2.4 Sequencing data

As a final confirmation, the plasmids generated by this work (pGEX-DL, pGEX-D1 and pGEX-DL1) were sequenced using the primers originally used for the amplification of respective inserts. The sequences were almost identical to the reported ovine calpastatin cDNA sequence (Collingwood 1994) and to other calpastatin sequences when submitted to GenBank for analysis. Moreover, there was no evidence of unexpected stop codons that can sometimes arise during PCR.

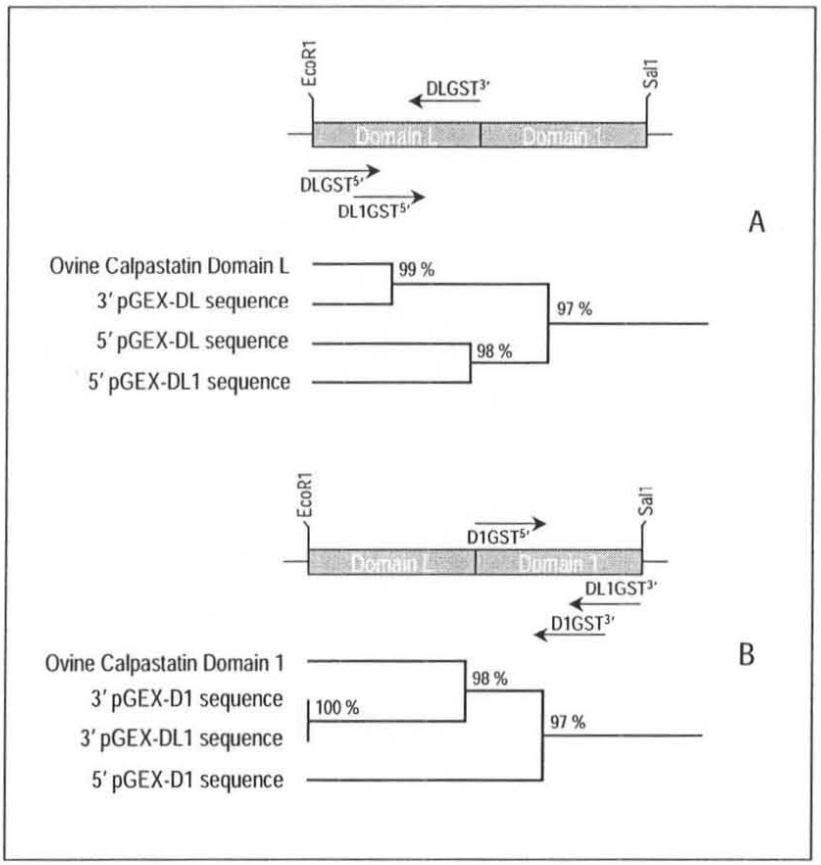

Figure 3.6. Homology trees comparing sequences contained within pGEX-DL, pGEX-D1 and pGEX-DL1 constructs, with domain L and 1 sequence from ovine calpastatin. A. Ovine calpastatin domain L compared to 5' sequence of inserts encoding domain L and domains L + 1, and the reverse complementary sequence of 3' sequenced domain L. **B.** Ovine calpastatin domain 1 compared to inserts encoding reverse complementary sequences of 3' sequenced domain 1 and domain L + 1, and 5' sequenced domain 1. Schematic diagrams show the application of primers used in the sequencing reactions. Percentage homology between closest pairings is displayed.

3.2.5 Summary

Regions of the ovine calpastatin cDNA sequence corresponding to domains L and 1 have been PCR amplified separately and in combination producing cDNA for domain L, 1 and L+ 1. Separately, the three sequences have been controllably incorporated into a GST expression vector ready for protein expression.

3.3 Expression and purification of GST - calpastatin fusion proteins

E. coli BL21 bacteria harbouring pGEX-4T-1, pGEX-DL, pGEX-D1 or pGEX-DL1 were used to produce GST - fusion proteins GST-DL, GST-D1 and GST-DL1 respectively. BL21 *E. coli* are excellent host bacteria for protein expression (Studier & Moffatt 1986) because they lack both the Ion and OmpT proteases (Phillips *et al.*, 1984; Grodberg & Dunn 1988). Cells deficient in these proteases accumulate recombinant proteins more quickly and are less likely to degrade some proteins during purification. BL21 *E.coli* have been previously used successfully for the expression of a variety of recombinant proteins including active calpastatin (Ma *et al.*, 1993; Ma *et al.*, 1994; Hitomi *et al.*, 1998; Kato *et al.*, 2000).

3.3.1 Induction of recombinant fusion proteins

Transformed bacteria were grown to log phase and induced to express protein using IPTG as described in section 2.2.10. There were no differences in growth rates or final density between untransformed and transformed bacteria (data not shown). A difficulty sometimes experienced with expression of large amounts of recombinant protein is the accumulation of inclusion bodies and resultant insolubility. A variety of approaches have been tried to obtain greater solubility through lower induction temperatures and IPTG concentrations, and longer induction periods. However, although protocols exist to retrieve insoluble protein (Frangioni & Neel 1993; Thatcher & Hitchcock 1994) there are some benefits from producing a protein that forms inclusion bodies. Target proteins are often protected from proteolytic breakdown and inclusion bodies are easy to isolate as a first step in a purification scheme. A comparison between the total insoluble and soluble protein

fractions was made and it was apparent that the majority of the fusion protein was expressed in a soluble form (see Figure 3.7).

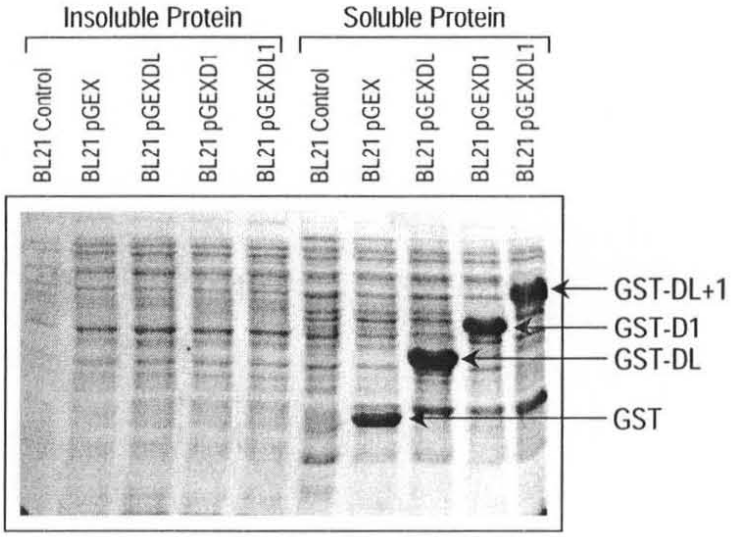

Figure 3.7 SDS-PAGE gel separating components of total soluble and insoluble protein fractions Intense bands of recombinant protein appearing 5 hours after induction of expression occur more prominently in the soluble fraction.

After centrifugation to remove the insoluble protein fraction, the recombinant protein was easily separated from the crude bacterial soluble fraction using affinity chromatography. Glutathione-agarose beads specifically bind GST and in so doing, separate the fusion protein from unbound proteins. Samples of protein supernatant taken before and after incubation with glutathione-agarose beads were electrophoresed and stained. These samples revealed, from subjective observation, that less than half of the expressed protein was being retained on the beads. A more precise measurement was obtained by exploiting the measurable activity of GST upon its chromogenic substrate; 1-chloro-2,4-dinitrobenzene (CBND). The reaction resulting from GST's action upon CBND produces a conjugate whose absorbance can be measured at 340 nm (see section 2.2.10.1). If required, this technique provides a way to estimate concentration (by weight) of expressed GST. Results from this analysis revealed that only 29 % of GST-DL was retained bound to the beads, 31 % of GST-D1 and 35 % of GST-DL1. Thus, two-thirds of the expressed protein was not recovered. Increasing the bead volume would be the simple solution to resolve

this but the objective of this work was to obtain sufficient pure protein for raising antibodies. Additional binding was therefore not performed.

Beads from the purification of each recombinant protein were washed repeatedly with PBS until unbound protein was eliminated from the liquid fraction. To test the purity of each GST fusion protein prior to thrombin treatment, an aliquot of beads from each purification was incubated with 10mM reduced glutathione (GSH) to elute bound proteins. In all cases SDS-PAGE analysis of the eluant revealed one major protein product corresponding to GST, GST-DL, GST-D1 or GST-DL1.

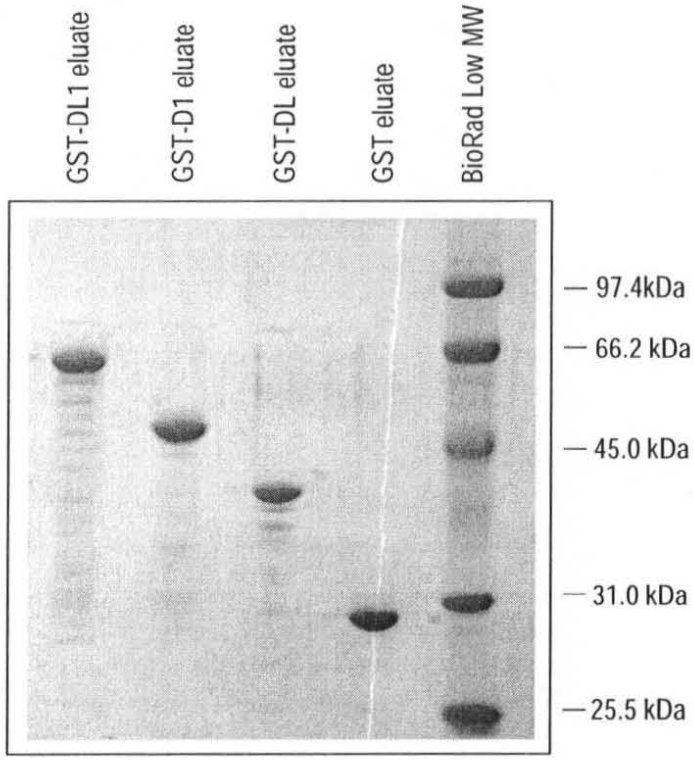

Figure 3.8 SDS-PAGE analysis of recombinant expression products. Fusion proteins, calpastatin domain + GST, have been eluted from glutathione agarose with GSH.

The expected sizes are 26.0 kDa GST protein, 37.4 kDa GST-DL, 40.6 kDa GST-D1 and 52.1 kDa GST-DL1 protein. These molecular weights are derived from the expected protein sequences, however the size calculated from PAGE analysis

indicates anomalous migration of the recombinant calpastatin. The molecular weights calculated from the migration of fusion proteins through the PAGE gel is 43.5 kDa, 54.5 kDa, and 70.0 kDa for GST-DL, GST-D1 and GST-DL1 respectively. GST alone migrated at 25.2 kDa which approximates its expected migration at 26.0 kDa, suggesting that the deviation from predicted size is due to the calpastatin component. This aberrant migration has been reported before and is thought to be due to an amino acid composition which is rich in Asp, Glu, Ser and Pro.

3.3.2 Purification of calpastatin domains

The pGEX GST expression vector encodes a thrombin protease specific site between GST and the multiple cloning site. Thrombin is a highly specific serine protease which, *in vivo*, is involved in the blood clotting cascade. Notably, thrombin is shown to be unable to cleave GST (Smith and Johnson 1988) which simplifies the extraction of the fusion partner. When GST-DL immobilised on GSH-agarose was incubated at 25°C in PBS containing thrombin, SDS-PAGE analysis showed a single major protein band released in the supernatant with an observed size of 16.6 kDa. On the other hand, immobilised GST-DL1 and GST-D1 treated with thrombin under similar conditions resulted in the appearance of two protein bands in each samples supernatant larger than would have been predicted (Figure 3.9).

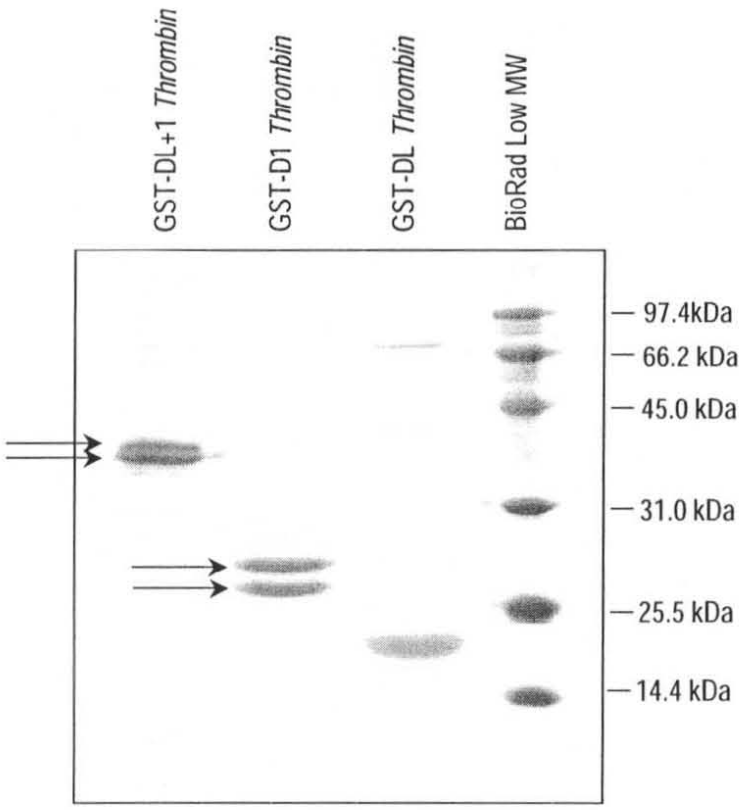

Figure 3.9 Protein extract after digestion of immobilised GST fusion proteins with thrombin. Thrombin digested recombinant calpastatin proteins domain L, domain 1 and domains L1. Doublet arrows emphasise original and truncated protein bands.

The sizes of the fragments in the GST-D1 supernatant are 22.7 kDa and 25.2 kDa, while those in GST-DL1's supernatant are 42.3 kDa and 44.8 kDa, note that in both cases the size difference is 2.5 kDa which approximates to 23 amino acid residues. The expected sizes (disregarding the unexpected 2.5 kDa differences) were 11.4 kDa for domain L, 14.6 kDa for domain 1 and 26 kDa for domains L1. The ratio of observed size (largest fragment) to predicted size is 1.72 for domain 1, 1.46 for domain L and 1.72 for the domain L and 1 combination. On average these are higher than the previously reported migration aberration ratios of 1.48 and 1.25 (Maki *et al.*, 1987a, Maki *et al.,* 1988), and 1.53 (Takano *et al.*, 1988).

With incubation time, the intensity of the larger molecular weight band in both GST-1 and GST-L1 decreased with concomitant increase of the smaller

molecular weight band until only the smaller protein moiety remained after 8 hours of exposure to thrombin. Furthermore, when using 5 units of thrombin, the larger molecular weight band was eradicated after only three hours of incubation (data not shown). This observation was consistent with specific degradation of full-length DL1 and D1 into truncated derivatives. To eliminate the possibility that there is a contaminating protease in the thrombin solution, enzymes from two suppliers (APB and Roche) were tested, however the outcomes from digestion were identical.

These derivatives were purified from an SDS-PAGE gel and subjected to N-terminal amino acid sequencing over six residues as described in section 2.2.12. The resultant sequences showed 100 % homology to the predicted residues of domains L and 1 respectively up to six residues. These observations strongly suggest the presence of a proteolytically labile site at the C-terminus of D1 resulting in DL1 and D1 polypeptides lacking carboxyl residues. It is unknown whether the proteolysis was due to secondary site recognition by thrombin or contaminating proteases that co-purify with the protease. Despite the observed dose dependent response to increasing concentrations of thrombin, the preferred thrombin cleavage site (Arg-X or the less common Lys-X, with Pro preceding Arg or Lys; Voet & Voet 1995) is not represented in domain 1. It maybe worth noting that the C terminal sequence contains a high number of PEST (proline, glutamic acid, serine and threonine) residues, which may form the basis of the recognition site. Thus truncation of calpastatin fragments containing D1 may be PEST-associated degradation attributable to a protease other than thrombin. Indeed, the cleavage of recombinant GST fusion proteins by unidentified proteases has been observed in BL21 *E.coli* (Hengen 1996). In future, it may be advisable to precipitate crude bacterial proteins with TCA and heat denature immediately upon rupturing the cells. This will eliminate any problematic proteases, while leaving calpastatin intact (Geesink *et al.*, 1998), and would represent a useful preliminary purification step.

3.4 Activity of recombinant calpastatin on calpain *in vitro*

Calpastatin is a constituent of the calpain proteolytic system and measurement of its activity is reflected through a decline in calpain mediated

proteolysis of a substrate protein. By employing such an assay system, it has been reported that recombinant proteins representing domains L and 1 - 4 show different inhibitory potential (Maki *et al.*, 1987b). No inhibition is witnessed from domain L, however domains 1 – 4 show comparable inhibitory action. In addition, other properties reported for native calpastatin were maintained, including inhibitory specificity and heat stability. Calpastatin is an unusually stable protein, and accurately assayable activity is preserved even after boiling for several minutes (Shackelford *et al.*, 1994). The activity and stability of recombinant calpastatin are two properties which were investigated to assess whether the expressed protein product was functionally active in a manner typical of native calpastatin.

3.4.1 Determination of recombinant calpastatin activities

Calpastatin activity is determined through its ability to inhibit calpain. The standard calpastatin assay used in this work is based on that described by Koohmaraie (1990) and is presented in section 2.2.11.2. In brief, "controls" measure calpain activity before addition of calpastatin. These values are compared with a series of "experimental values" which measure calpain activity following the addition of differing amounts of calpastatin. Figure 3.10 shows the effect of addition of recombinant calpastatin upon native purified m-calpain. Furthermore, the graph contrasts GSH eluted GST-fusions and thrombin digested calpastatin to assess whether the GST partner has any affect on inhibitory potential.

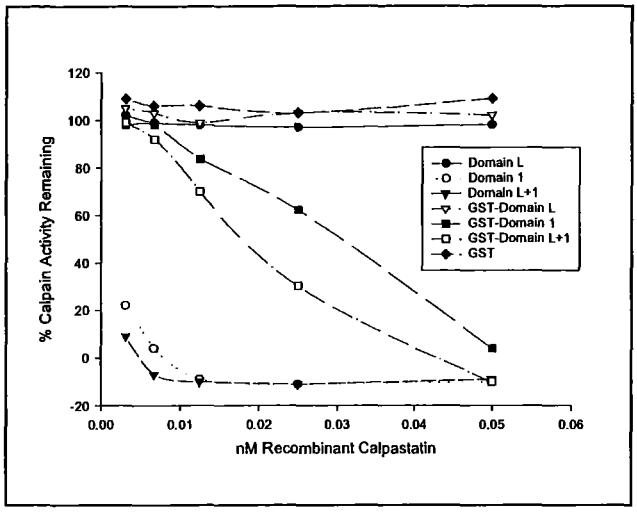

Figure 3.10 Graph showing the effect of different concentrations of recombinant calpastatin on m-calpain activity. Graph showing the effect of increasing molar concentrations of calpastatin on calpain activity. Represented are GSH eluted domains still partnered to GST (GST-L, GST-1, and GST-L1) as well as GST alone (GST), and thrombin digested domains lacking the GST tag (L, 1 and L1).

The assays were equalised on the basis of molarity, after calculating the molecular weights of the different samples and protein concentrations, and after performing preliminary assays to determine the range of sensitivities. This ensures that between assays, identical amounts of protein on a molecular basis are being compared, rather than total protein concentration which would see larger proteins under-represented. As expected, recombinant proteins containing the inhibitory domain 1 reduced the proteolytic activity of m-calpain, while domain L and GST failed to impact upon activity by themselves.

GST fusions with 1 and L1 have notably less inhibitory potential than their digested counterparts. This may indicate that the GST tag constrains the competitive binding between calpain and calpastatin through steric inhibition. Further examination of the data demonstrates that GST-DL1 has a greater inhibitory potential than GST-D1. When compared to GST-D1, the inhibitory domain of GST-DL1 is presumably more distant from the N-terminal GST due to the presence of the intervening L domain, which may result in easier coupling to calpain. Interestingly, an enhanced inhibitory potential correlating to the presence of domain

L is also suggested after removal of GST (Figure 3.10). This implies that domain L may improve domain 1's efficacy directly, as well as acting as a buffer region between GST and domain 1. The augmented inhibition attributed to domain L has also been reported by Melloni *et al.* (1998a). These authors observed that the addition of recombinant domain L to calpastatin assays reduced the inhibitory potential of active recombinant calpastatin isoforms. They suggest that domain L assists calpastatin in its recognition of calpain by binding to the protease.

To eliminate the possibility that the different inhibitions are due simply to inaccurate protein determinations western blot titrations were performed. Four SDS-Page gels were loaded with samples of GST-DL1, GST-D1, DL1 and D1 (two-fold serial dilutions from 0.025 nM to 0.049 pM). Gels were electrophoresed, transferred and western blotted identically with an antibody recognising domain 1 (Figure 3.11).

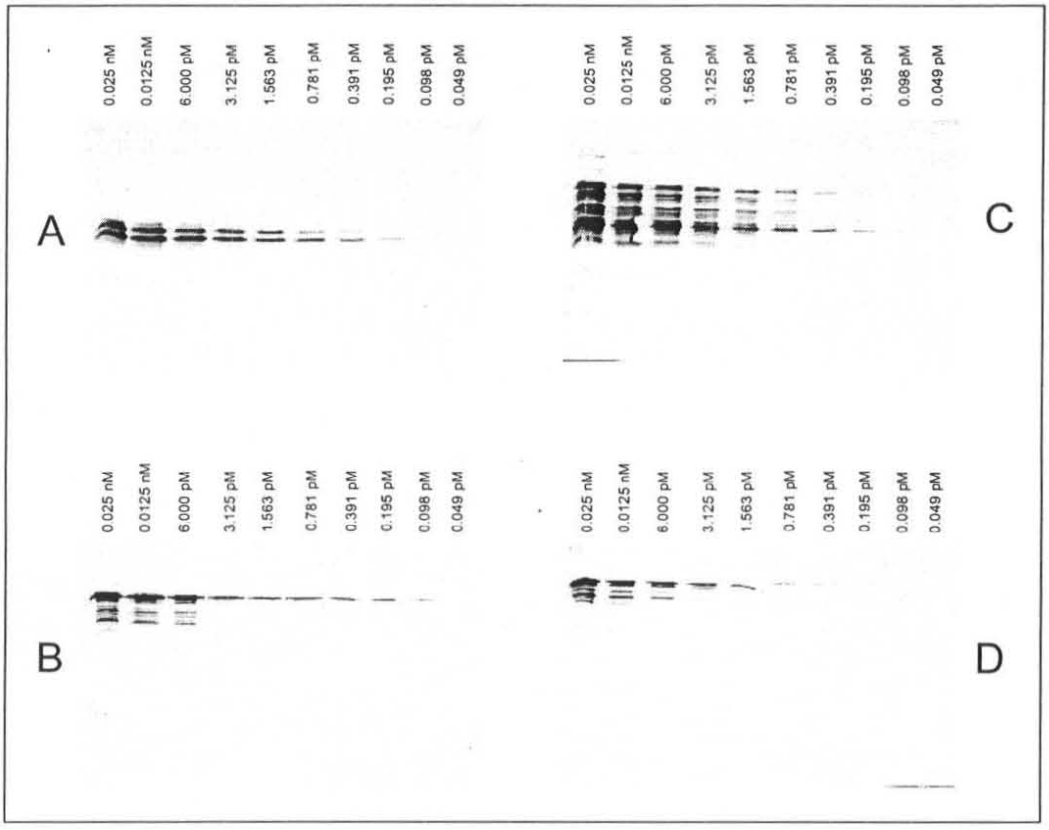

Figure 3.11 Western blot detecting recombinant calpastatin at a range of concentrations. Proteins possessing calpain inhibitory potential were assayed using the BioRad protein assay described in section 2.2.3.4, titrated to concentrations ranging from 0.025 nM - 0.049 pM, and loaded onto a 10 % SDS-PAGE gel. Specifically, domain 1 (**A**), domain 1 + GST (**B**), domains L+1 (**C**), and domains L+1 + GST (**D**) were loaded. These were transferred and probed with anti-domain 1 antibody (552) at 1:500 dilution.

Examination of the developed western blot shows relatively equal loading of all samples. The detection limit for both domain 1 (A) and domain L+1 (C) is at 0.195 pM and, consequently, these proteins are accurately quantified with respect to each other. Domain 1 + GST (B) is detectable at 0.098 pM which indicates that it may be present at slightly higher levels in relation to the other active calpastatin recombinants. Finally, domain L1 + GST (D) is undetectable below 0.391 pM and is probably under-represented in the inhibitory assay shown in Figure 3.10. However, since GST-DL1 already demonstrates more potency than GST-D1, this result strengthens the argument that domain L augments domain 1's activity.

Calculating the units of inhibition shows that the specific activity of D1 and DL1 was 27 units/mg total protein. Following thrombin digestion and boiling (10 minutes 95°C), calpastatin specific activity increased to approximately 7100 units/mg for domain 1 and 5000 units/mg for domain L1. This is a significant amount of activity. Bacteria are, therefore, a feasible vehicle to obtain large quantities of pure active protein which calpastatin characteristics. Furthermore, the results indicate that both active calpastatin fragments (D-1 & D-L1) exhibit the thermal stability previously reported for both full-length and derivative protein fragments of calpastatin. Consequently, in this work affinity-purified calpastatin fragments were subjected to heat treatment as a final purification step before being used as antigens for antibody production.

3.5 Generation of polyclonal antibodies

Polyclonal antibodies were generated by hyperimmunising rabbits with antigenic recombinant calpastatin as described in section 2.2.13. Briefly, two pairs of rabbits were injected over an 8-10 week period with either purified domain L or 1 recombinant protein emulsified with Freund's complete adjuvant. To confirm that a polyclonal antigenic response was occurring, blood samples were taken after 4 weeks, serum was collected and used as primary antibody in a Western analysis of antigen. In all instances an antigenic response was noted (data not shown). At the conclusion of the immunisation routine the animals were anaesthetised, sacrificed and bled out. Approximately 100–150 ml blood was collected. From this 70-100 ml serum was harvested and used without further purification as the primary antibody for Western analysis.

Immunological cross-reactivity of domain L and domain 1 polyclonal antibodies was assessed by Western Blotting (see section 2.2.14). Samples of antigen (ie. purified domain L and 1) were applied to an SDS-PAGE gel together with purified domain L1, the soluble protein fraction from untransformed *E. coli*, and GSH eluted GST, GST-L, GST-1 and GST-L1. After electrophoresis and transfer, membranes from four similar gels were blotted with serum from the four rabbits (see Figure 3.12).

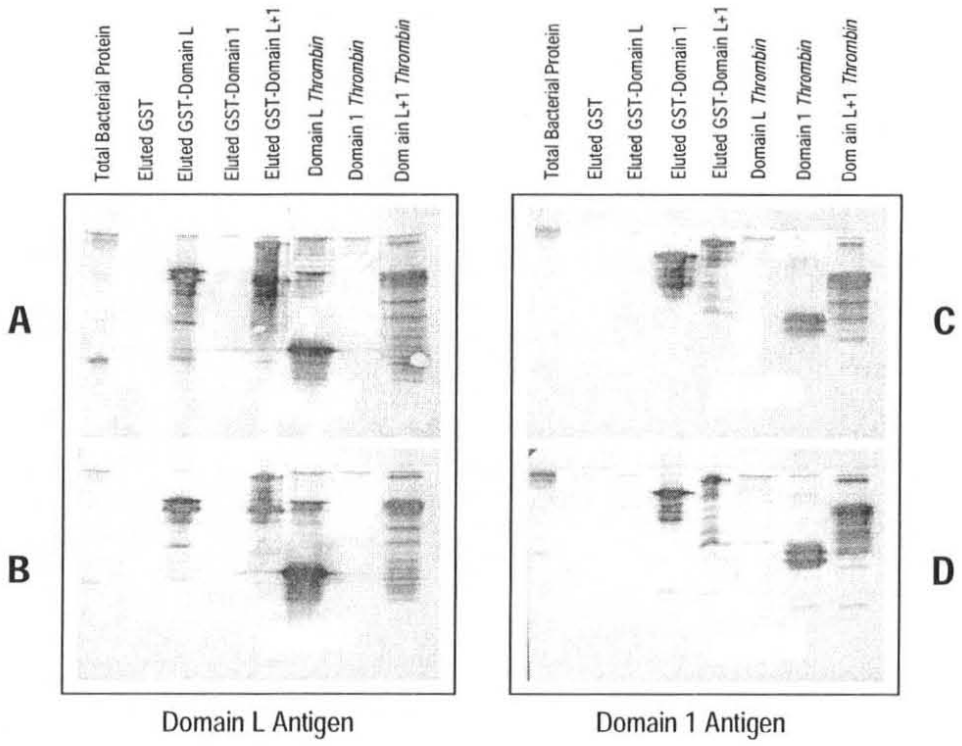

Figure 3.12 Western blots of cross-reactivity between the polyclonal antibody response from immunised rabbits. Lanes contain crude bacterial extract (1), GSH eluted GST (2), GSH eluted GST-L (3), GST-1 (4), GST-L1 (5), and thrombin digested domains L (6), 1 (7) and L1 (8). Serum (1:500 dilution) was used from rabbits 551 and 556 (A, B) immunised with pure domain L, and rabbits 552 and 557 (C, D) immunised with pure domain 1.

The affinity tag appeared to confer added stability to the recombinant protein. High product homogeneity in the GST fusion proteins is indicated by the presence of a single expressed band following extraction using a lysozyme/freeze-thawing approach (Figure 3.9). Previously, Takano *et al.* (1988) directly expressed full-length and partial fragments of pig calpastatin in *E. coli* and reported severe proteolytic degradation of the translation products during extraction using the lysozyme/freeze-thaw method. More recently, Hitomi *et al.* (1998) noted a significant degradation of recombinant calpastatin extracted through hypotonic disruption or sonication. In this work, the method of extraction did not appear to significantly disturb GST - fusion proteins although a degree of disruption might be indicated by the smear of reactivity seen in Figure 3.12.

Serum was validated for immunoreactivity against native calpastatin from sheep skeletal muscle prepared as described in section 2.2.3.3. Four duplicate gels were electrophoresed, protein was transferred and probed with one of the four serum preparations. The results from these western blots are shown in Figure 3.13.

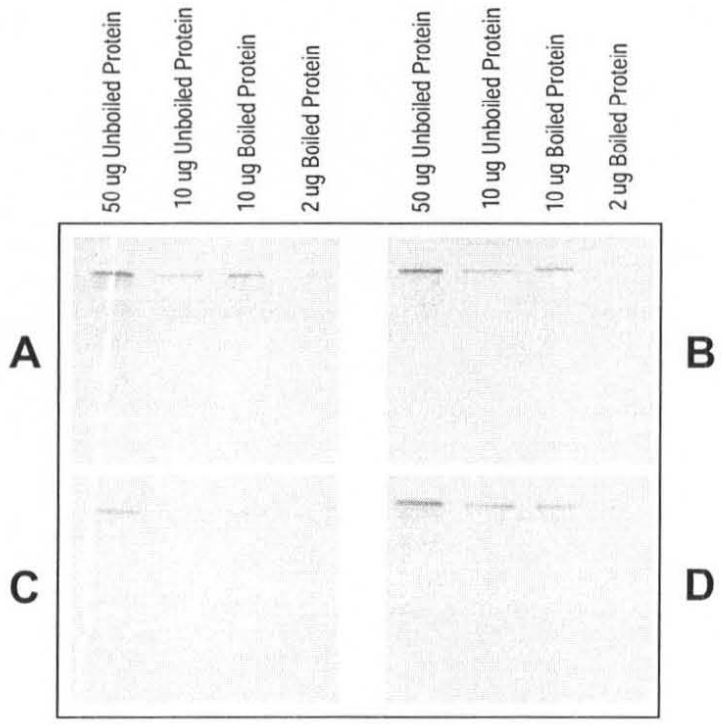

Figure 3.13 Western blots of demonstrating immunoreactivity of polyclonal anti-domain L and 1 antibodies against native ovine calpastatin. Soluble total protein was extracted from fresh ovine skeletal muscle as described in section 2.2.3.3 An aliquot was removed and heated at 100 °C for 5 minutes before being centrifuged at maximum speed in a bench top microfuge. The supernate was collected and, together with the unboiled soluble protein fraction, its protein concentration was determined. Samples were electrophoresed through a 10 % SDS-PAGE gel and transferred to nitrocellulose before being probed with either anti-domain 1 antibody 567 (A) and 522 (C), or anti-domain L antibody 566 (B) and 551 (D) at 1:100.

Figure 3.13 demonstrates that the four antibodies raised in these experiments specifically recognised a large protein in both boiled and unboiled ovine soluble protein extracts. Although the ideal confirmation would be from purified native calpastatin, the migration distance and the fact that the immunoreactive product is heat stable strongly indicates the band is calpastatin. In a similar experiment, Doumit

et al.(1996) used recombinant bovine calpastatin domains 2 – 4 expressed with GST to produce antibodies. When assessing immunoreactivity, he observed cross reactions with samples of muscle from beef, sheep and pigs. This serves to demonstrate the high degree of homology between calpastatin protein from different species.

3.6 Discussion

In order to cater for certain structural and biochemical analyses, sufficient quantities of purified calpastatin are required. This is also required in the instance when quantification of calpain or calpastatin is required in total protein units. Previously, native calpastatin has been isolated from various target tissues of different species using conventional techniques, specifically chromatography. Such techniques yield only small quantities from substantial starting raw material, requiring tedious processing. For example, Mellgren (1988) reported yields of 1 milligram purified calpastatin per kilogram of bovine heart. The methodology described in this chapter provides a useful and efficient approach for the production of large quantities of target calpastatin fragments in *E. coli* with yields of a few milligrams of purified recombinant protein per litre of bacteria culture.

Amplification and incorporation of ovine calpastatin cDNA sequences encoding domains L, 1 and L1 was successful. The three fragments of ovine calpastatin were efficiently expressed in *E. coli* strain BL21 as fusions together with GST using the highly efficient pGEX-4T-1 vector. Under the control of the IPTG-inducible tac promoter present in this vector, large quantities of soluble GST-fused calpastatin fragments were generated. After removal of the insoluble protein component, the recombinant fusion proteins were purified by affinity chromatography using glutathione-agarose.

Minor contamination of all three fusion proteins with a 70 kDa protein was observed. This contaminant was likely to be the *E. coli* chaperone DnaK which has been identified as a common impurity co-purifying with recombinant proteins (Hellebust *et al.*, 1990).

All three calpastatin fragments: DL, D1 and DL1, exhibited aberrant mobilities on SDS-PAGE. This is in agreement with previous reports of anomalous electrophoretic behaviour of calpastatin and its deletion derivatives (Takano *et al.,* 1988; Emori *et al.,* 1988). Examination of the primary amino acid sequence of DL, D1 and DL1 revealed a high proportion of charged residues and proline. Such unusual amino acid composition has been previously noted by other researchers to be characteristic of calpastatin in other species (Takano *et al.* 1988). It has been proposed that the abundance of charged residues and/or proline was responsible for the discrepancy between the calculated and observed molecular weight. The question of whether thrombin is responsible for the non-specific proteolysis of C-terminal domain 1 is unanswered. While the rate of appearance of truncated product is dose dependent upon thrombin concentration, there is no simple structure in the approximate region of cleavage that corresponds to thrombin's recognition site. In future, this problem may be avoided by using an alternative GST vector utilising an alternative protease recognition site, such as those recognised by Factor X (Smith & Johnson 1988) or protease 3C (Walker *et al.*, 1994).

There are several significant advantages of the GST fusion system. Firstly, consistent high yields of all three recombinant calpastatin fragments were obtained from a procedure that can be easily scaled up or down. Secondly, some stability may be provided by the GST tag, which serves to protect calpastatin during the extraction process. The inclusion of the *lacI*^{q} gene within the plasmid ensures tight repression of the *tac* promoter. Previously BL21 have been seen to perish due to strong protein expression (Miroux & Walker 1996). However, this self inhibition allows normal, unimpeded growth of bacteria until they reach the desired density, when addition of IPTG alleviates this restraint and permits expression. Finally, the immobilisation of calpastatin on an agarose column via the GST tag essentially provides a calpastatin affinity column. This could then be used to examine calpastatins interaction with other agents (eg. calpain) in a manner that has been successfully employed previously (Ma *et al.*, 1993).

Biological activity of recombinant calpastatin fragments was tested in calpain assays. Calpain was inhibited in a dose-dependent manner by DL1 and D1 fragments, whereas no inhibitory potential was apparent in the case of DL and control GST incubations. This agrees with numerous studies in which activity is ascribed to particular regions within domains 1 - 4, but no activity is attributed to the N-terminal domain L (Maki *et al.*, 1987a; Maki *et al.*, 1987b; Maki *et al.*, 1988; Ma *et al.*, 1993; Ma *et al.*, 1994). The relative activities before and after removal of the GST handle were compared. It was found that the attachment of GST reduced the potential activity of the calpastatin partner. The inhibitor activity of DL1 and D1 was not lost after heat treatment at 94°C for 20 minutes. In fact, this procedure appeared to increase the specific activity of these calpastatin fragments. Thus, heat treatment was found to be a useful ancillary step for purification of active calpastatin polypeptides produced in *E. coli*. This underscores the heat stability of calpastatin. A further advantage of the GST gene fusion approach is that GST activity can be assayed. This provides yet another tool for normalising the absolute amount of calpastatin added in calpain inhibition assays.

The immunoreactivity and specificity of the polyclonal antibodies was good. There was no cross reactivity between domain L and 1 antibodies with their respective antigen, nor was there any reactivity with GST or soluble bacterial protein. This indicates the procedure to prepare antigen free of contamination was highly successful. Part of this success is probably due to the boiling step which removes non-heat stable proteins. Finally, all four antibodies display specific immunoreactivity against ovine calpastatin.

Chapter 4

Characterisation of C2C12 fusion and transfection optimisation

4.1 Introduction

A growing body of evidence indicates the active involvement of calpains in fusion permissive events occurring at the plasma membrane (Cottin *et al.,* 1994; Hayashi *et al.,* 1996; Dourdin *et al.,* 1997). Calpastatin has the potential to influence the fusion process, through its action on calpain but there is relatively little information on the role of calpastatin in the fusion process. It is has been established that: (i) calpastatin mRNA and protein levels are down-regulated during fusion (Balcerzak *et al.,* 1998), (ii) calpain is associated with calpastatin during proliferation and fusion (Barnoy *et al.,* 1999) and (iii) microinjection of excess calpastatin prevents fusion (Temm-Grove *et al.,* 1999).

Several approaches are available to investigate the influence of calpastatin on fusion. A researcher may choose to examine the effects of agents that mimic calpastatin and inhibit calpain (Barnoy *et al.,* 1997; Barnoy *et al.,* 1998) or calpastatin may be introduced mechanically into an experimental system through microinjection (Temm-Grove *et al.,* 1999). A third approach is to manipulate calpain activity by introducing into cells expression plasmids encoding calpastatin (Huang & Forsberg 1998). This is arguably the least intrusive technique and provides the closest approximation to *in vivo* manipulation.

Since its early use as a marker for gene expression (Chalfie *et al.,* 1994), Green Fluorescent Protein (GFP) has been used as a cellular probe to monitor the expression and localisation of proteins *in vivo.* For excellent reviews of the properties, protocols and applications of GFP see Tsein (1998) and Chalfie & Kain (1998). GFP was discovered in its native form in the bioluminescent jellyfish *Aequorea victoria* and

converts blue light (emitted by the photoprotein aequorin) into green fluorescence (Morise *et al.,* 1974). GFP has been well characterised structurally (Ormo *et al.,* 1996; Yang *et al.,* 1996; Brejc *et al.,* 1997). The mechanism of its fluorescence has also been established (Boxer 1996; Youvan & Michel-Beyerle 1996).

The cDNA encoding GFP is commercially available and may be combined with cDNA's of interest to produce hybrid proteins (Rizzuto *et al.,* 1995). There are examples of GFP being introduced into plants (Haseloff & Amos 1995) Drosophila (Brand 1995) and mammalian cells (Pines 1995). A unique quality of GFP is that it only requires oxygen as a cofactor to generate its brilliant fluorescence. This has made it particularly versatile for use *in vivo* to monitor the movements of individual proteins (Pelham 1997; Presley *et al.,* 1997) and protein interactions (Ma *et al.,* 1996). Moreover, the proteins structure and chromophore has been carefully manipulated to generate several variants on the wild-type GFP including Red Fluorescent Protein (Matz *et al.,* 1999), Blue Fluorescent Protein (Heim & Tsein 1996), Yellow Fluorescent Protein (Ormo *et al.,* 1996) and the Enhanced Green Fluorescent Protein (EGFP) used in these experiments (Cormack *et al.,* 1996).

Chapter 4 reports two issues. Firstly, this chapter characterises "normal" myoblast behaviour. Specifically, it investigates the percentage of cells fused after induction of fusion and the typical changes in the level of distinctive proteins using Western Blotting. Secondly, in preparation for the experiments performed in Chapter 5 this chapter reports the construction of expression plasmids containing regions of calpastatin cDNA fused to EGFP. Chapter 4 also investigates the transfection of C2C12 myoblasts and the expression of a EGPF coding plasmid.

4.2 Characterisation of myoblast fusion

Myoblast fusion represents a useful model with which to examine developmental processes. It is possible to either purchase immortalised myoblasts such as C2C12, L6 and L8 cell lines or to extract a population of myoblasts from an embryonic source and construct a primary culture. The main advantages of using myoblast cell lines is that: (i) the populations are homogeneous, (ii) they may undergo

a significant number of mitotic cycles before perishing, and (iii) are usually well represented in the literature. The advantages of primary culture are the reduced cost, ease of access and, most importantly, the close physiological approximation to *in situ* myoblasts.

In the literature, both types of culture have been used to replicate fusion events using similar methods. Myoblast are usually propagated in a growth factor rich environment until they attain a density of 50 – 100 % confluence, then the media is changed to one containing lower concentrations of growth factors. This reduction in growth factors means that the myoblasts are no longer being forced to enter mitotic cycles and transcription of the myogenic regulatory genes can begin (Ludolph & Konieczny 1995). Failure to reduce the exposure of confluent cells to high levels of growth factors, causes cell overgrowth, depletion of nutrients, accumulation of waste products, loss of adherence and, ultimately, cell death (Temm-Grove *et al.,* 1999).

4.2.1 Fusion

Fusion and differentiation can be assessed in terms of morphology and/or the expression of characteristic proteins. The gross morphological change which characterises fusion is the formation of myotubes. These may be observed over a series of discrete time intervals and are most often quantified as "percent fusion". This value reflects the number of nuclei contained within myotubes divided by the total number of nuclei in the population. It is determined microscopically within a field of cells, by counting nuclei and subjectively classifying them into either "myoblast" (1 or 2 nuclei) or "myotube" (≥3 nuclei). Alternatively, a Flow Cytometer (or Fluorescent Automated Cell Sorter; FACS) may be used. This device is a laser based detection system and provides a multiparameter snapshot of individual cell characteristics. Specifically, the forward and side scatter of a laser beam, deflected by a single cell falling through space, reflects the cell size and complexity (granularity) respectively. For analysis by flow cytometry, cells are freed into a solution of PBS before being individually analysed by laser. This rapidly generates a description of the population. It is anticipated that large complex cells, representing myotubes, will

appear as a distinct sub-population over the fusion period and will be distinguishable from smaller myoblasts.

As described in section 2.2.15, C2C12 myoblasts were propagated in complete growth media (10 % Foetal Calf Serum (FCS) in Dulbecco's Modified Eagles Media (DMEM), 37°C, 5 % CO_2) where they were seen to double every 18 - 24 hours. Upon reaching confluence, the media was changed to differentiation media containing low levels of growth factors (2 % Horse Serum (HS) in DMEM). Within 24 hours of changing the media, multinucleate myotubes could be distinguished (see Figure 4.1).

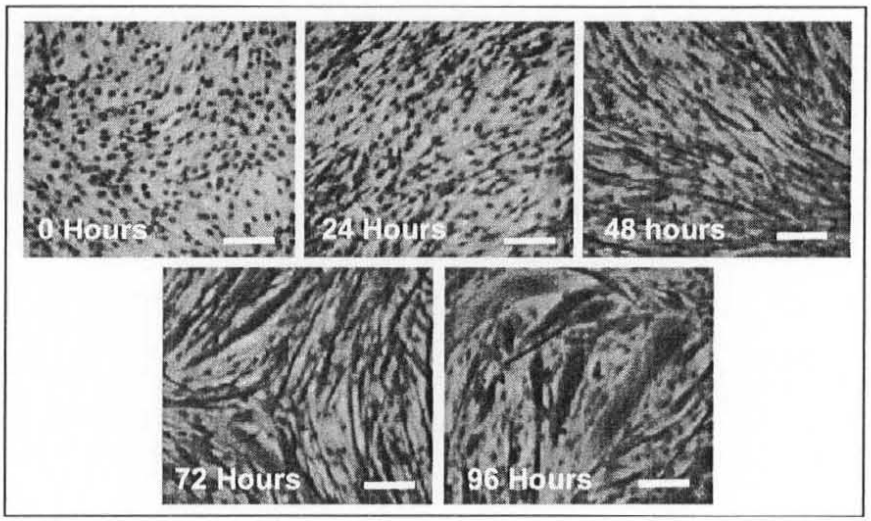

Figure 4.1. Photographs of myoblasts exposed to differentiation media. Times are expressed as hours after initial exposure. Cells grown on coverslips were fixed with methanol before being air dried and stained with Giemsa solution, bar represents 100 μm (400 x).

The conditions employed in these experiments appear suitable for the induction of fusion. Parallel alignment is evident by 24 hours and clear distinguishable multinucleate myotubes are visible by 48 hours. It is notable that the myotube nuclei aggregate within the cell structure and, generally, do not appear randomly distributed throughout the cytoplasm.

Fusion was quantified in two ways. Conventional microscopic cell counting demands that cells be grown on coverslips for ease of staining. FACS analysis requires cells to be freed from their growth surface into a solution of PBS which is drawn into the analyser tube. Cells are then rapidly (200 – 500 / second) quantified for size and granularity. The distribution of cells at 0 hours is defined as the typical distribution of myoblasts and called "region 1" (R1). At subsequent times, as fusion progresses, cells or events falling outside this defined region are observed. These events, which exhibit greater granularity and size, probably represent the myotubes and are contained in "region 2" (R2), as shown in Figure 4.2.

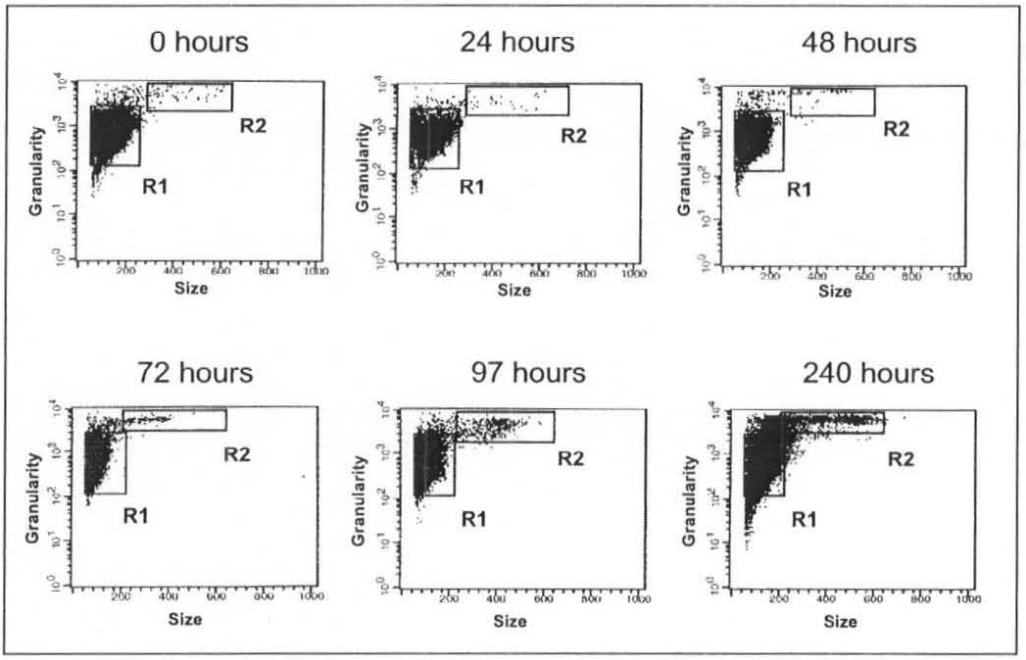

Figure 4.2 FACS results from cells harvested at 0, 24, 48, 72, 96 and 240 hours after induction of fusion. Each point on the scatter graph describes the side scatter (granularity) and forward scatter (size) of a single cell (event). Approximately 50 000 events are depicted in each graph. Events may be included in region 1 (R1) or region 2 (R2) groupings.

The majority of cells (> 99 %) at 0 hours are mononucleate myoblasts and are captured by the R1 "gate". The R2 gate was set to include the larger more complex cells. At the early time points (0 and 24 hours) there was little fusion and consequently the R2 group probably represents mitotically dividing myoblasts. The collection of events outside the R1 and R2 gates (especially those in the top left of

the plots) were not included in the analysis since their relatively small size and high complexity suggest they are dead or dying cells.

The fusion percentage determined by FACS was calculated by dividing the number of events in region 2 (R2) by the sum of all events (ie. R1 + R2 + non-gated events). Figure 4.3 displays the fusion trends determined by FACS and conventional microscopic cell counting approaches. It is apparent that conventional cell counting using coverslips produced a significantly different growth profile than the FACS method of determining fusion. Cell counting established that approximately 50 % of the nuclei were contained within fused myotubes by 96 hours. FACS analysis determined the fused percentage was only 1 %. Clearly, based on the microscopic observations, the FACS procedure grossly underestimated the extent of fusion.

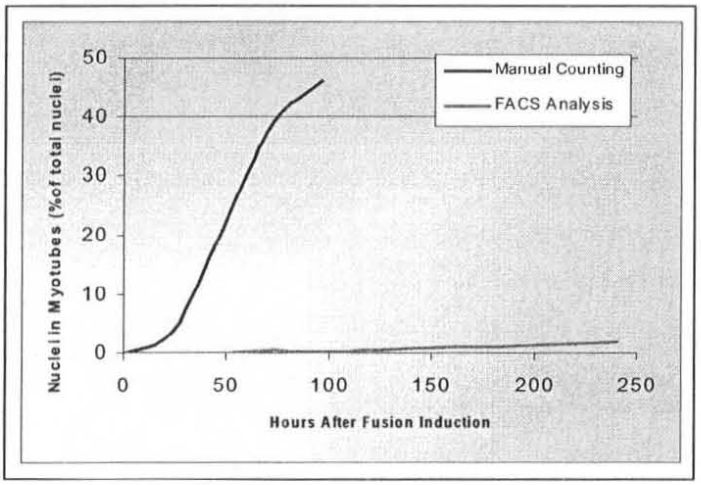

Figure 4.3 Graph comparing % fusion values determined by microscopic examination (manual counting) and FACS analysis. C2C12 myoblasts were grown to confluence in complete media, rinsed with PBS and media replaced with differentiation media to promote fusion.

C2C12 myoblasts growth and fusion rate has been compared by other researchers under a variety of culture conditions. It was observed that the most effective serum additive at promoting proliferation was 10 % FCS and 2 % HS was the most effective at promoting fusion (Temm-Grove *et al.*, 1999). The study also reported that with 2 % HS, fusion was initiated at 30 hours and after 65 hours between 50 and 60 % of the nuclei were contained within the myotubes. This was a

faster rate of fusion than was seen in this work. Namely, at 96 hours the fusion rate had declined and only 45 - 50 % of the nuclei were contained within the myotubes, as determined by microscopic examination. The difference between the studies could be due to several important variables. The most likely explanation is the variation in growth factors in the 10 % calf serum additive. Another explanation is the characteristics of the cell line under investigation. For example, rat L8 myoblast are a commonly used cell line. After growing L8s to 50 % confluence in DMEM supplemented with 15 % FCS, they are usually induced to fuse by changing the media to DMEM + 2 % HS and 4 units of insulin per 100 ml. After 24 - 48 hours of residual proliferation the cells reached confluence and by 72 – 96 hours the cells align and fusion starts. Between 120 – 168 hours 60 – 80 % of myoblasts have fused and formed myotubes (Barnoy *et al.,* 1996: Barnoy *et al.,* 1997). This process in the L8's is significantly slower than the C2C12 cells used in this work and illustrates the possibility of intrinsic but dissimilar biochemical qualities impacting upon fusion dynamics. Such idiosyncrasies are not be peculiar to cell lines. Primary cultures have also displayed variability in regards to the initiation and completion of fusion and to the extent that nuclei are distributed in myotubes (Cottin *et al.,* 1994; Elamrani *et al.,* 1995; Balcerzak *et al.,* 1998). A common theme is (i) the sigmoidal shape of the % fusion curve and (ii) the ultimate percentage of nuclei contained within myotubes is between 50 and 80 %. This latter observation indicates that over the time period examined a significant proportion of cells remain mononucleate.

FACS analysis does not seem to be a suitable technique for assessing the fusion percentage. Only 2 % fusion had occurred even after 10 days of culture in the differentiation media. This is a considerable underestimation relative to the manual cell count and is probably due to the change from adherent growth conditions to suspension in a liquid milieu. After extensive fusion, C2C12 myotubes appear as large as 1 mm in length (see Figure 4.1) and often multi-branched in structure. However, following trypsin digestion, the released myotubes altered from their elongated morphology into a more compact spherical form. It seems likely that the inability of the FACS to differentiate myoblasts from myotubes is largely due to the loss of their distinctive morphology under these circumstances and a failure to distinguish the subtle volume changes which accompany and are indicative of fusion. According to

microscopic examination, fusion approached a plateau at 96 hours. FACS analysis demonstrated the increase occurred between 96 and 240 hours. These differences may be attributed to the fact that the two approaches measure different parameters. The microscope quantifies the number of nuclei as a feature of cell type whilst FACS measures size and granularity of the cells. It is likely, therefore, that the increase in % fusion from 96 to 240 hours measured by FACS is due to a net increase in protein synthesis associated with the development of larger cells. These larger cells are then classified as myotubes on the basis of size and granularity and not because of significant additional fusion. This underscores the unsuitability of FACS analysis for the determination of % fusion compared to other techniques such as direct manual cell counting.

4.2.2 Protein indicators

Concomitant with fusion are fluctuations in protein content and specific changes in protein profile. For example, the release of [^{3}H] – tyrosine during protein degradation appears to decline by about 20 % in myotubes which are exposed to 10 % horse serum (Hong & Forsberg 1994) and stay repressed for 96 hours. Under the same conditions, protein synthesis can be stimulated and a net gain in total protein content can occur (Gulve & Dice 1989). A recent paper by Ertbjerg *et al,* (2000) the total protein content of C2C12 cells could be reduced by 16 % with the addition of epinephrine after fusion induction. Concomitant with the decline in protein content was a three-fold increase in assayable μ- and m-calpain, and a third increase in calpastatin activity. This indicates that calpains may be directly involved in hormonally regulated protein catabolism in myotubes.

It seems that once the drive for cell proliferation has been released, cells redirect their metabolic activity from the mitotic cycle into protein synthesis. Perhaps more significant, however, is the release of inhibition from myogenic regulatory factors such as MyoD1 and myogenin. These factors drive the expression of muscle specific proteins which biochemically distinguish the developing myotubes. Western blotting, which permits the identification and visualisation of specific proteins, allows the detection of muscle proteins which characterise the fusion period.

In this work, to enable Western analysis, cells were cultured to 100 % confluence in DMEM + 10 % FCS. Upon reaching confluence, the plates were washed twice with PBS and the media replaced with DMEM + 2 % HS to induce fusion. After addition of fusion media, the cells were collected at 0, 24, 48, 72 and 96 hours to provide a representative cross-section of fusion at different stages. Proteins were extracted, SDS-PAGE fractionated and used in Western analysis as described in sections 2.2.16, 2.2.4.2, and 2.2.14 respectively.

In a study by Dourdin *et al.* (1999), adherent myoblasts were grown for 36 hours under differentiation conditions, incubated for 30 minutes in PBS containing 1 % Triton X-100 and rinsed twice with PBS to leave the cytoskeletal remains. Electrophoresis of the harvested cytoskeletal remains revealed 7 major bands. The largest of the four bands were identified as myosin, vimentin, desmin and actin. In this work the representative myofibrillar proteins myosin, desmin and troponin T were selected as indicators of myotube differentiation. Myosin has been previously noted as a good indicator of biochemical differentiation (Okazaki & Holtzer 1966, Ludolph & Konieczny 1995) and desmin and troponin T as myogenic indicators. The latter, in addition, are substrates for calpain (Di Lisa *et al.,* 1995, Dourdin *et al.,* 1999, Geesink & Koohmaraie 1999). Desmin and troponin T may be useful to monitor since the objective of Chapter 5 is to perturb calpain activity which may alter the proteolysis of substrates such as desmin and troponin T. To investigate the calpain system thoroughly, μ-calpain, m-calpain, and calpastatin were also included in the Western analysis protocol.

4.2.2.1 Troponin T

Troponin T (TnT) is one subunit of the troponin complex which associates with actin and myosin and acts as a calcium sensitive switch during muscle contraction. TnT has been previously detected in C2C12 myoblasts 3 days after fusion induction (Crescenzi *et al.,* 1994). In this work, full sized TnT (35 kDa) was very faintly detected in the 0 hour samples and increased over the 96 hour fusion period (see Figure 4.4).

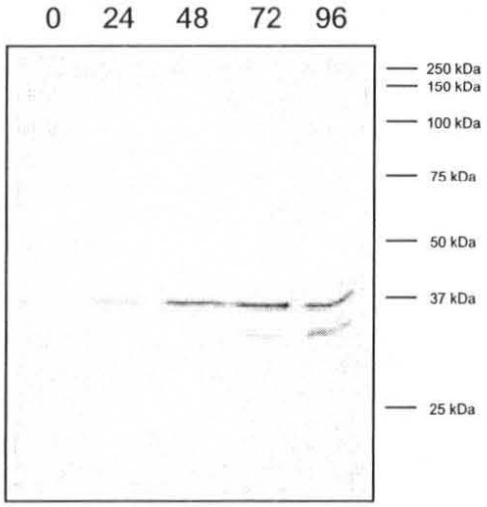

Figure 4.4 Troponin T levels over 96 hour fusion period. Approximately 100 ug of total protein extracted from C2C12's was electrophoresed through a 10 % SDS-PAGE gel and transferred to nitrocellulose before being probed with anti-troponin T antibody (CT3) at 1 : 100 dilution. An immunoreactive fragment appears below the full size troponin T at late stages of fusion.

In addition to full sized troponin T (35 kDa), immunoreactivity between the TnT monoclonal antibody (CT3) and a smaller fragment (approximately 30 kDa) became apparent after 48 hours. The intensity of the small fragment rapidly increased from 72 to 96 hours, while the full-sized TnT band remained relatively static. TnT is particularly susceptible to proteolysis by μ-calpain and, to a lesser extent, by m-calpain both *in vitro* and *in situ* (Di Lisa *et al.,* 1995). Calpain proteolysis of TnT produces several fragments with molecular weights from 27 to 30 kDa (Ho *et al.*, 1994). It is possible that the small fragment witnessed in this study were products of calpain proteolysis. The degradation of recognition epitopes may preclude, however, the detection of any additional fragments. Furthermore, the failure to detect the 30 kDa TnT isoform may be due to the absence of the putative proteolytic machinery responsible for its generation. TnT has been found to be expressed in C2C12 which have been prevented from fusing (Crescenzi *et al.,* 1994) demonstrating that TnT is not an indicator of fusion but an indicator of differentiation.

4.2.2.2 Desmin

Desmin (53 kDa) is a major protein subunit of muscle-type intermediate filaments. In mature muscle, desmin is found at the myofibril periphery and surrounding the Z-disc.

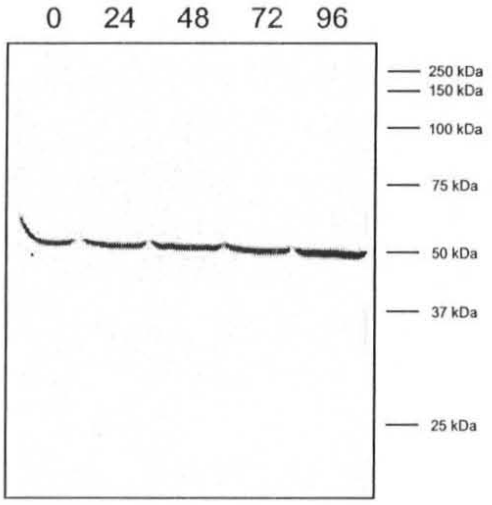

Figure 4.5 Desmin levels over 96 hour fusion period. Approximately 100 ug of total protein extracted from C2C12's was electrophoresed through a 10 % SDS-PAGE gel and transferred to nitrocellulose before being probed with anti-desmin antibody (D3) at 1 : 100 dilution.

In vitro studies on desmin extracted from primary muscle cultures have demonstrated that the proteolysis of desmin by m-calpain produces fragments ranging in size from 30 to 40 kDa (Dourdin *et al.,* 1999). In this thesis, the desmin antibody (D3) has successfully detected desmin in C2C12 extracts (Allen *et al.,* 1998) and, in other work, demonstrated the protective effect of the Callipyge phenotype (over-expression of calpastatin) on desmin degradation (Geesink & Koohmaraie 1999). Additionally, desmin has been used as a marker in primary cell preparations to differentiate between cultured muscle cells and contaminating cell types (Allen *et al.,* 1998; Temm-Grove *et al.,* 1999). In primary cultures of myoblasts, desmin levels at 36 – 44 hours (time of maximum fusion) decrease by 40 % compared to the levels at 20 hours and increase again when the rate of fusion declines (Elamrani *et al.,* 1995). In contrast, evidence in Figure 4.5 shows desmin levels increase by 300 % over the 96 hour culture period as myoblasts differentiate into myotubes. These differences may result from differing experimental procedures. Dourdin *et al.* (1999) and

Elamrani *et al.* (1995) used media containing 10 % HS to stimulate fusion in a primary culture and were able to demonstrate declines in desmin. The experiments described in this thesis used 2 % HS and an established cell line. The effect of the serum concentration is illustrated by the fact that 5 % HS is significantly less potent than 2 % HS at stimulating myoblast fusion (Temm-Grove *et al.*, 1999). By inference, 10 % HS may be even less effective. Moreover, primary cell cultures are at a disadvantage compared to cell lines, in that it is almost impossible to eliminate contaminating non-muscle cells which contribute their protein to total population extracts.

4.2.2.3 Myosin

Myosin (200 kDa) is one of the main protein components of muscle tissue where it functions as part of the contractile apparatus. Because myosin is only expressed in myotubes and myoblasts committed to myogenesis (Okazaki & Holtzer 1966) it has been used as a marker of myoblast differentiation (Ludolph & Konieczny 1995).

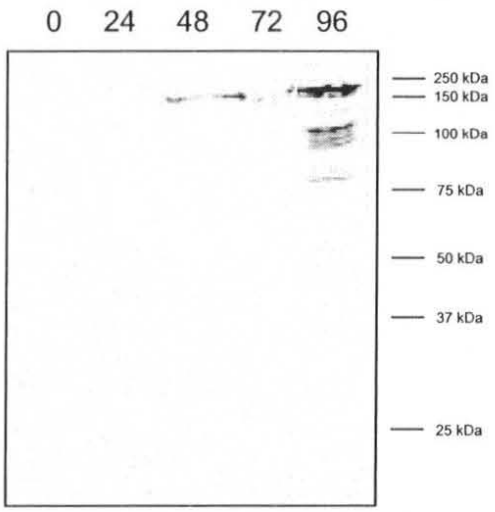

Figure 4.6 Myosin levels over 96 hour fusion period. Approximately 100 ug of total protein extracted from C2C12's was electrophoresed through a 10 % SDS-PAGE gel and transferred to nitrocellulose before being probed with anti-myosin antibody (MF20) at 1 : 2000 dilution.

In normal fusing C2C12 cells, myosin heavy chain protein was detected by immuno-fluorescence at 3 days (Fuentealba *et al.*, 1999). This agrees with the blot

shown in Figure 4.6, which initially detects myosin at 48 hours and shows myosin steadily increases over the 72 and 96 hours culture period. In a study by Hartley *et al.* (1991) the appearance of two myosin isoforms (embryonic and ventricular) in foetal chicken myoblasts and adult myotubes were compared. They found that in myoblasts, both myosin isoforms were expressed and that the expression was not prevented by eliminating fusion with EGTA. This suggests that the appearance of myosin indicates biochemical differentiation and not fusion. This assertion is supported by two other studies. Fuentealba *et al.* (1999) used antisense technology to indirectly hasten myogenin expression which resulted in accelerated myosin appearance, while Crescenzi *et al.* (1994) abolished C2C12 fusion by infecting cells with the *myc* oncogene without affecting the appearance of myosin. The anti-myosin antibody used in this thesis has been used successfully to detect myosin in extracts from primary muscle cell cultures (Hartley *et al.,* 1991) and in C2C12 cells (Crescenzi *et al.,* 1994).

4.2.2.4 μ-Calpain

μ-Calpain (110 kDa) is one of the two ubiquitous calpain proteases that is potentially involved in the fusion process. The appearance and expression of μ-calpain in the soluble cytosolic fraction is constitutive.

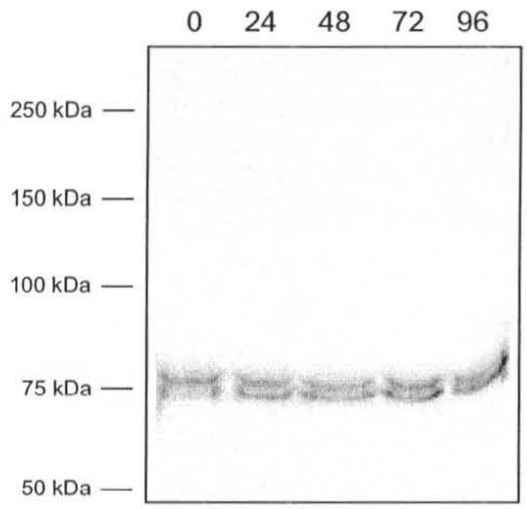

Figure 4.7 μ-calpain levels over 96 hour fusion period. Approximately 100 ug of total protein extracted from C2C12's was electrophoresed through a 7.5 % SDS-PAGE gel and transferred to nitrocellulose before being probed with anti-μ-calpain antibody (B2F9) at 1 : 25 dilution.

Several anti-μ-calpain antibodies were tested for their efficacy in detecting μ-calpain from C2C12. Antibody "2H2" is a monoclonal raised against domain II of human μ-calpain. The "Forsberg anti-μ-calpain" is a polyclonal antibody raised against the N-terminal sequence of human μ-calpain (SEEIITPVYCTGVSAQVQ KQRC; Forsberg personal communication). Neither of these antibodies demonstrated reactivity against C2C12 protein extract (data not shown). A third antibody, B2F9, was generated as a monoclonal against the 80 kDa subunit of native bovine skeletal muscle protein (Geesink & Koohmaraie 1999). As shown in Figure 4.7, this antibody successfully reacted against two protein bands of similar size and was, therefore, chosen for this investigation. The molecular weight marker indicates that these bands are approximately 78 kDa and 75 kDa in size. The double band pattern most likely represents full sized μ-calpain and an N-terminally truncated autolytic product. As noted previously, calpains undergo a two stage autoproteolysis with truncation of the 80 kDa isoform into 78 kDa and 76 kDa isoforms. The B2F9 antibody has been previously used to detect all three isoforms in ovine skeletal muscle extract (Geesink & Koohmaraie 1999). Close examination of the Western blot reveals a third band (which has not reproduced in Figure 4.7) about equal distance below the 78 kDa and 75 kDa bands, which probably represents the second

autolytic product. The slight size difference between the traditional 80 / 78 / 76 kDa isoforms and the 78 / 75 / 72 kDa shown in these data is probably due to inaccuracy in calibration against the molecular weight markers. There is no evidence in the literature of murine μ-calpain migrating at these small sizes. The intensity of the two bands apparent in Figure 4.7 increased over the 96 hour culture period which is a trend reported previously by Barnoy *et al.* (1996:1997). These authors observed that μ-calpain increases to 120 – 170 % of its initial level by 48 hours with no subsequent changes over the next 120 hour period. In contrast, some studies have detected μ-calpain in primary myoblast cultures only after 6 days of fusion (Cottin *et al.,* 1994) while others failed to detect μ-calpain in 50 % fused C2C12 extracts despite using large scale cultures (Temm-Grove *et al.,* 1999). In this latter study the authors concluded that the levels of μ-calpain were non-existent or fall below the sensitivity of the antibody.

The fact that this research was able to detect μ-calpain where other authors have failed suggests that attention should be directed at confirming the identity of the protein despite the validated specificity of the B2F9 monoclonal antibody (Geesink & Koohmaraie 1999). For this reason, samples of non-denatured protein were analysed for proteolytic activity using zymographic gels (see section 2.2.17) and transferred from non-denaturing gels for Western blotting as shown in Figure 4.8.

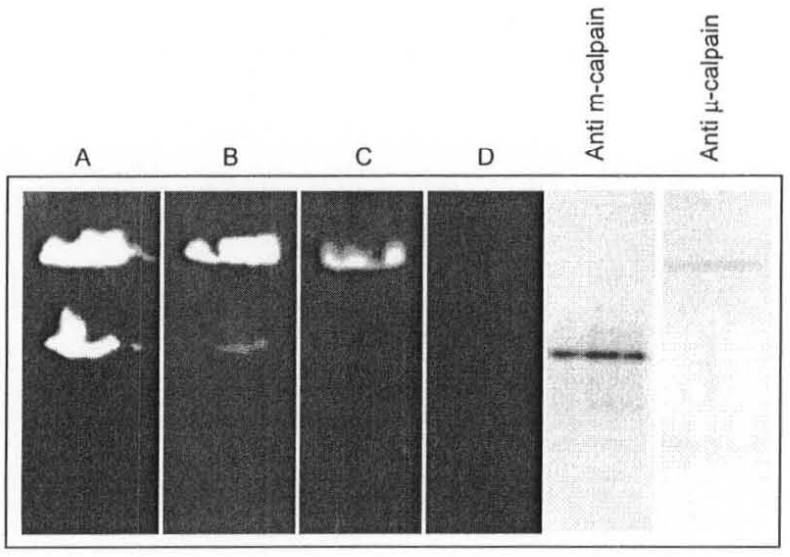

Figure 4.8 Casein zymographic gels and Western demonstrating activity and identifying μ- and m-calpain. Zymographic gels were loaded with 400 μg / lane C2C12 myoblast extract, gels were incubated with 400 mM CaCl2 (**A**), 100 μM CaCl2 (**B**), 10 μM CaCl2 (**C**) and 100 μM EDTA (**D**). Similarly loaded non-denaturing gels were electrophoresed and transferred, before being probed with anti-μ-calpain (B2F9) or anti-m-calpain (Forsberg anti-m-calpain) antibodies.

As has been previously shown, μ-calpain and m-calpain migrated differently through a non-denaturing gel milieu despite having similar sizes (Raser *et al.*, 1995). As a consequence of the greater negative charge carried by m-calpain (which is reflected in its late elution from weakly basic DEAE ion-exchange columns) m-calpain migrates further through the gel than μ-calpain. Its identity is indicated by the observation that proteolytic activity in this size range is essentially undetectable at calcium concentrations of 100 and 10 mM. Extra evidence is provided by the Western blot showing reactivity of anti-m-calpain antibody with the more negatively charged, high calcium demanding proteolytic activity, and reactivity between anti-μ-calpain with the proteolytic activity demanding low calcium concentrations. Note that the anti μ-calpain antibody reacted weakly against non-denatured μ-calpain relative to the denatured μ-calpain shown in Figure 4.7. This is possibly due to the fact that the original antigen was denatured μ-calpain large subunit which had been gel extracted from SDS-PAGE (Geesink - personal communication) and the epitope is less accessible to the monoclonal antibody, hence, the poor reactivity. The anti m-calpain antibody is, apparently, less affected by the non-denatured structure of its

target. This is not surprising because it is a polyclonal mixture of antibodies recognising many different epitopes some of which are likely to remain accessible. In conclusion, there is little doubt that both m- and μ-calpain are present in an active state in C2C12 myoblasts. Failure to detect these proteins with Western blotting may be a feature of sample preparation or antibody specificity.

4.2.2.5 m-Calpain

As noted by casein zymography the second ubiquitous component of the calpain system, m-calpain (110 kDa), is detectable in C2C12 extract.

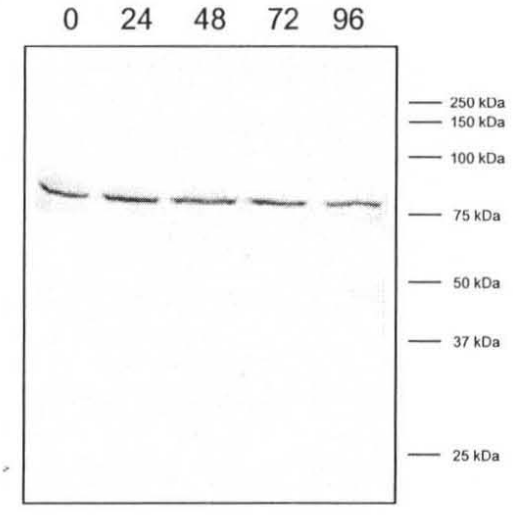

Figure 4.9 m-calpain levels over 96 hour fusion period. Approximately 100 ug of total protein extracted from C2C12's was electrophoresed through a 10 % SDS-PAGE gel and transferred to nitrocellulose before being probed with anti-m-calpain antibody (Forsberg anti-m-calpain) at 1 : 500 dilution.

Two anti-m-calpain antibodies were compared to determine which was the most suitable for detection of m-calpain in C2C12 extracts. Firstly, the monoclonal antibody 107-82 (Geesink & Goll 1995) which recognises domains III and IV of the large subunit was tested. In these studies this antibody displayed no immunoreactivity with C2C12 extract (data not shown), which contrasts to the experimental results of Temm-Grove *et al.* (1999) who found this antibody successfully reacted with C2C12 extracts. Differences in antibody dilution factor and protein load fail to explain these

differences suggesting that sample preparation may be a factor. An anti-m-calpain polyclonal antibody raised against mammalian large subunit N-terminal 18-mer polypeptide (Huang & Forsberg 1998) was also tested. This presented a clear banding pattern with no major cross-reactivity and was chosen for all subsequent experiments in this work. Western blots revealed, that from 0 – 24 hours m-calpain levels increased by 220 % and remained elevated from 24 – 72 hours but fell to 150 % of the initial levels at 96 hours (Figure 4.9). This result has been mirrored in previous experiments on C2C12's by Temm-Grove *et al.* (1999) where m-calpain levels paralleled the fusion rate. Levels, as measured by Temm-Grove *et al.* (1999), increased from 79 arbitrary units (au) to 111 au, and then decreased to 47 au. This has also been reported by Cottin *et al.* (1994) who observed a large increase in m-calpain from days 2 – 6 (18 – 100 au) and a decrease at day 8 to 70 au. Again these changes closely paralleled the fusion dynamics which showed a rapid but tapering fusion from 2 to 6 days, and a decline in fusion by day 8. In contrast, Barnoy *et al.* (1996:1997) found that the levels of m-calpain increased between 0 and 48 hours to 130 – 160 % of their initial levels. There were no subsequent significant changes. Kwak *et al.* (1993a) also observed a significant increase in m-calpain levels over 72 hours without a subsequent decline.

The fact that some of the researchers failed to observe a reduction in m-calpain, as observed in this thesis, could be due to differences in cell type and/or growth conditions. Furthermore, it is impossible to determine whether the decline in m-calpain is actually occurring in the studies or if the decline is masked by significant asynchronous fusion events. In addition, there are two factors which should be considered when attempting to accurately measure m-calpain.

First, Temm-Grove *et al.* (1999) found that a large proportion of m-calpain extracted from C2C12's existed as a large aggregate and failed to enter the separating gel, unless the percentage of β-MCE in the loading buffer was increased from 5 % to 8 %. The authors, however, did not speculate whether this phenomenon is a feature of the extraction process or a characteristic of *in situ* events. The β-MCE concentration of protein samples used in this thesis was < 1 %. There was no evidence of large immunoreactive protein failing to migrate into the gel.

Second, Brustis *et al.* (1994) detected m-calpain by immuno-fluorescence and found it was unevenly scattered around non-permeabilised myoblasts at 60 hours after fusion induction. The fact that the cells had not been made permeable to antibodies lead the authors to conclude that the detected m-calpain was externalised. This conclusion was reinforced by electronmicrographs which clearly depicted anti-m-calpain antibody associated with the extracellular matrix of myoblasts induced to fuse (Dourdin *et al.*, 1997). In summary, obtaining an accurate sample of m-calpain in a PAGE gel requires attention to sample preparation and may account for some of the differences reported in the literature.

4.2.2.6 Calpastatin

This thesis is built upon the premise that by manipulating levels of calpastatin it may be possible to alter myoblast fusion. For this reason several anti-calpastatin antibodies were tested to assess their abilities to detect endogenous calpastatin in non-heated and heated soluble protein extracts from murine muscle. Ovine samples were included as controls. The four polyclonal antibodies generated in Chapter 3 were tested along with monoclonal antibodies 1F7 and 3B9 (see Figure 4.10).

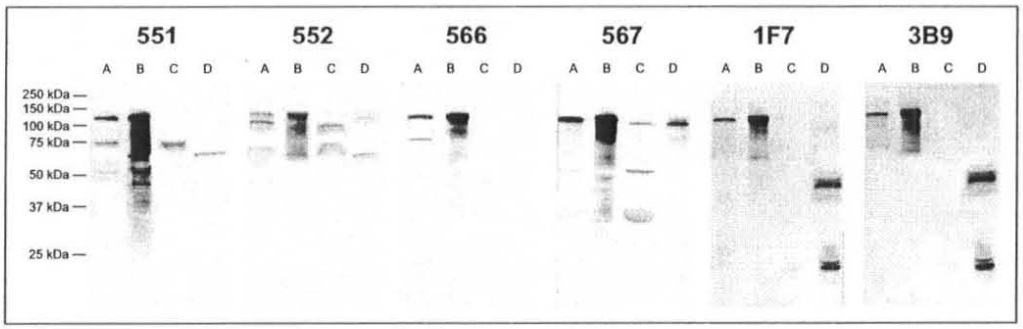

Figure 4.10 Western blots performed to determine effectiveness of anti-calpastatin antibodies. SDS-PAGE gels (10 %) were loaded with 50 µg total soluble protein from ovine skeletal muscle (**A**), 7.5 µg heated total soluble protein from ovine skeletal muscle (**B**), 50 µg total soluble protein from murine skeletal muscle (**C**) and 7.5 µg heated total soluble protein from murine skeletal muscle (**D**). Following electrophoresis, proteins were transferred for Western analysis using polyclonal antibodies produced from recombinant ovine domain L (551, 566; 1:100) and domain 1 (552, 567; 1:100), and using monoclonal antibodies raised against human domain 4 (1F7; 1:5000) and bovine domains 2 - 4 (3B9; 1:100).

Monoclonal antibody 1F7 was raised against domain IV of human calpastatin (Thompson & Goll, unpublished data). In these blots the antibody recognised a 120 kDa heat stable protein, which is likely to be calpastatin, from ovine tissue but was not able to detect this protein in total murine extract or in heated murine extract. In the latter sample, two pairs of similarly sized bands at approximately 50 kDa and 20 kDa were detected. An almost identical blot was obtained using monoclonal antibody 3B9 (Doumit & Koohmaraie 1999) which was raised against an undefined region of recombinant bovine calpastatin domains 2 - 4 (Doumit *et al.,* 1996). Again, immunoreactivity was seen with a 120 kDa protein in non-heated and heated ovine skeletal muscle extract, no reactivity was seen in non-heated murine extract. Two pairs of bands (50 kDa and 20 kDa) reacted in the heated murine extract. Monoclonal antibody 1F7 has been successfully used by Temm-Grove *et al.* (1999) to detect a 137 kDa isoform of calpastatin in C2C12 extract. Therefore, it is surprising that this antibody failed to detect anything in non-boiled murine extract or in C2C12 extract which was also tested (data not shown).

As noted, the only murine proteins recognised by the 1F7 and 3B9 antibodies were two pairs of closely related proteins at 50 kDa and 20kDa. Both were only detectable only in the heated extract. The main effect of heating is to purify the soluble extract. By loading the maximum allowable volume of calpastatin enriched extract onto a gel the detection of calpastatin is enhanced. It is possible, therefore, that these smaller bands represent calpastatin fragments which are present below the levels of detection in the non-boiled extract. These fragments may result from proteolysis of calpastatin. Notably, calpain has been demonstrated to have the ability to degrade calpastatin into fragments ranging from 29 kDa to 100 kDa (Doumit & Koohmaraie 1999). However, the nature of these bands remains uncertain and neither 1F7 or 3B9 appear suitable antibodies to detect full length calpastatin from murine tissue.

The four polyclonal antibodies described in Chapter 3 were also investigated for their efficacy in detecting murine calpastatin. As demonstrated in Figure 3.13 each of these antibodies was effective in detecting a 120 kDa heat stable protein in ovine samples. With respect to murine extracts their immunoreactivity varied.

Antibody 567 was able to detect a protein at approximately 110 kDa in both heated and non-heated samples. A recent paper by Takano *et al.* (2000) reports that an anti-calpastatin polyclonal antibody recognised two major bands (70 kDa and 110 kDa) in mouse testis, although they suggest that the smaller band is specific to this tissue. The relatively slight size difference between the immunoreactive ovine protein (120 kDa) and this immunoreactive protein is similar to other reported species specific differences (Takano *et al.,* 1986a). Antibody 552 which, like 567, was raised against recombinant domain 1 detected a 110 kDa protein, although only in the heated extract. As already mentioned, heating enriches the sample in favour of calpastatin. The lack of reactivity in the non-heated sample may be simply due to poor sensitivity. Antibodies 551 and 566 were also not successful in detecting 110 kDa proteins in either the heated or non-heated samples. All these results show that antibody 567 is suitable for the detection of full length murine calpastatin. The Western analysis of C2C12 extract using this antibody is shown in Figure 4.11.

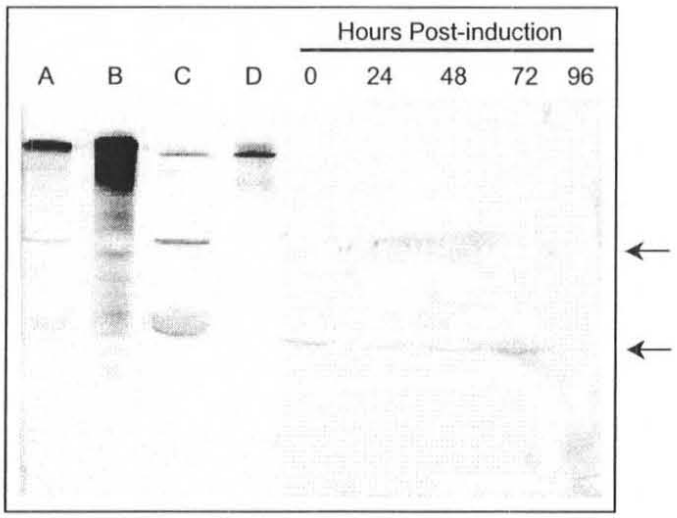

Figure 4.11 Calpastatin levels over 96 hour fusion period. Controls were included which contain: 50 μg total soluble protein from ovine skeletal muscle (**A**), 7.5 μg heated total soluble protein from ovine skeletal muscle (**B**), 50 μg total soluble protein from murine skeletal muscle (**C**) and 7.5 μg heated total soluble protein from murine skeletal muscle (**D**). Approximately 100 ug of total protein was extracted from C2C12's at from 0, 24, 48, 72 and 96 hours post-induction and, together with controls, was electrophoresed through a 10 % SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-calpastatin antibody (567) at 1 : 100 dilution. Immunoreactive bands in C2C12 extract are arrowed.

Figure 4.11 shows that the Western with antibody 567 failed to detect calpastatin in C2C12 extract. This demonstrates that full length murine calpastatin is either: (i) absent or (ii) present at very low levels or (iii) structurally different from native *in vivo* ovine and murine calpastatin. Two proteins were detected by antibody 567 (arrowed). These proteins correspond to the non-heat stable proteins detected in non-boiled murine extract and are unlikely to be calpastatin isoforms. Previously, and under different experimental conditions, Barnoy *et al.* (1996:1997) found that calpastatin levels declined to 20 - 30 % of their initial levels at the start of fusion (72 - 96 hours) and then increased to 70 - 80 % of the initial levels by 120 hours. This was also observed by Balcerzak *et al.* (1998) who, using a different cell preparation, observed a 60 % reduction in calpastatin protein at the time of maximum fusion and an increase back to normal levels after fusion was completed. In contrast, Temm-Grove *et al.* (1999) observed that calpastatin levels increased as cells passed from the proliferative to the fusion stage and continued to increase to the point at which fusion was represented by 50 % nuclei in the myotubes. It is not possible to speculate as to the cause of these experimental differences. Cell type, antibody specificity and growth conditions can all be contributing factors.

4.2.3 Conclusions

Several useful indicators of differentiation have been confirmed. Myosin, Troponin T and desmin all accrue in response to biochemical determination of C2C12 myoblasts. These modifications to the proteome are mediated by reducing the growth factor concentration in their immediate surroundings. An important point to note is that these proteins are markers of biochemical differentiation and are not representative of fusion.

Troponin T and desmin are both substrates for calpain. Proteolytic fragments of these proteins may provide a measure of calpain activity *in vivo*. With respect to desmin there is no evidence of proteolytic fragments in C2C12 extract. Previous work has used a monoclonal antibody (D3) in desmin blots and detected fragments from calpain proteolysis (Geesink & Koohmaraie 1999). Failure of D3 to detect proteolytic fragments in this work indicates a lack of proteolysis rather than an

inability of the antibody to recognise its epitope. The troponin T blots revealed the existence of a fragment resulting from its degradation. While the size of this fragment is similar to some of those generated by calpain *in vivo* (Geesink & Koohmaraie 1999), it cannot be concluded that calpain is responsible for the proteolytic products. While the appearance of a 30 kDa TnT fragment does not specifically coincide with the presence of either μ- or m-calpain, it is of interest to examine the dynamics of this fragment in cells transfected with calpastatin variants.

With respect to the calpain and calpastatin system, several differences are evident between published results and the data obtained in these experiments. Essentially, both μ-calpain and m-calpain were detected in C2C12 extracts at all time periods during the fusion process. μ-Calpain clearly displayed the products of autolysis which is evidence of its activity *in vivo*. Autolytic products of m-calpain were not discernible in the Western blots although Brown & Crawford (1993) noted that the autolytic products of m-calpains large subunit were difficult to resolve on SDS-PAGE. Both proteases are present in an active native form as demonstrated by casein zymography.

Endogenous calpastatin was not detected in the C2C12 extract. Several antibodies were examined for their ability to detect calpastatin but all were unsuccessful despite some having immunoreactive responsiveness to C2C12 or murine skeletal muscle extracts. It was concluded, that endogenous calpastatin is either absent or present below the levels of detection.

4.3 Construction of calpastatin – EGFP expression plasmids

The second issue this chapter addresses is the functionality of calpastatin domain L and its inhibitory regions 1-4. Reports suggest that domain L may facilitate the localisation of calpastatin to the plasma membrane. This could be an important consideration with respect to myoblast fusion, particularly if calpain is active in this region. To this end, plasmids were designed which would express domains of calpastatin with attached EGFP protein. A vector permitting C-terminal attachment

was chosen to ensure that the interactions between domain L and any potential membrane components would not be disrupted.

As described in Chapter 3, an appreciation of the ovine calpastatin cDNA sequence (Appendix B) facilitated construction of PCR primers which specifically amplify domains L and 1 or, when used in combination, domains L+1. Again, primer sequences were designed to include restriction enzyme sites which match those contained in the multiple cloning site of the GFP vector. After amplification and restriction digestion, sticky-ended ligation allowed insertion of the amplimer into the vector, in both the correct orientation and reading frame with respect to the C-terminally attached GFP. Figure 4.12 provides a schematic overview of the process.

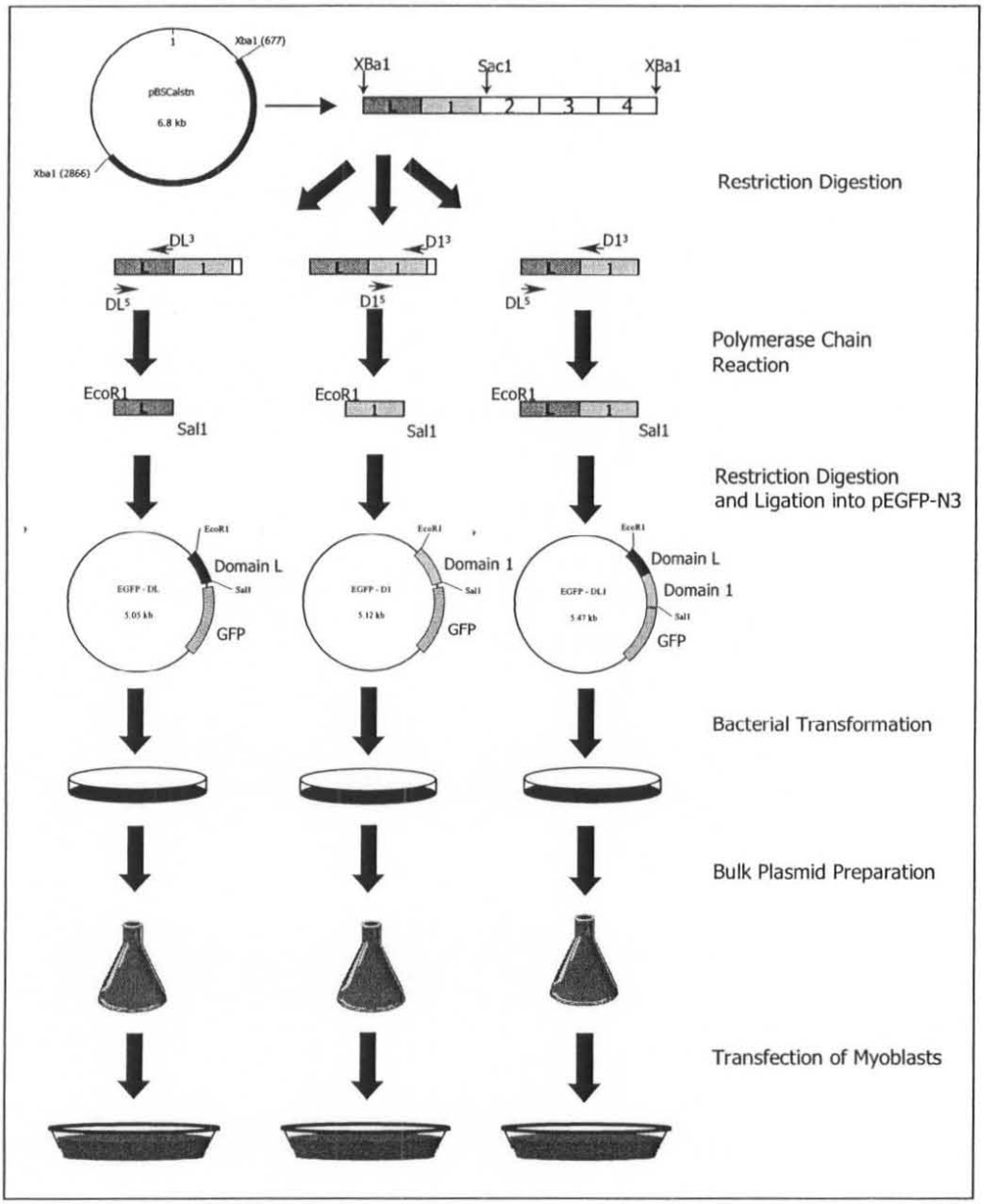

Figure 4.12 Construction of GFP-calpastatin plasmids. Ovine calpastatin cDNA contained within pBluescript II KS+ plasmid (pBSCalstn) was extracted from transformed bacteria, and purified. Digestion with Xba1 and Sac1 released a fragment containing domains L-1; this was used as template DNA in PCR reactions. Three reactions were performed using primer combinations of DLGFP$^{5'}$ & DLGFP$^{3'}$, D1GFP$^{5'}$ & D1GFP$^{3'}$ and DLGFP$^{5'}$ & D1GFP$^{3'}$, generating amplimers of domains L, 1 and L+1 respectively. The PCR products were gel purified and digested with restriction enzymes to generate sticky-ended products. These could then be ligated into a similarly digested pEGFP-N3 vector. The resultant construct is then suitable for introduction into *E. coli* for propagation. Following extraction, plasmid is purified and transfected into myoblasts to achieve expression of recombinant fusion proteins.

4.3.1 Template preparation

Generation of calpastatin-GFP encoding constructs starts with the preparation of a template DNA embracing the domain L and 1 regions. This was performed as described in section 3.2.1. Briefly, a double digestion of pBSCalstn using the restriction enzymes Xba1 and Sac1 produced three fragments. The largest of the fragments represents the pBSKS vector. The smaller fragments represent the totality of the calpastatin insert. Agarose gel electrophoresis and DEAE membrane extraction permitted isolation of the smaller 862 bp fragment containing domains L and 1 and a short sequence of domain 2. After purification, band homogeneity and size was confirmed by gel electrophoresis and the fragment was used in PCR. Quantity and purity of DNA template was determined according to section 2.2.6.

4.3.2 Amplification of calpastatin domains

Three PCR reactions were performed as described in section 2.2.9 using the 862 bp fragment as template DNA and the primers shown in Figure 4.13.

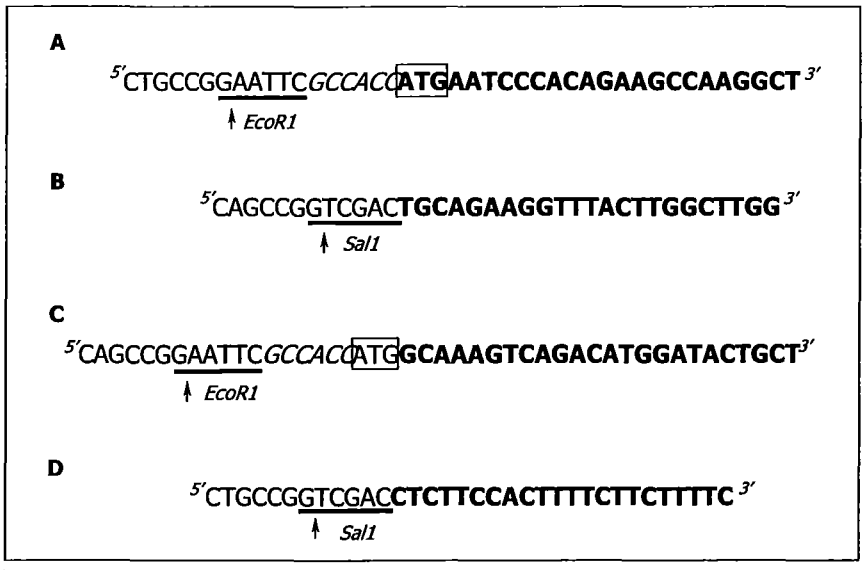

Figure 4.13 PCR primer sequences used for the amplification of domains L and 1 prior to insertion into pEGFP-N3. Bold nucleotide sequence is homologous to ovine cDNA, boxed "ATG" represents upstream start codons, restriction enzyme locations are noted together with cut points (arrowed), italicised nucleotides represent a Kozak consensus translation initiation site. Remaining nucleotides are non-homologous to ovine cDNA but are included to provide an "overhang" region.

In producing primers for the construction of GST-calpastatin constructs there was no need to include start codons because the upstream GST cDNA contained its own ATG sequence. In this instance polymerase activity would continue downstream through the calpastatin cDNA until reaching a stop codon; accordingly such codon sequences were included in both 3' primers (see section 3.2.2). In the instance of pEGFP constructs, the translation product will be N-terminal calpastatin with C-terminally attached EGFP. Thus, it is necessary to guarantee the presence of appropriate start codons (ATG) at the commencement of the calpastatin coding sequence and the absence of intervening stop codons. In the case of domain L, a start codon is recognisable which demarcates the beginning of the domains translated sequence (see Appendix B). After scrutinising domain 1, an in-frame ATG was found at nucleotide position 10. However, for completeness it was resolved to include a start codon immediately upstream of the domains first codon. This ensures the inclusion of the three amino acids which would otherwise have been lost. Furthermore, the context of the ATG at nucleotide position 10 was not characteristic of the archetypical Kozak consensus translation initiation sequence. The Kozak sequence (5'...CC$^{A}/_{G}$CC**ATG**G...3') is known to be present in the majority of eukaryotic sequences and be the peptide chain initiation site (Kozak 1987). To accentuate the desired peptide initiation site in the EGFP constructs, Kozak sequences were included before the start codons in both 5' primers. No stop codons were found within either domains coding sequence. Thus, termination of translation would be effected by the GFP cDNA stop codon.

After PCR, amplimer fragments were gel electrophoresed and extracted using DEAE membrane, ethanol precipitated, and digested with EcoR1 and Sal1 to produce sticky-ends. The pEGFP-N3 expression vector (see Appendix A.4) was similarly digested before gel fractionation and ethanol precipitation. After precipitation, all products (PCR amplimers and digested pEGFP-N3) were subjected to phenol / chloroform extraction (see section 2.2.2.3) and quantified prior to ligation.

4.3.3 Ligation and transformation

PCR products were ligated separately into a digested pEGFP-N3 vector and the resultant products used to transform competent DH5α *E. coli* (see section 2.2.1). Bacteria were selected through their resistance to kanamycin antibiotic encoded by their plasmid. After 24 hours the plates were examined. Based on "blue-white" selection the procedure appeared to have been 100 % successful in producing plasmids containing inserts. The plasmid DNA from 5 colonies peculiar to each transformation treatment were harvested according to the miniprep protocol (section 2.2.2.1). After a double restriction enzyme digestion designed to remove the insert, the nature of the insert was assessed by gel electrophoresis. Bacterial colonies grown from individual bacterium successfully transformed with each of the three plasmids, were used to inoculate volumes of "Terrific Broth". A very high purity plasmid DNA was extracted from these bacteria according to the midiprep protocol (see section 2.2.2.2) and used in subsequent transfection experiments. DH5α strain *E. coli* were used since they have a defective *endA1* gene (Grant *et al.,* 1990*)*. The absence of this gene product (endonuclease 1) is thought to improve the quality of plasmids, and is a vital concern for successful transfection.

The positioning of the inserted DNA within the multiple cloning site means that there are 36 bp (12 amino acid residues) between the C-terminus of calpastatin and the N-terminus of GFP. These so-called "linker" regions can have significant effects upon the stability of the green fluorescent protein fusion proteins. Recently, Prescott *et al.* (1999) examined the effect of different lengths of linker regions (23 - 28 bp = long linker, and 5 – 7 bp = short linker) on GFP fusion proteins. While a long linker limited degradation of one fusion partner, a short linker seemed to favour another. Thus, while there are no rules on the ideal linker length between different fusion partners, an optimum length should be investigated when designing plasmids.

4.3.4 Sequencing data

As a final confirmation, the plasmids generated by this work (pEGFP- DL, pEGFP-D1 and pEGFP-DL1) were sequenced using the primers originally used for

the amplification of respective inserts. Once again, the structural organisation of these sequences was similar to the reported ovine calpastatin cDNA sequence and to other calpastatin sequences when submitted to GenBank for comparison. The detailed sequence data depicted in Figure 4.14 is provided in Appendix D.

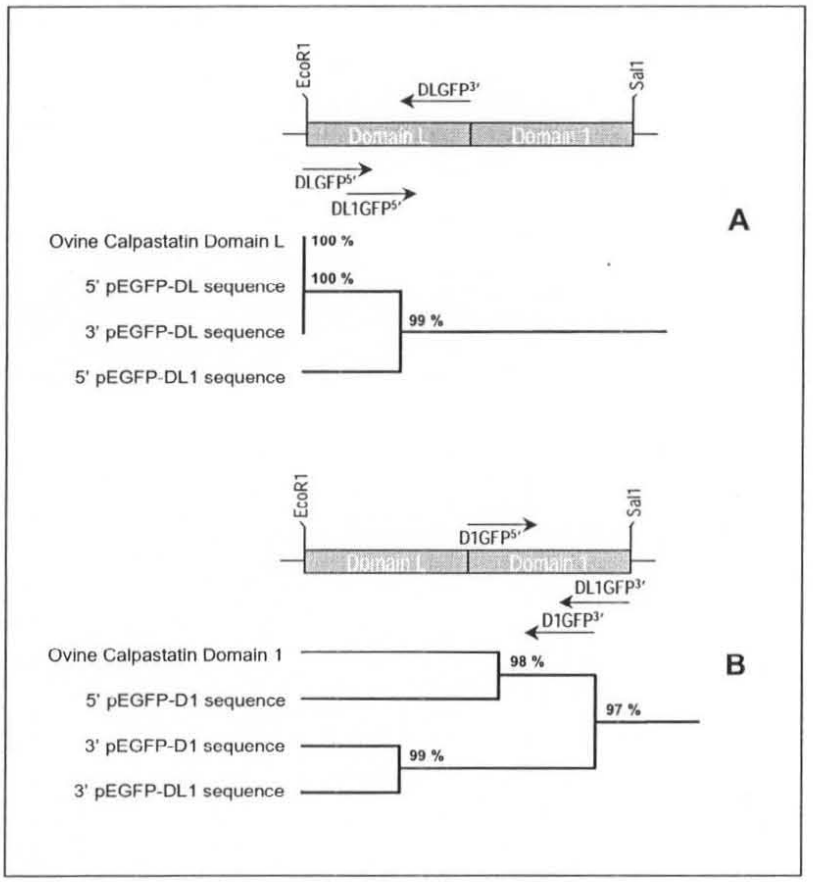

Figure 4.14 Homology trees comparing insert sequence data with domains of ovine calpastatin. Ovine calpastatin domain L compared to 5' sequence of inserts encoding domain L and domains L + 1, and the reverse complementary sequence of 3' sequenced domain L. Ovine calpastatin domain 1 compared to inserts encoding reverse complementary sequences of 3' sequenced domain 1 and domain L + 1, and 5' sequenced domain 1. Percentage homology between closest pairings is displayed.

4.3.5 Summary

Regions of the ovine calpastatin cDNA sequence, corresponding to domains L and 1, have been PCR amplified separately and, in combination, to produce cDNA for domain L, 1 and L+ 1. Separately, the three sequences have been incorporated

into a mammalian expression vector for protein expression. The recombinant product will consist of domains of calpastatin and C-terminally attached GFP.

4.4 Optimisation of C2C12 myoblast transfection with FuGENE 6 reagent

Transfer of DNA into eukaryotic cells is a standard technique in molecular biology. Mechanical, electrical or retroviral methods have been used to accomplish this transfer. Protocols which employ "uptake – enhancing" chemicals have been substantially refined and are now commonly used. The basic principle is to form a complex from negatively charged DNA and positively charged chemicals (usually polymers). Subsequently, the complexes are taken up by the cell through endocytosis and subjected to lysosomal processing. Entrapped DNA is released from the complex, either before or after entering the nucleus, and gene transcription takes place.

The most common examples of this approach are protocols using calcium – phosphate or cationic lipid. In the former, after mixing $CaPO_4$ and DNA in a HEPES buffer, a precipitate is formed and taken into the cells by an endocytic mechanism. With cationic lipids, an association is formed between the lipids and the negatively charged DNA which produces a net neutral or positive charged complex. The complex then associates with the negatively charged cell membrane and is assimilated into the cell through endocytosis.

Transfection efficiency describes the percentage or proportion of cells which receive and express the exogenous DNA. It is dependent upon the efficiency of DNA delivery (ie. the number of DNA molecules that penetrate the nucleus) and the efficiency of expression (ie. fraction of DNA molecules that undergo transcription). The efficiency of transcription can be improved with the use of strong promoters and enhancers. However, significant improvements in the efficiency of delivery remains elusive. The reason for this is that successful DNA delivery must overcome three barriers. First, a low uptake over the plasma membrane. Second, an inadequate release and limited stability of the DNA once within the cell. Third, a lack of nuclear targeting (Luo & Saltzman 2000).

A transfection experiment may be classified as either "transient" or "stable" in nature. As already noted, transfections are rarely 100 % successful. Transient transfections produce a mixed population of transfected and non-transfected cells. In stable transfections, attempts are made to isolate and propagate clones containing the transfected DNA. Typically, this isolation is accomplished through transfectants expressing resistance to antibiotics, displaying altered morphological characteristics or being furnished with an essential gene missing from the host cell line. These protocols are time consuming and may have detrimental effects on cells. Furthermore, because myoblasts have a finite number of mitotic cycles before senescence (Webster & Blau 1990), the potential survival time of successful transformants is low. Therefore, unless a population needs to be enriched, transient transfections are preferable.

4.4.1 FuGENE 6 transfection of C2C12 myoblasts

The literature contains numerous examples where DNA has been introduced into muscle cells (Dickson 1996). Transfection techniques that have been used include: microinjection of DNA (Hansen *et al.,* 1991); the use of calcium - phosphate (Donoghue *et al.,* 1988; Cribbs *et al.,* 1989; Rosenthal *et al.,* 1989; Ernst *et al.,* 1991; Bishopric *et al.,* 1992; Edmondson *et al.,* 1992; Huang & Forsberg 1998); viral vectors (Crescenzi *et al.,* 1994; Kessler *et al.,* 1996) and liposomes (Blanton *et al.,* 2000). The calcium – phosphate method is the preferred technique but it has been demonstrated that there is considerable replicate variability which is inherent with the technology (Song & Lahiri 1995). Thus, different transfection techniques yield variable results. The main sources of variation is cell type and methodology.

FuGENE 6 from Roche Molecular Biochemicals is a relatively recent addition to the suite of available transfection reagents. The manufacturers do not provide composition details but state that FuGENE 6 is a "proprietary blend of lipids and other components". Several articles refer to it as a lipid based delivery system (Ritter 1998). It is, in essence, a chemical delivery system.

Such lipid based delivery systems have been successful in delivering GFP reporter plasmids into primary myoblast cultures and established cell lines. The system has produced both stable and transient transfections (Moss *et al.,* 1996; Turnacioglu *et al.,* 1997). FuGENE 6 has also been successful in transfecting a wide variety of established and primary mammalian cells lines including L8 rat skeletal myoblasts (Zelzer *et al.,* 1998) and cardiac myocytes (Noah *et al.,* 1998). FuGENE 6 was selected for these experiments on the grounds of its relative ease of use and recorded efficiency.

4.4.2 FuGENE 6 transfection optimisation

In accordance with the manufacturer's recommendations, a variety of DNA and FuGENE 6 concentrations were compared to determine the best combination of the components. Very pure EGFP-N3 plasmid DNA was quantified by spectrophotometry and agarose gel electrophoresis according to section 2.2.6 and diluted to a concentration of 1 μg/μl FuGENE 6, supplied in an ethanol solution, was used directly. However care must be taken to minimise the possibility of evaporation from this volatile solution. Titrations of FuGENE 6 solution were added to serum free DMEM to a final volume of 100 μl and incubated at RT for 5 minutes. During this time, titrations of DNA were pipetted into microfuge tubes and maintained on ice until the FuGENE 6 solution could be added drop - wise. The mixture was incubated at RT for a further 15 minutes before being pipetted into the growth media of cells grown in 24 well plates with 1.0 ml media. After 24 hours, the cells were trypsinised from the plate according to section 2.2.15.1, washed once with DMEM + 10 % FCS, washed again with PBS and finally resuspended in a volume of 200 μl PBS. The total volume of cell suspension was pipetted into individual wells of a 96 well black plastic microtitre plate. These plates are designed to minimise the background by being non – reflective which prevents "spill - over" of illumination from adjacent wells. Fluorescence was measured with a BMG Fluorostar plate reader, arranged with overhead excitation (480 nm) and emission (520 nm) fibre-optic cables. Ten measurements of each well were taken at 20 seconds intervals with lateral shaking of the plate between measurements. Values were averaged and plotted in Figure 4.15.

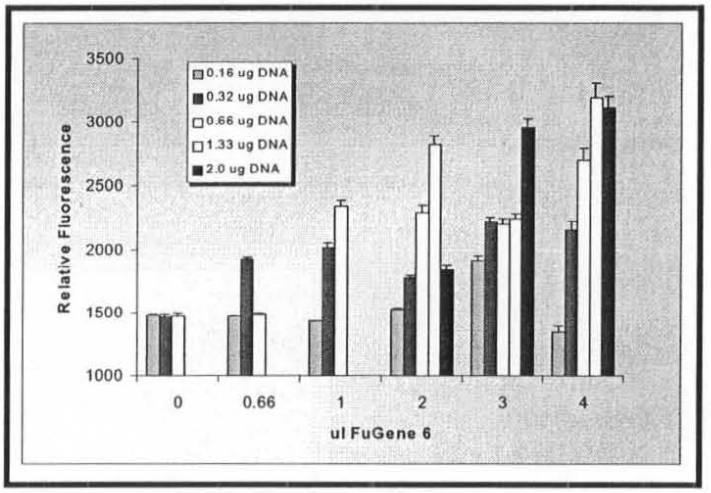

Figure 4.15 Optimisation of conditions for FuGENE 6 transfection. Plasmid EGFP-N3 DNA was transfected into C2C12 myoblasts (50 - 80 % confluent) at concentrations from 0.16 - 2.0 μg with FuGENE 6 at volumes from 0 - 4 μl.

The relative fluorescence values were determined by the BMG plate reader and are presented in Figure 4.15 without modification. On average, an empty well presented a relative fluorescence of 380. Non-transformed healthy cells showed an average fluorescence of 1400. These values represent the background well and cell fluorescence or auto – fluorescence. Only values greater than these values would indicate transformation. Values less than the background indicate a reduction in auto – fluorescence and may be result from cell death. Across a particular volume of FuGENE 6, fluorescence generally increases with increasing DNA concentration. The exception to this is when the concentration of DNA equals or exceeds the volume of FuGENE 6. This agrees with the manufacturer notes, which state that excess DNA, with respect to FuGENE 6, can be detrimental to cell transformation.

The greatest relative fluorescence was achieved by combining 4 μl FuGENE 6 with 1.33 μg DNA to be diluted in 1.0 ml of growth media. However, for the purpose of this experiment, transfection optimisation was defined as those conditions in which the minimum amount of reagent achieves the greatest acceptable fluorescence. As a result, relatively large increases in reagent consumption which results in only slight improvement in fluorescence, are inefficient. An example of this

is illustrated by comparing the fluorescence from [3 µl FuGENE 6 + 2 µg DNA] versus [4 µl FuGENE 6 + 1.33 DNA] versus [4 µl FuGENE 6 + 2.0 µg DNA]. The 25 % increase in FuGENE 6 usage resulted in a relatively small improvement in fluorescence; 17 % ± 8.4 for [1.33 µg DNA] and 12 % ± 6.7 for [2.0 µg DNA] when compared to [3 µl FuGENE 6 + 2 µg DNA] fluorescence. It is notable that [2 µl FuGENE 6 + 1.33 ug DNA] resulted in only slightly less fluorescence than [3 µl FuGENE 6 + 2 µg DNA], and represents an even more significant saving in both DNA and FuGENE 6. However, [2 µl FuGENE 6 + 1.33 ug DNA] achieves only 73 % fluorescence attained using [4 µl FuGENE 6 + 1.33 DNA] and represents a substantial decrease from potential efficiency. Therefore, while this loss was considered too great, the compromise in transfection efficiency resulting from using [3 µl FuGENE 6 + 2 µg DNA] is acceptable given the saving in FuGENE 6 which is the most expensive reagent (DNA is inexpensive to produce). It was decided that these volumes represent the optimised combination when transfecting C2C12 growing in 24 well plate with 1.0 ml growth media. Reagent volumes are increased or decreased proportionately when transfecting cells in larger or smaller volumes of media.

4.5 Discussion

Myogenesis describes the process by which precursor muscle cells (myoblasts) differentiate biochemically and morphologically into determined muscle tissue (myotubes). By controlling the environment in which myoblasts grow, it is possible to alter the conditions and promote this progression.

Morphological differentiation is manifested as myoblast fusion which is an easily inducible process monitored by quantifying the chronological appearance of myotubes. Direct observation and manual counting is the preferable method for determining percent fusion over time. This procedure revealed that under the experimental conditions in this study, approximately 50 – 60 % of myoblasts fuse to form myotubes over the first 96 hours of fusion induction, and that the most rapid fusion occurred between 24 and 72 hours. FACS analysis is not sensitive enough to discern myoblasts and myotubes once they are suspended in solution. It is not a suitable technique for quantifying fusion.

Several muscle specific proteins are only apparent in biochemically differentiated muscle cells. Myosin, troponin T and desmin all exhibit clear associations with differentiation. Both μ- and m-calpain are detectable in C2C12 extracts throughout the fusion period. Although components of the calpain system have been implicated in regulating the fusion process, the trends in calpain levels do not relate to any fusion event. Endogenous calpastatin is an important parameter in this work, since the initial objective of this thesis was the manipulation of levels of this protein. Several attempts using Western blotting were made to detect calpastatin in C2C12 extracts, however, all were unsuccessful. While this does not mean that calpastatin is completely absent from C2C12's, it does mean that the effects from expressing calpastatin variants (described in Chapter 5) should not be influenced by endogenous calpastatin.

As described previously three calpastatin variants were used in this work. They represented domain L, domain 1 or domains L and 1 together. In Chapter 3, these cDNA sequences were combined with GST cDNA to provide an affinity tag with which to purify the expressed product. In this chapter, the intention was to generate calpastatin constructs with a factor which will enable visualisation of the hybrid protein. GFP is marketed as a fluorescent tag for tracing proteins within living systems. Furthermore, the robust and non-reactive nature of GFP make it an ideal fusion partner. Controlled mutations have since produced enhanced GFP (EGFP) which emit higher levels of light than their wild-type progenitors. This chapter described the successful creation of eukaryotic expression plasmids whose products combine the calpastatin variants and N-terminally associated EGFP.

Several techniques are available to facilitate the introduction of plasmid DNA into living cells. Efficacy, expense, ease of use and effects upon cell viability are all criteria on which a particular technique may be selected. For these experiments FuGENE 6 reagent was selected. This is a liposomal reagent which combines with DNA to produce a cell penetrating complex. The optimal ratio of reagent to DNA was determined for transfection of C2C12 cells with pEGFP-N3 plasmid.

Chapter 5

Expression of calpastatin variants in fusing myoblasts

5.1 Introduction

Several papers have described the impact of calpastatin on myoblast fusion (Brustis *et al.,* 1994; Temm-Grove *et al.,* 1999). The examination of fusing cells revealed that calpastatin mRNA and protein levels are down-regulated during fusion (Barnoy *et al.,* 1996; Balcerzak *et al.,* 1998). This thesis sought to express calpastatin in a manner which would inhibit calpain activity and fusion. The approach used was influenced by two reported experimental findings. First, Mellgren showed that certain regions of the protein (specifically domain L) facilitated the binding of calpastatin to plasma membranes (Mellgren *et al.,* 1987a; Mellgren 1988; Mellgren *et al.,* 1989). Second, phosphorylation of calpastatin's domain L changes the inhibitory potential (Salamino *et al.,* 1994a; Salamino et al., 1994b; Averna *et al.,* 1999). While this data underscores the importance of domain L in regulating the function of calpastatin, it is inconclusive about whether these attributes have an *in vivo* function. Thus, when investigating the impact of calpastatin on fusion, it seems prudent to investigate both the structure and function of calpastatin. Expressing domains L, 1 and L1 in combination with EGFP allows several questions to be addressed. The first question is "what is the effect of expressing calpastatin on myoblasts during fusion?". The second question is "are there any differences in effect between the calpastatin variants?" and the third question is, "do the calpastatin variants show differential subcellular localisation at any stage during fusion?".

This Chapter reports the successful expression of calpastatin variant / EGFP hybrids in myoblasts during fusion. The first segment of the chapter reports on using FACS analysis to calculate transfection efficiency. The effect of calpastatin expression upon the rate and extent of cell fusion is then determined by counting cells directly with a microscope. Fluctuation in the levels of certain proteins may

indicate whether there are subtle changes associated with biochemical differentiation and changes to *in vivo* proteolysis. Analysis of these proteins will be performed using Western blotting. Finally, fluorescent microscopy will be used to detect evidence of differential subcellular localisation of the EGFP - calpastatin variants.

To perform these experiments, five batches of cells were growth on 100 mm tissue culture plates until they reached 50 – 80 % confluence. Four of these batches were then transfected with plasmid pEGFP-N3 (control EGFP), and plasmids pEGFP-DL, pEGFP-D1 or pEGFP-DL1 (encoding calpastatin variants and EGFP). The fifth batch was left untreated to serve as a negative control. The pEGFP-N3 transfection is intended to control any effect due to the transfection process and for the expression of EGFP itself. Each batch of cells was grown for a further 24 hours before being washed, passaged and re-plated at equivalent densities over five 175 cm^2 flasks and five 35 mm plates containing sterile coverslips. Cells were cultured with complete growth media and reached confluence simultaneously. At this time the coverslip from one 35 mm plate, from each batch, was recovered and washed. Cells were then fixed, stained, and counted for the determination of percentage fusion (see section 2.2.15.6). Similarly, one 175 cm^2 flask from each of the five batches was washed and the cells collected for protein extraction and Western blotting as described in section 2.2.15.5. Note that prior to homogenisation of the cells, a sub-sample was removed for immediate FACS analysis (to determine transfection efficiency) as described in section 2.2.17. The remaining four flasks and plates from each batch were washed with PBS and the complete growth media replaced with differentiation media. At 24 hour intervals, a flask and plate were selected from each batch and the cells were either extracted or stained as described above. Thus, after four days, samples of protein had been collected from cells transfected with each of the four plasmids and from control cells at 0, 24, 48, 72 and 96 hours after fusion induction.

5.2 Transfection efficiency of C2C12

As noted in section 4.4, transfection efficiency describes the proportion of cells which receive and express exogenous DNA. It is rare for transfections to be 100% efficient and significant variation is not uncommon. In any experiment using transfection it is desirable to measure the extent of transfection efficiency. To obtain this information FACS analysis was particularly suitable since the technique is reproducible and accurately measures the fluorescence emitted by individual cells within a population of cells. The data can also be directed to reflect specific subpopulations and can be compared to control cells.

As described in the introduction, sub-samples of cells for FACS analysis were collected from treatments at each time interval. Because these cells cannot be stored for extended periods of time, the samples were analysed daily, shortly after being harvested. Any "between-day" variation, which may arise from fluctuations in the FACS equipment, was controlled for by applying a sample of sub-confluent "calibration" myoblasts cultured in complete media each day. The fluorescence of both the control and treated cells was investigated using the parameters described in section 2.2.17. As noted in Chapter 4, each analysis performed by the FACS generates a scatter plot showing size and granularity of all the cells (raw FACS data is provided in Appendix C). Within the scatter of individual cells or "events" two regions were defined; R1 and R2. The intensity of fluorescence emitted by cells falling within either R region can be plotted as shown in Figure 5.1.

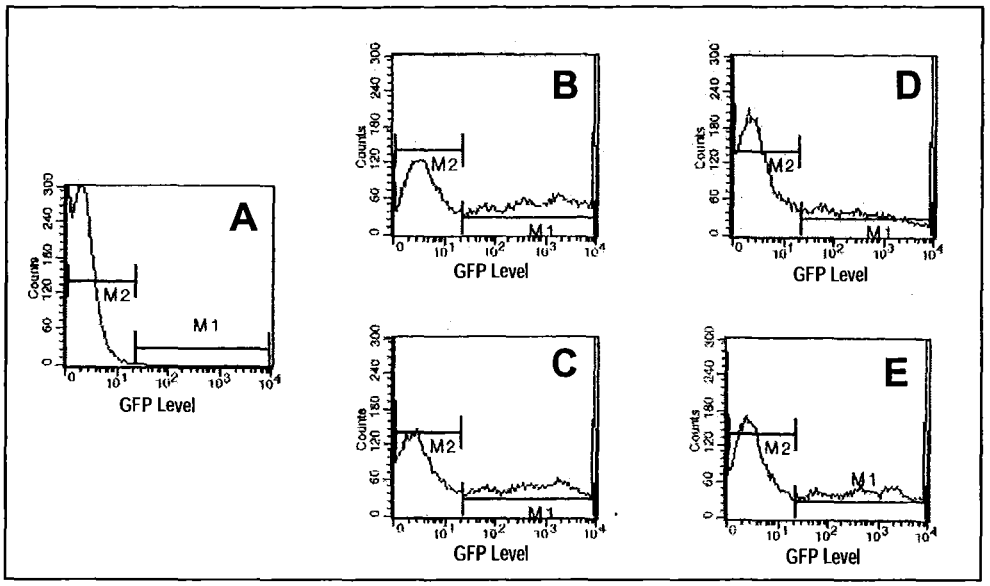

Figure 5.1 Fluorescence of the R1 region at 0 hours as determined by FACS analysis. The results contrast control (**A**) and transfected C2C12 cells at 0 hours of fusion. Transfected cells are represented by **B** (pEGFP-DL), **C** (pEGFP-D1), **D** (pEGFP-DL1) and **E** (pEGFP-N3). The X axis describes the level or intensity of emitted light in arbitrary units from 0 - 1000. The Y axis is a direct count of cells, the average total number of cells counted in each trace is 50 000. Note that while this figure depicts 0 hour R1 plots only, both R1 and R2 plots can be drawn at any time point.

Two arbitrarily positioned markers have been included within each "R" plot. The M2 marker encompasses the total range of fluorescence intensities emitted by control cells. Cells falling within this region are defined as non-fluorescing and any detected emissions are considered to represent background noise. The M1 marker embraces all cells displaying greater levels of fluorescence and defines transfected cells. Control cells were analysed at each time interval. Since there were no significant changes in their background profile, the M1 – M2 boundaries were not altered during the experiment. Because the M1 range determined at 0 hours for R1 encompassed all non-fluorescing events in both R1 and R2 regions, these M ranges were applied to the R1 and R2 plots for all cell treatments at each sampling time.

Transfection efficiency is expressed as a percentage and is calculated from the whole cell population by summing all M1 events and dividing by the total (M1 + M2) events. It is apparent that transfection resulted in a significant proportion of the cell population expressing EGFP. As shown in Table 5.1, the percent transfection at 0 hours of fusion ranged from 56 % (pEGFP-DL) to 33 % (pEGFP-DL1).

Table 5.1 Characteristics of transfected and control C2C12 myoblasts populations at the initiation of fusion (0 hours). Shown are the total cell counts, the cell count of the M1 fraction, the percentage transfection derived from these two values and the mean fluorescence of the M1 fraction in arbitrary units (AU).

	Total Cell Count		% Transfection	M1 Mean Fluorescence (AU)
Control	41026	10	0	27
pEGFP-N3	49078	22236	45	1692
pEGFP-DL	49681	27615	56	2218
pEGFP-D1	49377	24226	49	1682
pEGFP-DL1	49616	16339	33	1107

The data indicates that the transfection was successful in producing a mixed population of transfectants and non-transfectants and that the mean fluorescence at 0 hours is 50 – 100 times higher than background levels. These results indicate that the calpastatin containing constructs have the capacity to drive expression of EGFP. The percentage of cells transfected within each treatment over the experimental period is graphically represented in Figure 5.2.

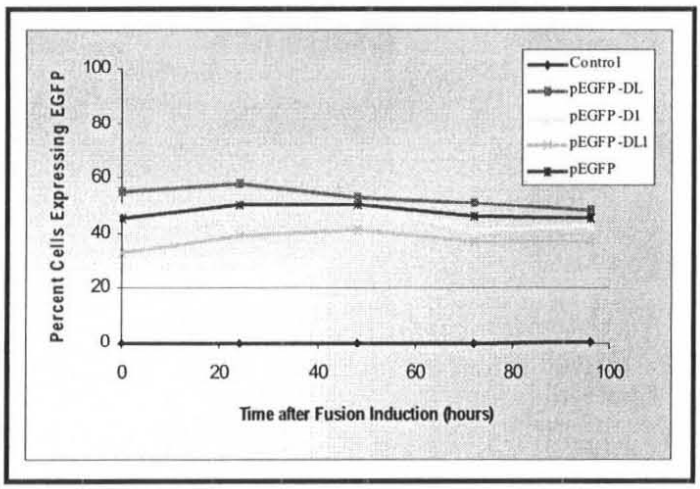

Figure 5.2. Graph showing fluorescence over the experimental period. The percentage of the total cell population fluorescing was calculated by summing the M1 counts from R1 and R2 groups and dividing by the total cell counts.

FACS represents a simple and accurate method for determining transfection efficiency. An alternative approach is to count fluorescent cells directly using microscopy. This approach, however, was not used for three reasons. First,

differentiation of fluorescing and non-fluorescing cells is subjective and only possible when the difference is relatively large. Since there is a broad range of fluorescence intensities, weakly fluorescing cells may not be scored and the resulting transfection efficiency will be skewed. Second, to calculate the transfection efficiency both the number of fluorescent cells and the total number of cells must be determined. In these experiments, examination of fluorescence and the counting of cells required two different slide preparations. This is because Geimsa, which is commonly used to identify and count muscle cell populations (Kosower & Barnoy 2000), involves methanol fixation which destroys GFP fluorescence. The distribution of transfected cells is uneven, and the two slide preparations required to calculate transfection efficiency are not guaranteed to harbour representative cell populations. Third, the counting of fluorescent cells can be difficult when they are clustered together. Identifying whether a particular fluorescent body is comprised of one large cell or several smaller cells requires good subject definition. Combining fluorescence with another mode of microscopy (eg. phase contrast or differential interference contrast) may solve the definition problem. Indeed, the use of alternative stains and the use of modern microscopic equipment may have made direct observation a viable approach for calculating transfection efficiency. For example, staining a transfected cell preparation with Hoechst 33342 will not perturb EGFP fluorescence but would allow nuclei to be distinguished. Multimode microscopes with digital cameras and image analysis software will now permit fluorescent images to be overlayed with high contrast light images.

5.3 The effect of transfection upon the rate and extent of fusion

A successful transfection makes it possible to address whether an active recombinant GFP-calpastatin has impacted upon the rate or extent of fusion. As asserted in section 4.2, the direct counting of stained cells is the most simple and accurate method to estimate the percentage of nuclei contained in fused cells.

5.3.1 Microscopic examination and quantification of fusion

Cells from each transfection were grown on coverslips. At appropriate times the coverslips were removed and the cells fixed, stained, and brightfeild microscope photographs taken. These are presented in Figure 5.3.

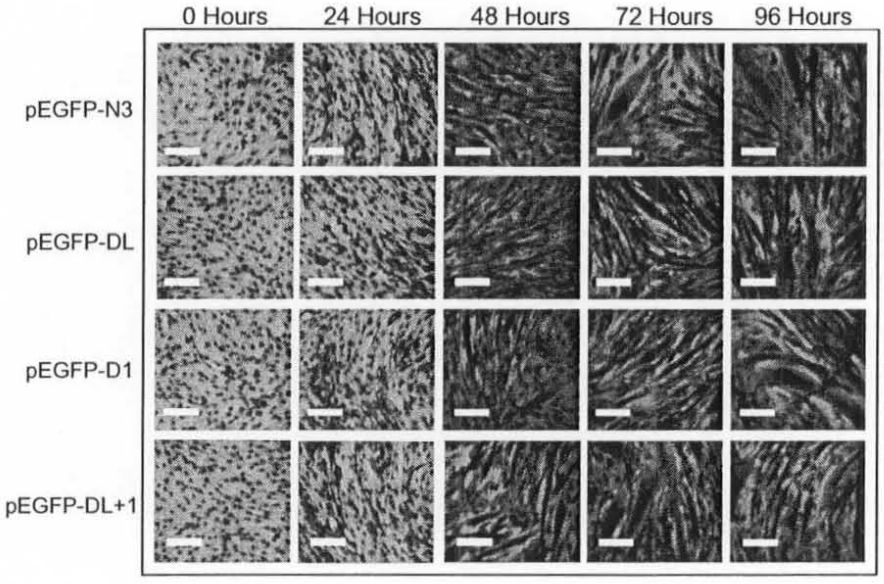

Figure 5.3 Photographs of experimental cells induced to fuse. Transfected and non-transfected C2C12 cells were, plated onto coverslips and induced to fuse as described. At 24 hour intervals, cell were fixed and stained (Bar = 100 μm).

Subjectively, all the cell populations appear to exhibit similar "normal" morphologies, alignment and fusion. Moreover, there were no obvious differences in cell density, plate coverage or cell mortality. The percentage fusion was calculated from these images as described in section 4.2.1. These data are presented in Table 5.2 and graphically represented in Figure 5.4.

Table 5.2 Estimation of the extent of fusion over 96 hours determined by direct cell counting. Cells falling within the microscopes field of view were counted and scored as myoblasts or myotubes. The stained nuclei within these cells were counted. The percent of fused cells is estimated by dividing the number of myotube nuclei by the total number of nuclei.

		Number of nuclei in Myoblasts	Number of nuclei in Myotubes	Total number of nuclei	
pEGFP-N3	1	657	26	683	3.8
pEGFP-DL	1	412	13	425	3.1
pEGFP-D1	1	439	15	454	3.4
pEGFP-DL1	1	557	21	578	3.7
pEGFP-N3	2	455	111	566	19.6
pEGFP-DL	2	382	98	480	20.4
pEGFP-D1	2	383	94	477	19.7
pEGFP-DL1	2	399	103	502	20.5
pEGFP-N3	3	325	231	556	41.5
pEGFP-DL	3	361	227	588	38.6
pEGFP-D1	3	348	226	574	39.4
pEGFP-DL1	3	416	273	689	39.6
pEGFP-N3	4	467	346	813	42.6
pEGFP-DL	4	475	381	856	44.5
pEGFP-D1	4	448	345	793	43.5
pEGFP-DL1	4	559	490	1049	46.7

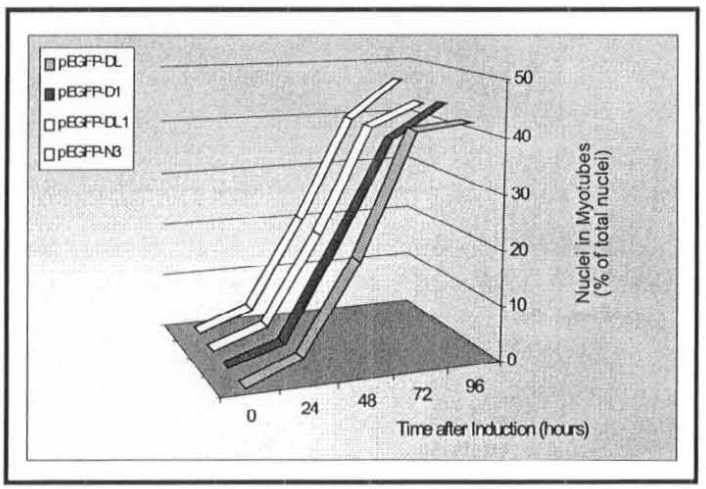

Figure 5.4 Graph showing percent fusion of treatment cells over the culture period. After fixation and staining, random fields were viewed with a light microscope and nuclei were counted and allocated into myoblast or myotube categories.

The percentage fusion of the control cells (those transfected with pEGFP-N3) is almost identical to the growth profile of untreated cells shown in the previous Chapter (see Figure 4.2). This indicates that the experimental protocol is robust and reproducible. Unfortunately, there were no conspicuous differences between the rate or extent of fusion in myoblasts expressing the calpastatin variants.

5.3.2 FACS analysis of R2 region fluorescence

Despite transfection being successful, the expression of calpastatin – EGFP failed to significantly influence the overall rate or extent of myoblast fusion. This may indicate that calpastatin is totally ineffective or that the impact of calpastatin is minor and cannot be discerned through direct microscopic examination. Sections 4.2.2.4 and 4.2.2.5 established that μ- and m-calpain are present in C2C12 cells during the fusion process. It seems reasonable to assume, therefore, that for calpastatin to prevent fusion, it must be present in C2C12 cells in sufficient amounts to repress calpain activity below that required for myoblast fusion. However, if only a few cells are expressing sufficient active calpastatin – EGFP to eliminate calpain activity then their presence may escape detection on examination of the entire cell population. Moreover, since the level of calpastatin expression is likely to be correlated to the intensity of EGFP fluorescence the mean fluorescence of myotubes should be compared between the treatments.

At this stage of experimentation, the most simple approach was to compare the mean fluorescence of the R2 groups between treatments. The FACS designated R2 region was an identifiable cell population presenting a large and granular morphology. In section 4.2.1, R2 was explored as a measure to quantify percent cell fusion. It was revealed that the R2 values were not numerically representative of fusion, and that the FACS device was unable to isolate all the myotubes from mixed cell populations. Nevertheless, the cell bodies that were separated are likely to be myotubes since they are, by definition, large and complex. Within this framework, the mean fluorescence of the transformed myotubes were compared in Figure 5.5.

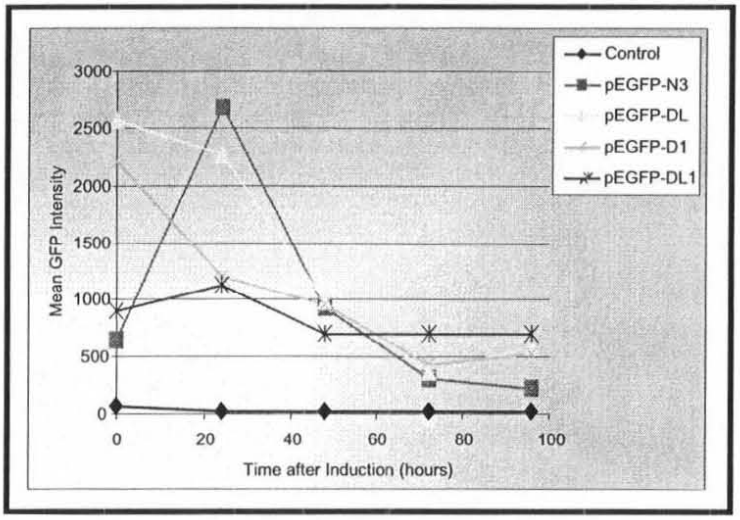

Figure 5.5 Mean fluorescence of M1 cells falling within the R2 region. The M1 subdivision within R2 describes cells whose fluorescence exceeds background levels determined in non-transformed cells. The X axis describes the level / intensity of emitted light in arbitrary units from 0 - 3000. The Y axis shows time after fusion induction.

The data presented in Figure 5.5 seeks to reveal any effect of treatment on the intensity of fluorescence emitted by transfected myotubes. At each time interval, and for each treatment, the mean fluorescence was calculated by summing the total fluorescence of the M1 cells and dividing this figure by the number of M1 cells. As already noted, the M1 division embraces cells whose fluorescence exceeds the background levels defined by non-transformed cells. All cells within M1 must, therefore, be transfected. By selectively examining the M1 mean fluorescence, as opposed to M1+M2 mean fluorescence, the impact of different transfection efficiencies on mean fluorescence is avoided.

There are no clear treatment effects on the mean fluorescence of myotubes. The large variations observed 0 and 24 hours are difficult to explain or associate with a treatment effect since little or no fusion takes place at this time. Indeed, over the period of greatest fusion (48 and 72 hours) the mean fluorescence of the transformed myotubes converge. These trends may be due to the relatively small number of cells used to calculate 0 and 24 hour means (from 19 to 50 cells) compared to the greater number used at later times (up to 300 cells at 96 hours). The larger sample size at

later times gives an accurate mean value compared to the more variable early mean values.

A common trend in treatments is that the mean levels of fluorescence converge from 72 hours. When expressed as a percentage, this decline is almost uniform across the R1 regions as shown in Figure 5.6. This is not unexpected since the experiment seeks only to transiently transfect cells. It would appear that whatever system is responsible for the reduction in fluorescence in these cells, it is not affected by the treatment.

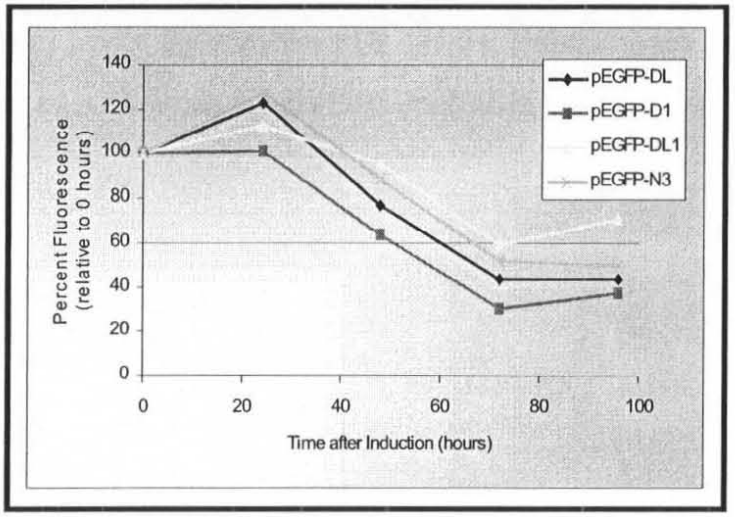

Figure 5.6 Percent decline in mean fluorescence of R1 across treatments. Values were calculated relative to 0 hour mean fluorescence.

5.3.3 Summary

FACS is a suitable method for determining transfection efficiency, and reveals that a significant proportion of C2C12 cells growing in culture were successfully transfected with pEGFP plasmids. The percentage of cells expressing EGPF was similar between the treatments and did not fluctuate significantly over the 96 hours of fusion culture. Importantly, however, direct microscopic examination of fixed cells did not reveal any differences between treatments with respect to the rate or extent of fusion. This indicates that the calpastatin variants are either completely

ineffective or have only a minor impact on the fusion process. To investigate whether strong expression levels reduced fusion, the mean fluorescence of transformed myotubes was compared. The differences which were seen are probably due to differences in the number of observations and the inaccuracies associated with this rather than a treatment effect upon fusion. Another direct approach is to microscopically examine transfected cells with fluorescence. While the potential to observe and track cells by microscopy during the fusion process would have been advantageous, at the time that these experiments were undertaken there was no readily available inverted fluorescent microscope. This restricted experiments to using an upright microscope whose objectives were above the stage, and permitted examination of slide mounted samples only.

5.4 Western analysis to compare protein dynamics

In the previous chapter, the level of several proteins were seen to change in association with myogenesis. Although there is disagreement in the literature concerning fluctuations of specific proteins, differences may be explained by variations in the sample or the method of analysis. The use of a consistent methodology in these experiments has permitted the effect of a treatment to be determined.

The following series of Western blots were performed under consistent conditions. Each Western blot examines the dynamics of a particular protein over the 96 hours of fusion from cultured cells transfected with one of the four experimental plasmids. While every attempt was made to equalise sample recovery, the sources of variability were the protein load, success of transfer and, most importantly, the antibody titre. Several of these variables are not relevant to a "within blot" comparison but are important when analysing "between blots". Westerns, therefore, focussed upon identifying qualitative and not quantitative differences between the treatments.

5.4.1 Troponin T

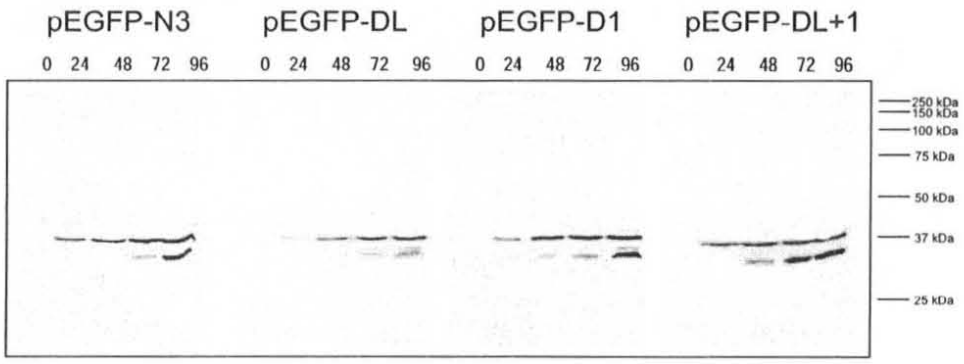

Figure 5.7 Comparison between experimental treatments of Troponin T levels over 96 hours. Samples of protein (100 ug) were electrophoresed on an 10 % SDS-PAGE gel before being probed with anti-troponin T antibody (CT3) at 1 : 100 dilution.

The Western blots in Figure 5.7 show little variation from the control blot in Figure 4.3. A single band migrating at around 35 kDa became apparent after 24 hours of fusion induction and became more intense during the incubation period. A smaller (30 kDa) immunoreactive band was apparent after 48 hours and increased from 48 to 96 hours. As described in section 4.2.2.1, this 30 kDa protein may be a proteolytic product resulting from calpain action. However, there is no indication from Figure 5.7 that the expression of active regions of calpastatin has impeded or reduced the rate of appearance of this fragment.

5.4.2 Desmin

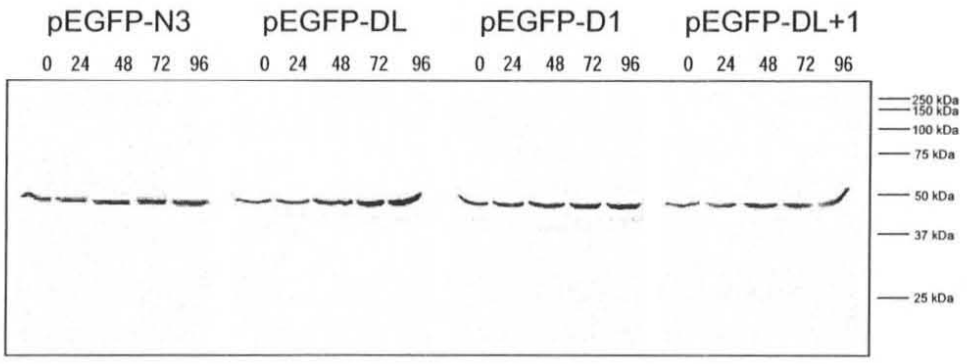

Figure 5.8 Comparison between experimental treatments of Desmin levels over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-desmin antibody (D3) at 1 : 100 dilution.

As shown in Figure 4.4 and reflected in the blots in Figure 5.8, the desmin levels in the C2C12's increased over the culture period. While this protein is a substrate for calpain (Dourdin *et al.,* 1999) there is no indication that proteolysis occurred and, consequently, there is no evidence of a protective effect from the expression of calpastatin.

5.4.3 Myosin

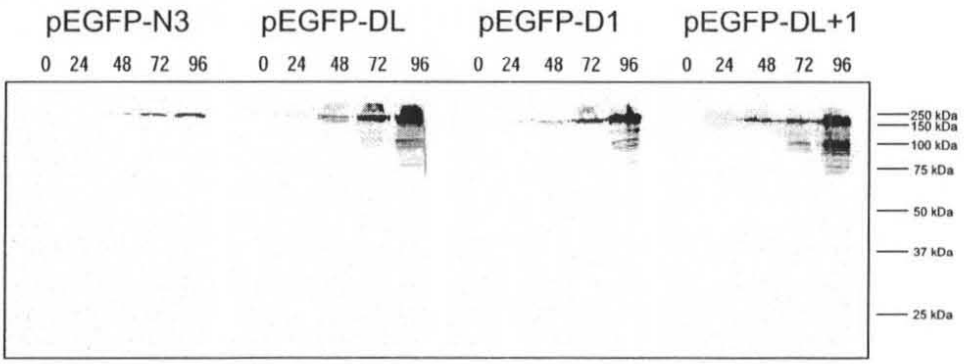

Figure 5.9 Comparison between experimental treatments of Myosin levels over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-myosin antibody (MF20) at 1 : 2000 dilution.

Myosin is a good indicator of myoblast differentiation. The blots shown in Figure 5.9 reflect the trend shown in non-transfected fusing myoblasts. There is no evidence of qualitative differences which can be attributed to the expression of the transfected plasmid. The reduced appearance of immunoreactive protein in the pEGFP-N3 myosin blot is an example of between blot variation. The lightness of this blot is probably due to incomplete colour development rather than significantly reduced levels of myosin.

5.4.4 μ-calpain

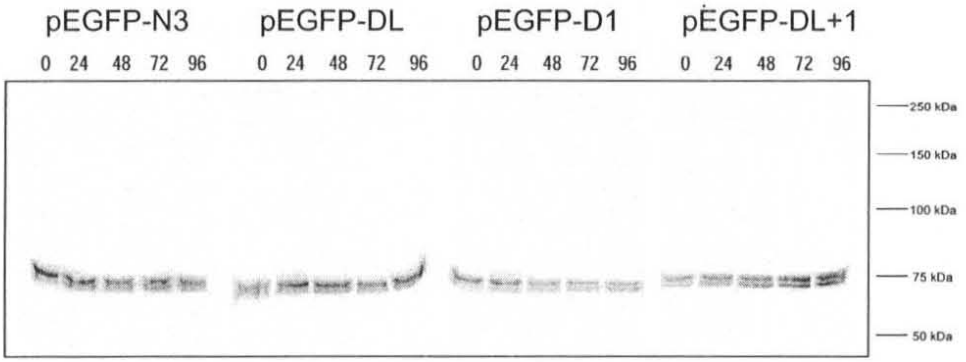

Figure 5.10 Comparison between experimental treatments of μ-calpain levels over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-μ-calpain antibody (B2F9) at 1 : 25 dilution.

Figure 5.10 demonstrates that μ-calpain from transformed cells consistently displayed a double banding pattern throughout the culture period. This pattern was previously seen in non-transformed cells (see Figure 4.7). The larger band represents the 80 kDa isoform of μ-calpain. The smaller 78 kDa band results from autolysis. A faint band, below the 78 kDa isoform, is not clear in Figure 5.10 and probably represents the second truncation product at 76 kDa. During the 96 hour fusion period, the intensity of the two prominent bands increased. There are no conspicuous differences between the treatments.

5.4.5 m-calpain

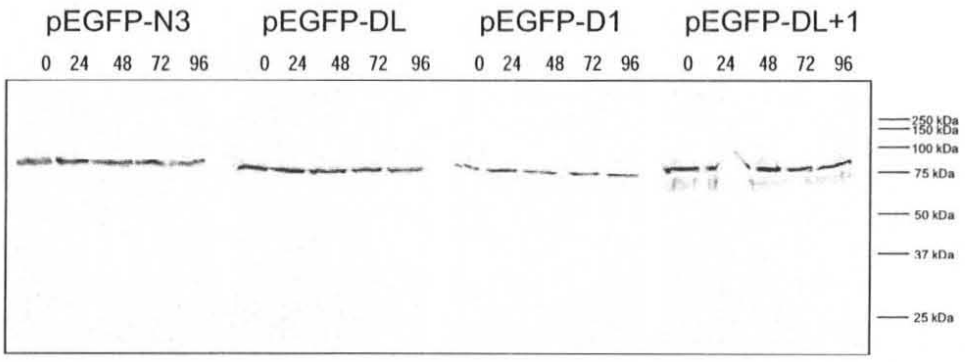

Figure 5.11 Comparison between experimental treatments of m-calpain levels over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-m-calpain antibody (Forsberg anti-m-calpain) at 1 : 500 dilution.

In accordance with the results from non-transformed control cells, m-calpain is detectable throughout the culture period (Figure 5.11). Between 0 and 24 hours, the detectable levels increased between 50 and 200 % (relative to 0 hours) and then gradually declined over the culture period until they reached the original levels.

5.4.6 Calpastatin

As noted in Chapter 4, polyclonal antibodies 567 and 552 have the capacity to detect murine skeletal muscle calpastatin. However, these antibodies and a selection of other anti-calpastatin antibodies were unable to detect a calpastatin-like protein in non-transformed C2C12 extract. This probably indicates that the levels of endogenous calpastatin fall below the sensitivity of the antibodies. Endogenous calpastatin is, therefore, unlikely to have any significant impact on the results obtained from transfections. Westerns to detect calpastatin variants are presented in the following subsections 5.4.8 and 5.4.9.

5.4.7 GFP

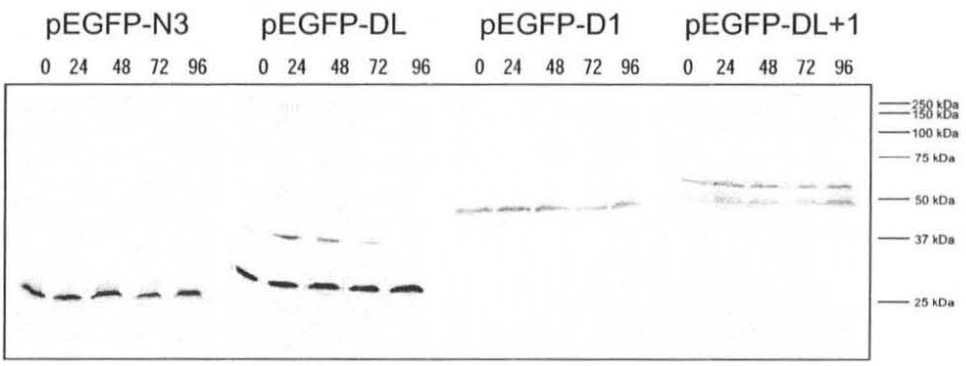

Figure 5.12 Comparison of EGFP levels between experimental treatments over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-GFP antibody at 1 : 1000 dilution.

In each transfection the GFP antibody reacted with a protein species which was absent in non-transformed control cells. As expected, EGFP expressed alone migrates at the smallest size while a combination of larger bands are apparent in domain L, 1 and L1 transfectants (Figure 5.12).

pEGFP-N3 - GFP antibody reacts with a single band migrating at approximately 25 kDa in the pEGPF-N3 extract. This size corresponds to the 25 – 26 kDa size range previously reported for recombinant GFP isoforms (Ward 1998). The levels of this protein remain unchanged over the experimental period despite large fluctuations being observed in detectable fluorescence. This implies that some factor(s) contributes to the relationship between protein and fluorescence.

pEGFP-DL - The pEGFP-DL transfectant protein extract contains two immunoreactive bands migrating at approximately 38 kDa and 26 kDa. The predicted molecular weight of a DL-EGFP hybrid is 38.4. Based upon densitometric examinations, the larger fragment increased by 200 % from 0 to 24 hours and then declined to 25 % of the initial levels by 96 hours. In contrast, the smaller fragment, which is almost 4 fold stronger than the larger at 0 hours, increased to 150 % of the initial levels over the fusion period.

pEGFP-D1 - Two immunoreactive bands were visible in the total protein extracts from pEGFP-D1 transfectants with molecular weights of 49 kDa and 46 kDa. The predicted molecular weight of a D1-EGFP hybrid is 41.6 kDa. The similarity of the sizes of the bands indicates they have not reproduced well in Figure 5.13. Both bands declined between 48 and 96 hours to 50 % of their initial levels.

pEGFP-DL1 - Probing the protein extract from pEGFP-DL1 transfectants with anti-GFP antibody revealed three bands. While the predicted size of DL1-EGFP is 53.1 kDa, these bands migrate at 63 kDa, 52 kDa and 49 kDa. Once again the small size differences between the two smaller bands has not reproduced very well in Figure 5.13. The 63 kDa band increases in intensity steadily to 200 % of its initial levels over the experimental period. Concurrently, the smaller 52 kDa and 49 kDa bands increase by 250 % and 180 % respectively.

The relative complexity of the blotting results indicate that some factor(s) was shaping the products expressed by the EGFP vectors. The observed size differences between the predicted and observed proteins were not unexpected. Aberrant migrations have been observed in other published experiments as discussed in Chapter 3. In each transformation, the largest band was considered to be the intact protein product corresponding to the transfected plasmid. Truncated immunoreactive proteins in these blots must retain the EGFP epitope. They could arise from proteolysis, structural instability or inappropriate transcription / translation. These blots suggest that proteolysis is a potential mechanism because the ratio of 38 kDa to 26 kDa protein in the pEGFP-DL transfectants varies over the 96 hour culture period. If the truncation was caused by instability or improper protein synthesis it would be constant, irrespective of the level of expression. However, in the pEGFP-DL transformants, a peak of EGFP expression (reflected by fluorescence) corresponded with the maximum amount of 38 kDa protein. This indicates that a mechanism with limited capacity (such as proteolysis) is responsible for truncation of the protein products. A hypothesis which can be developed from the key observations is:

(1) The control pEGFP-N3 plasmid produces a single band of 25 kDa which is an appropriate size for recombinant GPF (Wood 1998). This establishes that EGFP is not a substrate for the suspected proteolytic mechanism.

(2) The pEGFP-DL product migrates as two fragments. Both contain the GFP epitope. The larger 38 kDa band represents a full sized DL-EGFP while the smaller 26 kDa band retains GFP immunoreactivity but migrates 1 kDa larger than the control EGFP. This suggests there is a truncation site 1 kDa upstream from the N-Terminus of EGFP which leads to the separation of domain L from EGFP. As noted in 4.3.3, ligation of the calpastatin cDNA into the pEGFP multiple cloning site results in the inclusion of a 12 amino acid linker region between the end of each calpastatin variant and the C-terminally attached EGFP. This linker encodes a polypeptide with a predicted molecular weight of 1026 Da. It is possible, therefore, that the 26 kDa band represents a protein composed of all or part of the linker peptide attached to EGFP.

(3) The pEGFP-D1 protein also migrates as two bands at 49 kDa and 46 kDa. Since both bands retain GFP immunoreactivity and EGFP does not appear to be affected by the truncation mechanism, it is feasible that the size difference is due to the liberation of a short peptide region from the N-terminus of domain 1. The nature of some of these products may be confirmed by probing Western Blots with anti-domain L and domain 1 antibodies.

(4) The GFP reactive protein product from pEGFP-DL1 transfections migrates as three bands at 63 kDa, 52 kDa and 49 kDa. There is no obvious explanation for the appearance of these bands. Two possible interpretations exist. First, the 63 kDa band probably represents full size domain L1 + EGFP protein. Second, the 3 kDa difference between the smaller bands is analogous to the 3 kDa difference seen in domain 1 + EGFP which possibly results from N-terminal truncation of domain 1. Therefore, the 49 kDa protein may represent domain 1 + EGFP **after** the 3 kDa truncation. The 52 kDa protein may represent domain 1 + EGFP **before** the 3 kDa truncation. The 63 kDa protein represents **full**

size domain L1 + EGFP. A significant issue raised by this proposal is that the 52 kDa and 49 kDa proteins are larger than the 49 kDa and 46 kDa proteins seen in pEGFP-D1 transfectants. There is no apparent reason for this difference.

5.4.8 Recombinant Calpastatin Domain L

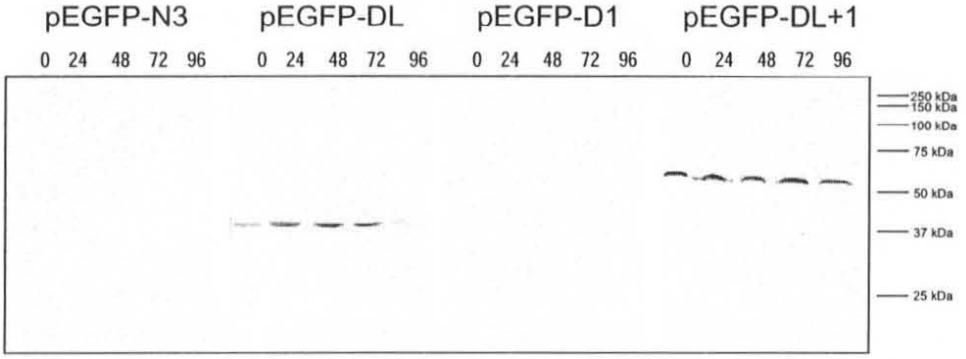

Figure 5.13 Comparison between experimental treatments of Calpastatin Domain L levels over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-calpastatin domain L antibody (552) at 1 : 100 dilution.

Antibody 552 was raised against domain L antigen and, as shown in Figure 5.13, cross-reacted with protein species from pEGFP-DL and pEGFP-DL1 transfectants only.

pEGFP-N3 and pEGFP-D1 - Protein extracts from both pEGFP-N3 and pEGFP-D1 transfectants did not demonstrate any cross-reactivity with anti-domain L antibody.

pEGFP-DL - Protein extracted from pEGFP-DL transfectants contained one 38 kDa immunoreactive band which migrated and fluctuated in a manner identical to the 38 kDa protein detected by anti-GFP. Specifically, protein levels increased by 250 % between 0 and 24 hours and subsequently declined to 50 % of the initial levels. The immunoreactivity of this band, with both the GFP and domain L antibodies, suggests that this is the expressed product from the pEGFP-DL plasmid. As shown in Figure 5.13, the rise and fall of the 38 kDa band, which has now been identified as

domain L + EGFP may be caused by proteolysis. Proteolysis near the C-terminus of domain L would liberate a relatively large peptide migrating at 12 kDa. Unfortunately, bands of 10 kDa and 15 kDa in the molecular weight marker migrated from the 10 % gel. On this basis, the domain L remnant would also have migrated from the gel.

pEGFP-DL1 - Figure 5.13 shows that the level of a 63 kDa GFP immunoreactive protein increases over the experimental period. In contrast, this blot identifies the same protein using anti-domain L antibody but demonstrates a decline in its levels to 50 % of the initial levels over the experimental period. This contradiction may result from inaccuracy of the image analysis especially of the lightly contrasted GFP blot.

5.4.9 Recombinant Calpastatin domain 1

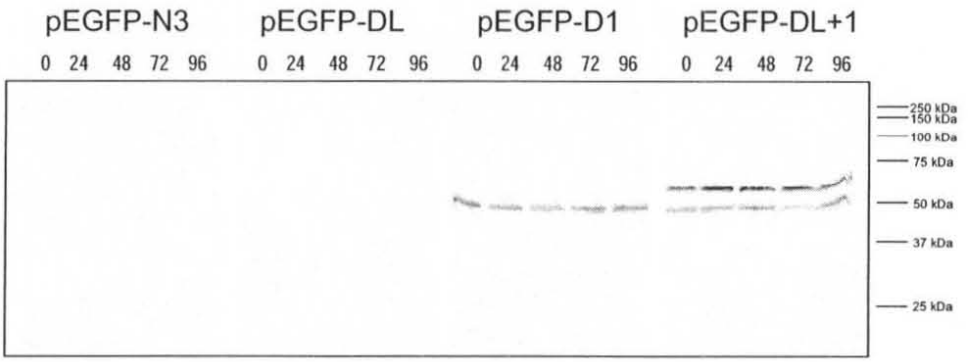

Figure 5.14 Comparison between experimental treatments of Calpastatin Domain 1 levels over 96 hours. Samples of protein (100 ug) were electrophoresed on a 10 % SDS-PAGE gel before being probed with anti-calpastatin domain 1 antibody (566) at 1 : 100 dilution.

Antibody 566 was raised against domain 1 antigen and, as shown in Figure 5.14, cross-reacted with protein species from pEGFP-D1 and pEGFP-DL1 transfectants only.

pEGFP-N3 and pEGFP-DL - Protein extracts from the pEGFP-N3 and pEGFP-DL transformants should not contain immunologically reactive domain 1. The light banding patterns in these Westerns are due to non-specific cross-reactivity.

pEGFP-D1 - The anti-domain 1 polyclonal antibodies raised in Chapter 3 have demonstrated a poor reactivity against the domain 1antigen. Nevertheless, two unique bands are apparent in pEGFP-D1 extract. These bands correspond to a 49 kDa and 46 kDa doublet using anti-GFP. The bands are too faint to be measured accurately using densitometry.

pEGFP-DL1 - The poor reactivity is repeated when probing the extract from pEGFP-DL1 transfectants. The Western blots revealed three bands at 63 kDa, 52 kDa and 49 kDa. While the bands not reproduce well they are very similar in migratory pattern to those seen with the anti-GFP antibody. They probably represent the full sized L1-EGFP and two truncated products.

5.4.10 Summary

A selection of proteins were examined in order to monitor biochemical differentiation and *in vivo* proteolysis. The Western blots presented in this chapter do not provide any evidence of significant effects of transfection on biochemical differentiation. The way in which myosin, desmin and troponin T appear, is similar to non-treated cells and the negative control pEGFP-N3 treated cells. Desmin and troponin T were included in the analysis because they are efficient indicators of differentiation and, in addition, are substrates for calpain *in vivo*. The transfection of C2C12 myoblasts with calpastatin variants and the expression of recombinant protein has the potential to reduce calpain activity *in vivo*. This may be reflected in altered substrate proteolysis. It was established in Chapter 4 that desmin was not subject to proteolysis in fusing C2C12's. Troponin T, however, showed a banding pattern which may result from proteolysis. The pattern was also detected in the transfected cell extract. Unfortunately, there were no apparent differences between control and treated cells. A simple conclusion is that calpain is not responsible for the proteolysis of troponin T. However, several other interpretations are possible: (i) calpastatin may be separated from any calpain - troponin T associations (ii) the troponin T fragments may not be produced by proteolysis (iii) the calpastatin protein produced from the vector may be inactive.

There were no significant differences between the treatments with respect to μ- and m-calpain protein levels. It is not known whether expression of calpastatin variants will impact on the expression of endogenous calpain. However, calpain autolysis is an indicator of *in vivo* activity and elevated levels of calpastatin can delay the appearance of autolytic products (Geesink & Koohmaraie 1999). Therefore, μ- and m-calpain were investigated, not only because they represent integral components of the fusion process, but also because their autolysis provides another measure of calpastatin activity. Unfortunately, the autolytic products of m-calpain were not resolved by SDS-PAGE in these experiments. Moreover, μ-calpain autolysis was similar in all the cell extracts and was independent of the treatments. There are two possible interpretations of this data: (i) inhibitory calpastatin is physically isolated from active calpain and (ii) recombinantly expressed calpastatin is inactive.

The Western blots exploring the products of the EGFP vectors revealed some unexpected result. It appears that recombinant calpastatin is either structurally unstable or susceptible to proteolysis in C2C12's. The principal observations have been gathered and an explanatory model is proposed.

As discussed in each section (5.4.7 – 5.4.9) EGFP itself appears to be stable throughout the fusion process. A point located in or around the EGFP linker is subject however to truncation in domain L + EGFP hybrids. A small portion of domain 1's N-terminus is also susceptible to truncation. Finally, the region demarcating domain L from domain 1 appears unstable in pEGFP-DL1 transfectants. The putative sites of instability or proteolysis are shown schematically in Figure 5.15.

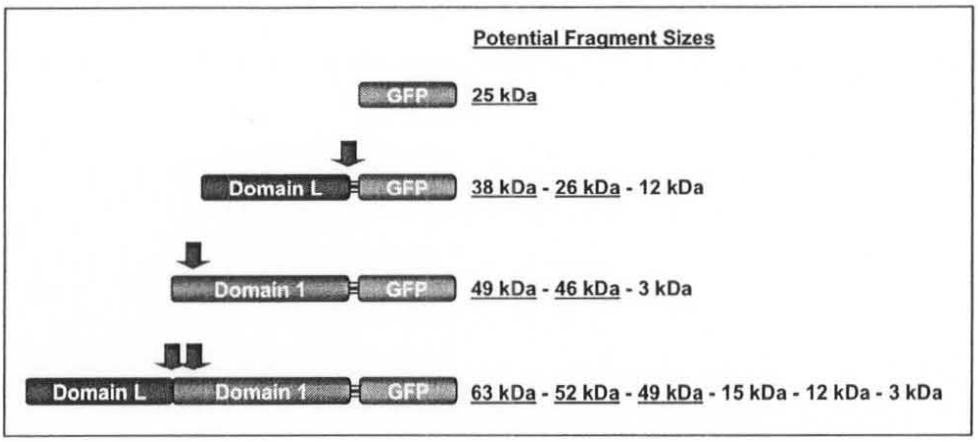

Figure 5.15 Schematic diagram showing the hypothetical cut points in recombinant calpastatin expression products. Four separate protein products are encoded by the EGFP plasmids used in this thesis. Protein products from plasmids combining regions of calpastatin with EGFP include an additional 10 amino acids between the C-terminus of calpastatin and the N-terminus of EGFP (≡). Points of structural instability or proteolytic susceptibility in these products are denoted with red arrows. The potential fragments resulting from truncation at these points are reported, those that have been detected in Western blots are underlined.

Recombinant calpastatin variants are also prone to truncation. The diagram (Figure 5.15) depicts the hypothesised points within the protein where the truncations occur. To confirm the accuracy of this model, representative samples of each protein were on the same gel and probed with anti-GFP (see Figure 5.16). The anti-GFP antibody will detect full-sized products from all four transfectants as well as the truncated products which have been difficult to resolve. Samples of the transfectants were compared to provide representative banding patterns proposed by Figure 5.16.

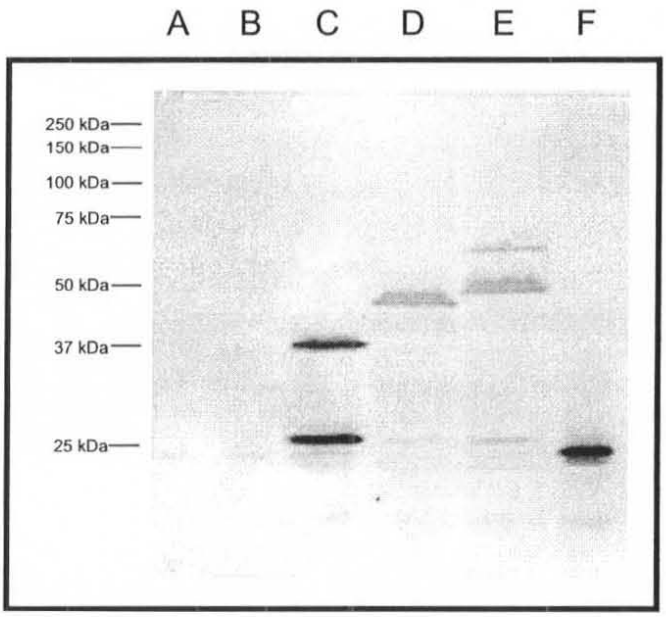

Figure 5.16 Anti-GFP Western blot of representative samples from each pEGFP transfection. Protein samples (100µg) from control cells at day 1 (A) and day 4 (B) were compared with samples of protein from day 1 pEGFP-DL (C), day 4 pEGFP-D1 (D), day 4 pEGFP-DL1 (E) and day 1 pEGFP (F). The particular days were chosen for each transfection to provide the best representation of protein fragments. Proteins were electrophoresed through a 10 % SDS-PAGE gel, transferred and probed with anti-GFP antibody (1:100).

Figure 5.16 provides information that contributes to the model presented in Figure 5.15. As well as resolving the close band pairings within pEGFP-D1 and pEGFP-DL1 transfectants, the presence of a 26 kDa band was revealed. Due to its relatively faint presence, this 26 kDa band was not detected in Figure 5.12. On a size basis, this fragment corresponds to the pEGFP-DL transfectant band resulting from truncation at the linker. Previously, it was not clear whether this truncation took place within the linker region or was at the N-terminus of domain L. An inability to detect the 26 kDa protein in either pEGFP-D1 or pEGFP-DL1 extract inferred that the cleavage was occurring at domain L's N-terminus. However, Figure 5.16 refutes this by exposing the 26 kDa protein in pEGFP-D1 and pEGFP-DL1 extracts, and thus establishing that the truncation point lies within the linker region.

The model proposed in Figure 5.15 relies on several untested assumptions. Several issues need addressing in order to reconcile the observations made in this chapter. They are:

(i) why is a large portion of domain L1 + EGFP's N-terminus removed (63 kDa ➔ 52 kDa),

(ii) why is the 3 kDa of domain 1's N-terminus removed (49 kDa ➔ 46 kDa for pEGFP-D1 transfectants, and 52 kDa ➔ 49 kDa for pEGFP-DL1 transfectants),

(iii) why, does domain 1 + EGFP from pEGFP-DL1 (52 kDa) transfectants migrate differently to domain 1 + EGFP from pEGFP-D1 (49 kDa) transfectants.

Clearly, the model proposed in Figure 5.15 is inadequate and does not fully explain the complex expression patterns apparent in transfected cells. Furthermore, it is not possible to speculate whether the appearance of the unexpected fragmentation patterns is associated with the apparent failure of the calpastatin variants to impact on fusion. Potentially, if calpastatin is subject to proteolysis or suffers from structural instability then these effects may explain the poor inhibitory potential.

5.5 Microscopic examination to detect EGFP

To date, the results have shown that the expression of calpastatin – EGFP hybrids have failed to have any effect on the fusion of C2C12 myoblasts. Despite the FACS analysis and Western blots demonstrating that there is expression of EGFP, efforts to detect calpastatin activity in the C2C12 extracts were inconclusive. An important aspect in the conception of this work was to design calpastatin variants containing the three possible arrangements of domains L and 1 ie. DL, D1 and DL1. The design was based on the evidence that *in vivo* calpastatin localises to the plasma membrane via domain L. In addition, domain L is apparently the region preferentially phosphorylated (Averna *et al.,* 1999) and leads to accumulation of the protein at the plasma membrane (Adachi *et al.,* 1991). As a consequence, fluorescent microscope photographs were taken to establish the intracellular location of EGFP. The

expectation is that cells transfected with DL or DL1 would preferentially localise to the plasma membrane and that D1 would be dispersed throughout the cytosol. As shown in Figure 5.17 this was not the case.

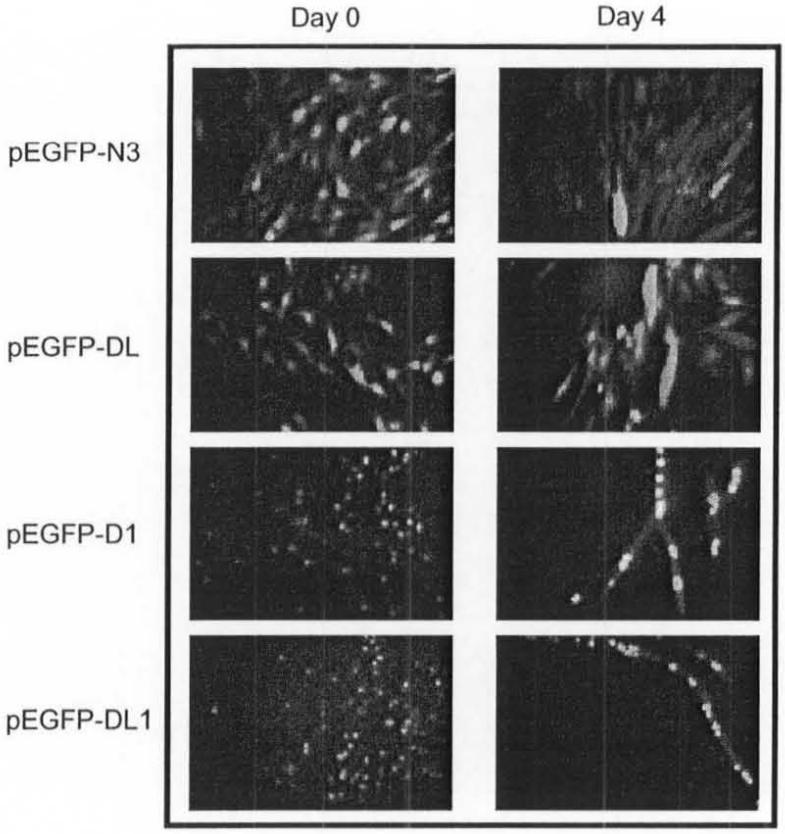

Figure 5.17. Photographs of fusing C2C12 cells transfected with calpastatin - EGFP hybrids. Photographs were taken at 0 and 96 hours after fusion induction. Cells were transfected with pEGFP-N3 (**A**), pEGFP-DL (**B**), pEGFP-D1 (**C**) and pEGFP-DL1 (**D**). Photographs used Fujichrome Sensia II 400 film, an automatic light meter and were photographed at x400 magnification.

Despite the results in Figure 5.2 which indicated that transfection efficiency was high, microscopic examination revealed there were relatively few representative muscle cells which fluoresced brightly. While C2C12 cells were evenly dispersed over the slide surface, sub-populations of fluorescent cells were not evenly dispersed and appeared in localised regions. This may reflect an uneven distribution of the transfection mix and / or pockets of sibling cells derived from the transfected parents.

The photographs shown in Figure 5.17 indicate differential subcellular localisation. It would appear that at all stages of fusion, EGFP is evenly dispersed throughout the cells transfected with pEGFP-N3 and pEGFP-DL. It is not possible from these images to be certain if the diffuse cytoplasmic fluorescence is throughout the cytoplasm or associated with the plasma membrane. These distributions appear similar when observing the whole cells from above although a plasma membrane distribution may produce a ring effect where the cross sectional area of the cell is the greatest. In contrast, pEGFP-D1 and pEGFP-DL1 transfectants demonstrate localised EGFP fluorescence. By comparing the size of the localised fluorescence, relative to the cell size, with a Giemsa stained cell and images of nuclear stained C2C12 myotubes (Crescenzi *et al.,* 1994), it is possible to speculate that the fluorescence is in or around the nucleus. Examination of pEGFP-D1 and pEGFP-DL1 transfectants reveals a small amount of evenly dispersed fluorescence. This is not unexpected since protein expression takes place within the cytoplasm.

In both distribution profiles, diffuse and localised, the fluorescent agent may predominantly bind to membranes. As noted, direct observation of the whole cell from above does not provide enough detail to resolve this question. Confocal microscopy is a technique whereby the whole cell or tissue sample is exposed to lasers. This permits the investigator to virtually section the sample in three dimensions. Focussing the lasers up and down provides images of the cell on a single plane and establishes whether the fluorescence shows any membrane association.

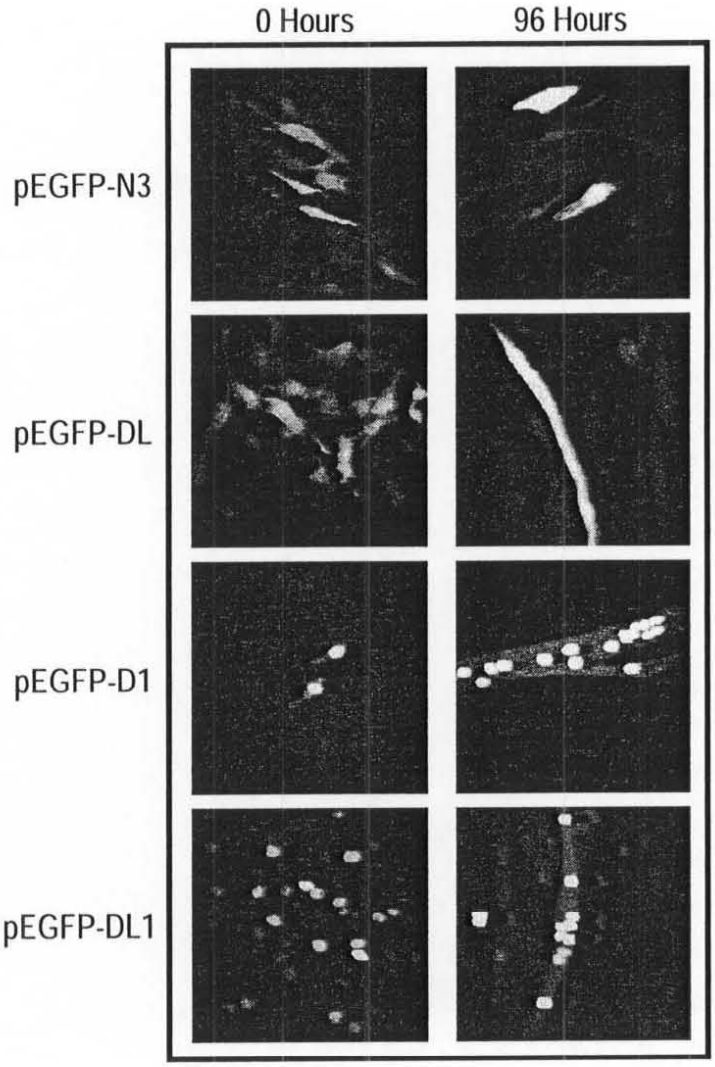

Figure 5.18. Confocal images of transfected C2C12 at 0 and 96 hours after fusion induction. In each instance, images were captured from the transect plane providing the greatest information.

The pictures in Figure 5.18 verify that the distribution seen with pEGFP-N3 and pEGFP-DL transfectants is diffuse and throughout the cytoplasm. There is no preference for the plasma membrane. In the case of pEGFP-D1 and pEGFP-DL1 transfectants, fluorescence was throughout the subcellular compartment and was not restricted to the internal phospholipid membranes.

Contrary to expectations, in these experiments, domain L did not demonstrate potential to direct calpastatin's localisation to the plasma membrane.

Remarkably, it appears that domain 1 may play a significant role in directing calpastatin to the nucleus. This was demonstrated by EGFP hybrids including domain 1 which exhibited preferential localisation to the nucleus. The model proposed to explain the complex recombinant calpastatin patterns (Figure 5.15) suggests that domain L is removed from the EGFP component, while domain 1 remains attached. If this model is accurate, then domain L may exhibit membrane associations which would not be reflected in the localisation of EGFP. In contrast, the bulk of domain 1 remains attached to the EGFP component. It seems likely that the failure of expressed calpastatin to perturb fusion may be due to this distribution. That is, domain 1 is the only region of the introduced cDNA with the ability to influence calpain activity, and sequestering domain 1 within the nucleus prohibits it from influencing calpain mediated proteolysis at the plasma membrane or within the cytoplasm in general.

Previously, the calpain and calpastatin system has been associated with the nucleus and nuclear events. Gilchrist *et al.* (1994) wrote an excellent review which discussed the importance of calcium and calcium binding proteins, including calpain, in the nucleus. It has been observed *in vitro* that μ- and m-calpain are able to solubilise the nuclear protein H1 kinase and that the calcium concentration required for m-calpain activity was reduced to 3 μM (Mellgren & Rozanov 1990; Mellgren 1991). This is close to physiological concentrations of calcium and is dependent upon the presence of DNA (Mellgren *et al.,* 1993). These results indicate that DNA has the capacity to regulate the action of the calpain system and proteolysis.

Moreover, nuclear protease activity has been detected in a variety of rat tissues (Gopalakrishna & Barsky 1986) and in differentiating cells where it is thought to play a role in nuclear function by regulating transcription factor turnover (Scholtz *et al.,*1996). For example, the c-*mos* proto-oncogene product $pp39^{mos}$, accumulates in mature oocytes and arrests meiosis. Upon fertilisation there is a large transient increase in calcium and $pp39^{mos}$ levels decline. The inclusion of calpain inhibitors (leupeptin, E-64, antipain or calpastatin) prevents degradation of $pp39^{mos}$ (Watanabe *et al.,* 1989; Watanabe *et al.,* 1991) which strongly indicates that the $pp39^{mos}$ cytostatic factor is a target for selective calpain proteolysis. Other transcription factors have

also been shown to be sensitive to calpain (Watt & Molloy 1993). In particular, *c-fos* and *c-jun* proto-oncogene products are short lived proteins with a high susceptibility to calpain proteolysis (Hirai *et al.,* 1991, Carillo *et al.,* 1994; Carillo *et al.,* 1996). As a heterodimer they form a large part of the AP-1 translational activator protein. The latter is a critical transcriptional activator protein (Gutman & Wasylyk 1991) with myogenic regulatory potential. Specifically, the AP-1 protein suppresses the transcriptional activation of certain muscle specific proteins by myogenin and MyoD1 (Li *et al.,* 1992). Moreover, co-expression of *c-jun* with calpastatin sense or anti-sense cDNA resulted in a decrease and increase, respectively, in the transcription of c-Jun induced CAT expression (Hirai *et al.,* 1991). Note that *in vivo* calpain mediated proteolysis of c-*FOS* and c-*JUN* is probably a cytoplasmic event regulating levels of full length protein that is available for nuclear transport (Carillo *et al.,* 1994). More recently, nuclear factor κB, which regulates a wide variety of viral and cellular genes, has demonstrated a specific sensitivity to both μ- and m-calpain (Liu *et al.,* 1996). As well as nuclear functions, calpain has displayed a potential involvement in structural changes taking place in the nucleus. Leupeptin inhibited proteolytic activity has been associated with lamin proteins which contribute to the nuclear scaffold (Tokes & Clawson 1989). Also, the microinjection of m-calpain into PtK_1 cells accelerates mitotic spindle disassembly and the start of anaphase (Schollmeyer 1988). Both events demand the disruption of the karyoskeleton. In these experiments, specific localisation of m-calpain to the chromosomes, nuclear membrane and mid-body regions were witnessed at different phases of the mitotic cycle.

Several other studies have reported distinct nuclear subcellular localisation. Mellgren and Lu (1994) demonstrated, using digitonin permeabilised A431 cells, that FITC labelled μ-calpain was localised to the nucleus in an energy (ATP) dependent manner. Interestingly, the translocation could be repressed by co-incubation with a Nuclear Localisation Signal (NLS) peptide suggesting that the mechanism responsible for translocation of μ-calpain recognises this sequence in this protein. In another study, examination of a human primary myoblast cell culture demonstrated a specific nuclear distribution of μ-calpain, during maturation into myotubes. However, m-calpain was diffusely distributed throughout the cytoplasm

(Moraczewski *et al.,* 1996). The lack of m-calpain nuclear translocation was also witnessed by Mellgren & Lu (1994) who saw FITC labelled m-calpain dispersed through the cytoplasm and Dourdin *et al.* (1997) who reported extracellular distribution of m-calpain.

With respect to calpastatin, localisation experiments have generated a variety of results. Calpastatin has been found associated with myocardial sarcolemma and sarcoplasmic reticulum vesicles (Lane *et al.,* 1985; Mellgren *et al.,* 1987a; Mellgren *et al.,* 1987b). Recently, FITC labelled calpastatin introduced into permeabilised A431 cells demonstrated no selective transport to the nucleus or any other compartment (Mellgren & Lu 1994). Calpastatin has been localised to two granular, non-membrane bound structures, roughly 1 μm in diameter close to nuclear invaginations (De Tullio *et al.,* 1999). This pattern was also found in several murine and human cell lines. The authors noted that following differentiation induction of LAN-5 neuroblastoma cells, calpastatin is displaced from its granular form into the soluble fraction where its absolute levels decline. It is unclear why different studies produce such widely differing results. Sample type and preparation, antibody characteristics and other variables could possibly explain some of the differences.

Examination of the downstream domain 1 primer depicted in Figure 4.13 revealed a similarity with reported nuclear localisation consensus signals. This primer encodes domain 1's C terminal amino acid residues, as defined in Appendix B, which are PPKEKRRKV. The basic nature of this sequence exhibits the characteristics of other NLS. Specifically, NLS are: (1) short sequences of 8-10 residues, and (2) contain a high proportion of charged residues such as K and R (Garcia-Bustos *et al.,* 1991; Silver 1991). An archetypal example of NLS is PKKKRKV, this is apparent in SV40 T antigen (Matheny *et al.,* 1994) and shares similarities with a multitude of putative NLS (Boulikas 1994).

As noted earlier, studies examining the subcellular localisation of calpastatin have generated variable results. Certainly there is no clear evidence of calpastatin's nuclear localisation as seen in this chapter. This is despite the PPKEKRRKV sequence being an integral part of this protein, and being present in a number of

other calpastatin sequences (Takano *et al.*, 1986b; Mainelli *et al.*, 1996; Takano *et al.*, 2000). The reason for this may be the absolute requirement for the NLS to be presented on the surface of a targeted protein. Strong NLS sequences have no ability to direct proteins when buried within the quaternary structure of the protein (Garcia-Bustos *et al.*, 1991). The amplification of domains L1 and 1 resulted in the relative relocation of this NLS to the C-terminus of the protein. After folding, this sequence may be exposed in a manner which enables it to interact with the nuclear pore complex proteins, and culminates in its active importation.

5.6 Discussion

The experimental work in Chapter 4 was preparatory for the experiments reported in this Chapter. Plasmids combining regions of calpastatin cDNA were combined with EGFP cDNA in mammalian expression vectors. These were successfully introduced into proliferating myoblasts using FuGENE 6 reagent. FACS analysis allowed the transfection efficiency to be determined along with a variety of other physical parameters.

Despite a relatively high proportion of cells expressing EGFP, there were no clear indications of reduced fusion at the gross level. Western analysis also failed to detect any significant effects from calpastatin expression either as altered proteolytic patterns of calpain substrates or as reduced calpain autolysis. What it did reveal, however, was evidence that recombinant calpastatin is susceptible to truncation following expression in C2C12's.

This is a crucial result since it fails to support the hypothesis that expression of calpastatin perturbs fusion events. It is not possible to determine if the structural decomposition of the calpastatin isoform is related to its lack of physiological impact.

Microscopic examination revealed that recombinant calpastatin variants combining domains 1 or L1 with EGFP were preferentially located within the cell nucleus. This is probably a critical observation which explains why the inhibitory calpastatin isoforms did not affect fusion. Some mechanism must be responsible for targeting

and transporting domain 1 containing isoforms to the cell nucleus after their manufacture. Once retained within this intracellular body calpastatin is effectively isolated from proteolytic events which occur in the cell, including those taking place at the plasma membrane. Unfortunately, the presence of a potential NLS in domain 1 may have predestined this localisation outcome. Changing the position of the domain 1 downstream primer could have been used to eliminate this short region and would have contributed to confirming its importance in nuclear targeting. Equally, however, there is potential to investigate the role of this sequence in regulating the targeting of proteins to the nucleus.

Chapter 6

Conclusions and Future Work

Despite the ubiquitous nature of the calpains and their endogenous inhibitor calpastatin, the physiological function(s) of this protease system remains uncertain. The developmental process of myogenesis is dramatic and tightly regulated. Available evidence strongly supports a role for calpains in proteolytic events accompanying plasma membrane reconstruction. Moreover, fluctuations in the level of calpastatin expression seem to be a permissive requirement for the fusion process. This thesis sought to investigate the potential physiological role that calpastatin plays in myoblast fusion. In addition, investigation into proteolysis regulation at the plasma membrane was inspired by reports that calpastatin's domain L may be responsible for targeting calpastatin to this region and / or be involved in regulating its inhibitory activity.

A major objective of this thesis was to produce fusion competent myoblasts expressing regions of ovine calpastatin hybridised to EGFP. This outcome has been achieved through a linear experimental approach. Initially, the particular regions of ovine calpastatin (domain L, domain 1 and domains L1) were expressed in prokaryotes, purified, assayed *in vitro*, and used as antigens in the production of polyclonal antibodies. The approach used to generate prokaryotic expression vectors was also employed to produce eukaryotic expression vectors combining calpastatin with EGFP. After optimising the transfection conditions, these vectors were introduced into cultured myoblasts (C2C12's). Cells were grown under conditions that stimulated both morphological and biochemical differentiation. Microscopic examination, FACS analysis and Western blotting were used to monitor aspects of myogenesis in control cells and treated cells.

While regions of calpastatin have been expressed in myoblasts together with EGFP, they produced no detectable effects upon the fusion process aspects or biochemical differentiation. Based upon the results there are two potential

explanations for this: (i) the truncation of recombinant calpastatin may indicate a loss of functionality or (ii) the nuclear localisation of the expressed calpastatins embracing domain 1 may prevent the inhibitor from acting upon free cytosolic or plasma membrane bound calpain.

The failure of recombinant calpastatin to interfere with fusion does not negate the likely role of this protein in regulating the processes via calpain inhibition. Significant data exists in the literature which provides convincing evidence for calpastatins to have a potential impact in fusion events. However, further research should be directed at establishing the validity of the two explanations mentioned above.

Extraction and purification of recombinant calpastatin from large scale cultures of transfected myoblasts followed by *in vitro* assays should establish the functional capacity of the truncated protein. Care should be taken to limit any proteolysis immediately following extraction. Purification of recombinant calpastatin may employ traditional chromatographic techniques together with heat denaturation. The EGFP polypeptide may provide the opportunity to employ an additional affinity chromatographic step. Alternatively, zymography may be a suitable technique to detect the calpastatin truncation products using an approach that has not been attempted previously. Non-denatured protein samples extracted from myoblasts could be electrophoresed through a zymographic gel prepared from a fluorescent substrate, such as BODIPY-FL (Thompson *et al.,* 2000b). This gel could then be incubated in the presence of excess calpain and calcium over a period of time which allows diffusion of the protease into the gel matrix. Calpain that is not inhibited by electrophoresed calpastatin will proteolyse the casein substrate resulting in fluorescence. This approach, while not establishing the quantity of calpastatin, may qualitatively assess the truncated isoforms.

The second issue addresses whether calpastatin's localisation in the nucleus reduces its potential to modify calpain activity in the cytoplasmic compartment. The most effective way of answering this question is to prevent recombinant calpastatin from entering the nucleus. Although nuclear pores allow the diffusion of relatively

large proteins into and out of the nucleus, the differential inclusion of only recombinants containing domain 1 implies that the mechanism of relocation is selective and active. Previously, ATP depleting reagents and expression with nuclear localisation sequence peptide have demonstrated an ability to retard nuclear importation (Mellgren & Lu 1994). Either of these approaches could be used to restrict the activity of recombinant calpastatin in the cytoplasm.

While this thesis was not successful in detecting an effect of recombinant calpastatin upon myoblast fusion, it has reported some valuable observations and developed some useful techniques. These have the potential to be applied in other experiments.

A significant product from the initial work was the expression and purification of relatively large quantities of recombinant calpastatin from bacteria. The ability to manipulate the cDNA sequence with, for example, site-directed mutagenesis permits the production of defined proteins. This enables very precise exploration of structure and function. Moreover, the generation of pure proteins creates an opportunity to produce specific antibodies, which in tern can be used in diagnostic tests (eg. Western) *in vivo* manipulation (eg. applying "anti-x" antibodies to systems in tissue culture) and purification approaches (eg. calpastatin affinity chromatography). Significantly, Chapter 3 revealed that domain 1 activity *in vitro* was enhanced when associated with domain L. The mechanism behind this observation is unclear, although the domain L specific phosphorylation has been associated with augmentation of activity (Salamino *et al.,* 1994a; Salamino *et al.,* 1994b; Averna *et al.,* 1999).

While Chapter 4 was primarily directed towards establishing methods that quantify aspects of "normal" myoblast differentiation, it was able to assess the validity of several published observations. Significantly, in these experiments μ-calpain was detected in fusing myoblasts. This contrast with previous reports which found μ-calpain at late stages of fusion (Cottin *et al.,* 1994) or not at all (Temm-Grove *et al.,* 1999). Additionally, after testing a selection of antibodies, only one (created during the course of this work) demonstrated immunoreactivity against

murine calpastatin. Moreover, this reactivity was witnessed only in mature skeletal muscle while myoblasts in culture did not appear to contain detectable levels of this protein. This observation is contrary to a number of other studies which report detecting calpastatin in muscle cultures.

The final segment of work in this thesis developed techniques for analysing the fusion of myoblasts and for detecting EGFP. The opportunity to directly visualise proteins *in vivo* is attractive to cell biologists interested in investigating the function of cellular machinery. To my knowledge, this is the first example of this labelling technique being employed to explore the calpain system. The potential to apply this technology to answer other questions is considerable. The availability of GFP mutants emitting fluorescence at differing wavelengths means that differentially labelling calpain isoforms and calpastatin and observing their interactions is potentially possible. Together with labelled substrates, this type of data could establish a complete description of the action *in vivo* of the calpain system under a variety of conditions. Artificial vector systems would also permit controlled protein expression through the inclusion of inducible promoters or temporally regulated promoters (eg. myosin light chain promoter).

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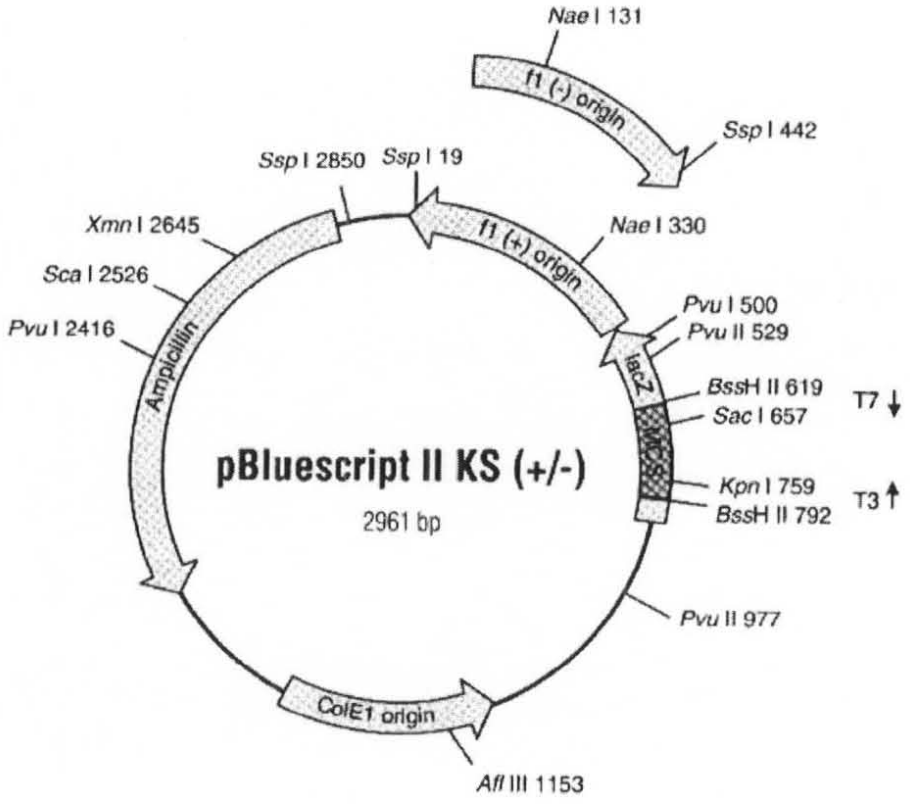
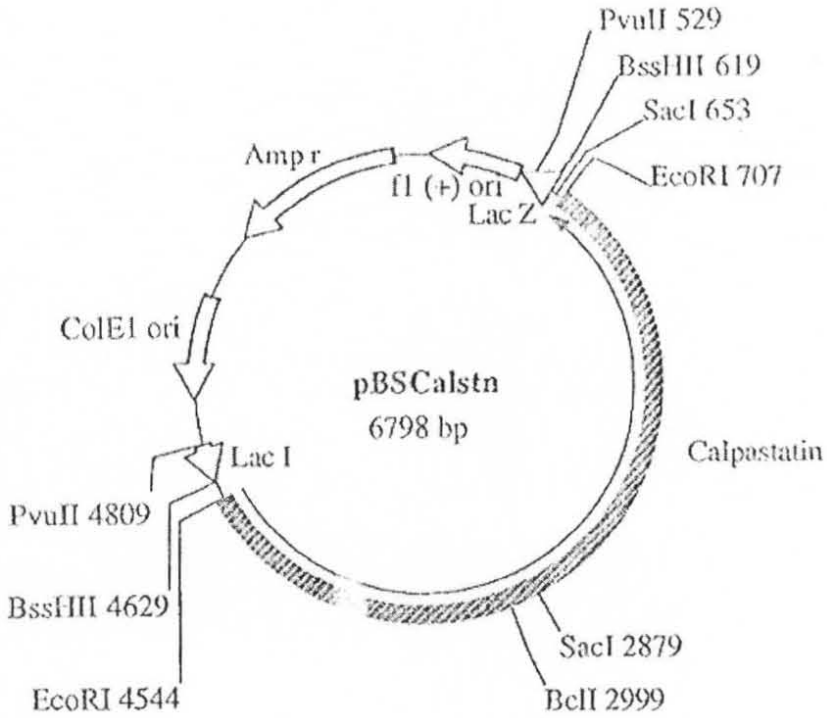
Appendix A.1 – pBluescript Plasmid Map



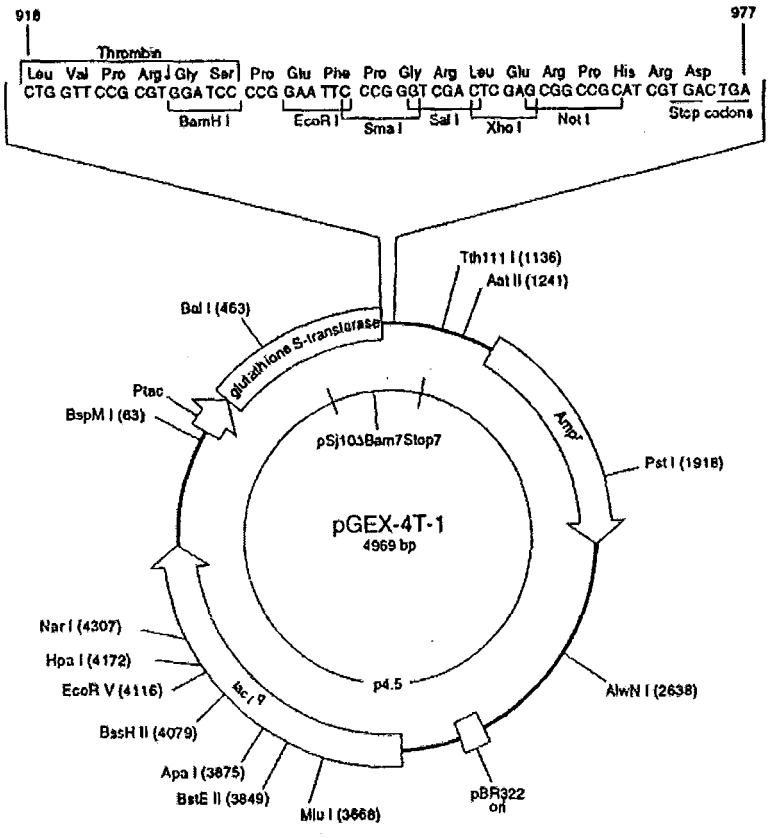
Plasmid map of the pBSKS II phagemid commercially available from Stratagene. GeneBank accession number #X52327.

Appendix A.2 – pBSCalstn Plasmid Map



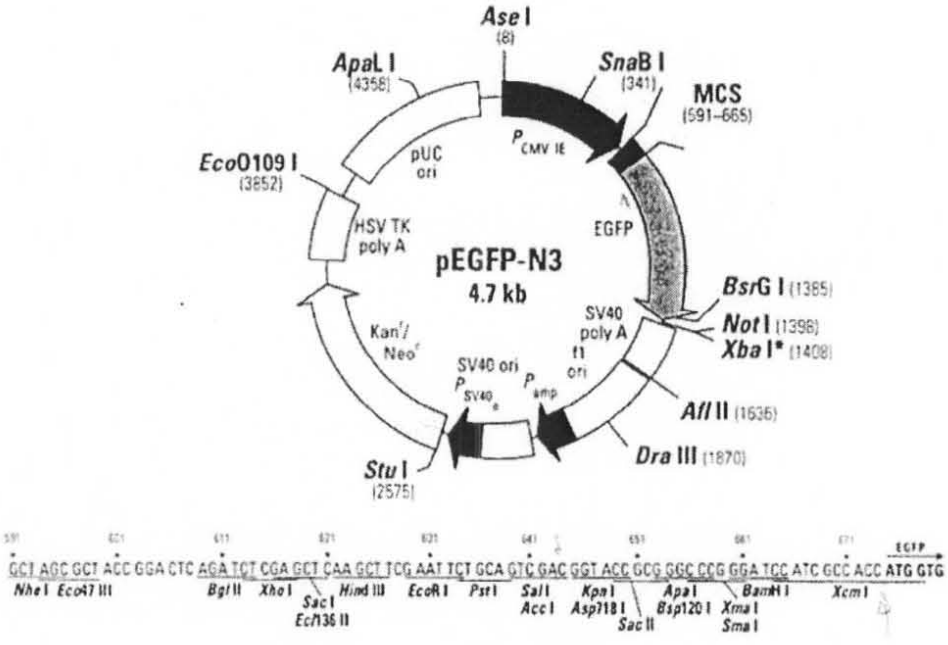
Plasmid map of pBSCalstn. Plasmid is composed of the complete ovine cDNA inserted into the pBSKS vector (Collingwood 1994).

Appendix A.3 – pGEX-4T-1 Plasmid Map



Plasmid map of the commercially available pGEX-4T-1 vector (APB). Included is an expanded view of the plasmids multiple cloning site. GeneBank accession number U13853.

Appendix A.4 – pEGFP-N3 Plasmid Map



Plasmid map of the pEGFP-N3 plasmid commercially available from Clontech. Included is a description of the plasmids multiple cloning site. GeneBank accession number U57609.

Appendix B – Ovine Calpastatin cDNA Sequence

SEQ New: 2199 bp;
Composition 744 A; 557 C; 526 G; 372 T; 0 OTHER
Percentage: 34% A; 25% C; 24% G; 17% T; 0%OTHER

Molecular Weight (kDa): ssDNA: 679.99 dsDNA: 1355.7
ORIGIN

↓Xba1 cut site
ACTG*TCTAGA* CCGGTCACCA AGGTAACTAC TTCCTCCGCT TCAGCCAGCA AGTCTTCCAG
↓Start of Domain L
T|ATGAATCCC ACAGAAGCCA AGGCTGTAAA AACAGAACCT GAGAAGAAGC CGCAATCATC
↑ DL 5′ primers binding site
TAAGCCAAAA AGCCAACCCA AGCACCCCTC AGATACAAGA AGCAAGCATG CTCCTAAGGA

AAAAGCTGTT TCCAAGTCAA GCGAGCAGCC ACCATCAGAG AAATCAACAA AACCAAAGAC

CAAGTCACAG GACGAGATTT CCGGTGGTGG AAAGAGCGCT GTTCCTGCTG TTGCAGCTGC

AGCATCTGCC GAACCAGCTG ACAAGAATAA AGAAAGTAAA TTGTTAACAT CGGCCGTGCC
Domain L ↓ Domain 1 boundary
AGTTGAATCC AAGCCAAGTA AACCTTCTGC A|AAGTCAGAC ATGGATACTG CTCAGGATGA
DL 3′ primer binding site↑ ↑ D1 5′primer binding site

CTTAATAGAC ACTTTAGGAG AACCTGAAGA GACAAAAGAA GATACCACAA CATATACTGG

ACCTGAAGTT TCGGATCCAA TGAGTTCTAC CTACCTCGAG GAACTGGGTA AAAGAGAAGT

CACACTTCCT CCAAAATATA GGGAACTTTT GAATAAAGAA GAAGGGATCG CAGGGCCTCC

TCCAGACTCC TCGAAACCCC TGGGGCCCAA TGACGCCATC GATGCCTTGT CATCAGACTT

CACCTGCAGT TCCCCTACAG CTGATGCAAA GAAAACTGAG AAAGAGAAAT CTACAGAAGA

GGCTTTAAAA GCTCAGTCAG CTGGGGTGAT CAGAAGTGCT GCTCCACCCA AAGAGAAAAG
Domain 1 ↓ Domain 2 boundary
AAGAAAAGTG GAAGAG|GATA CCATGACTGA GCAAGCCCTG GAGGCCCTGT CTGCCTCCCT
↑ DL 3′ primer binding site
↓Sac1 cut site
GGGCACCCGG AAGCCAGAGC CA*GAGCTC*GA CCCCAGCTCC ATTAAGGAGG TCGATGAGGC

AAAAGCCAAA GAAGAGAAAG TAAAGAAATG TGGTGAAGAT GAGGAAACAG TCCCATCGGA

GTATAGATTA AAGCCGGCCA CAGATAAAGA TGGAAAACCA CTCTTGCCAG AGGCTGAAGA

AAAACCCAAG CCCCTGAGTG AATCAGAACT CATCGATGAA CTCTCAGAAG ATTTTGACCG

GTCTAAGTGT AAAGAAAAAC AATCTAAGCC AACTGAAAAA ACAGAGGCAT CCCCGGCCGC

TGCCCCTGTG CCTGTGGCAG AGGATGTGCC TCGGACCTCC ATGTGTTCTG TGCAGTCAGC
Domain 2 ↓ Domain 3 boundary
TCCACCCACA GCAGCT|CCAG CGAAGGGCAT GGTGCCAGAC GATGCTGTGG AAGCCTTGGC

TGGAAGCCTG GGCAAAAAGG AAGCAGATCC AGAAGACGGA AAGCCTGTGG AGGATAAAGT

CAAGGAGAAA GCCAAAGAAG AGGATCGTGA GAAACTTGGT GAAAGAGAAG AAACGATTCC

TCCTGATTAC AGATTAGAAG AGGCCAAGGA TAAAGACGGA AAACCACTGC CGCCAAAAGA

GGTCAAGGAA CCGCTCCCAC CCTTGAGTGA AGACTTCCTC CTCGATGCTC TGTCCAAGGA

CTTCACTGTC CCCTCAGACA CGTCATCGCC TCAATTTGAA GATGCTAAAC TTTCAGCTGT

CGTCTCTGAA GTGGTTTCCC AAACCCCAGC TCCAACCACC CAGGCAGCCG GTCCACCCCG

Domain 3 ↓ Domain 4 boundary

CGACTCTGCG CAGCGTGAC|A ACAAAGAACT TGACGATGCC CTGGATCAAC TTTCTGACAG

TCTCGGGCAA AGACAGCCTG ATCCAGATGA GCATAAACCT GTAGAGGATA AAGTCAAGGA

AAAAGCCAAA GCTGAACACA GAGACAAGCT GGGAGAAAGA GATGACACCA TCCCACCTAA

ATACCAGCAT CTTCTGGATG ATAACAAGGA GGGCACACCC GGGAAGCCAA AGGCATCAGA

GAAGCCCGAG GCATCAGAGA AACCTGCAGG TGCCCAGGAC CCCATTGACG CCCTCTCAGG

GGACTTGGAC AGCTGTCCCT CAACTACAGA AGCCTCAACA AACACAGCAA AGGACAAAGA

CAAGAAGCCT GCTTCCAGTG ACAAAGCACC CAGGAATGGC GGGAAAGCAA AGGATTCCAC

End of Domain 4 ↓

AAAGGCAAAG GAGGAAACT|T CCAAGCCAAA AGCTGATGGA AAAAGTACAA GTTAAAATTC

ACAGTATTTG GTTCTGCATA TACAATCTGC AGCAGGTAGA AGAACGTCTG AAGAACAAAA

↓XbaI cut site

GCCTTTGACA ACAGAAACAA TTCCTGGGTG GCT*TCTAGA*

Complete ovine calpastatin sequence encoding domains L, 1, 2, 3 and 4. Domain boundaries are noted in Blue, restriction enzyme sites are labelled in red and regions to which the primers shown in Figures 3.3 and 4.13 are underlined.

Appendix C – Raw FACS Results

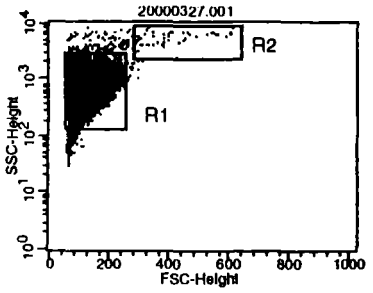
The following pages present raw FACS results obtained from C2C12 cells at 0, 24, 48, 72, 96 and 240 hours (Days 0, 1, 2, 3, 4 and 10). At each time interval five sets of data are presented. These represent control cells (**C**), and cells transfected with pEGFP-DL (**DL**), pEGFP-D1 (**D1**), pEGFP-DL1 (**DL1**) and pEGFP-N3 (**N3**). Within each data set three graphical plots are presented. The scatter plot reflects all measured events (cells) in terms of size (Y axis) and granularity (X axis), and depicts the R1 and R2 regions described in section 4.2.1. Events captured by R1 and R2 regions were further analysed for their fluorescence intensity. The two line plots within each data set depict the number of events (Y axis) versus fluorescence intensity (X axis). In all instances, the left-hand line plot describes R1, while the right-hand plot describes R2. Line plots also present the M1 and M2 divisions described in section 5.2.

Below each graph are tabulated results which describe that particular graph. Parameters described in the **scatter plot** tables are :

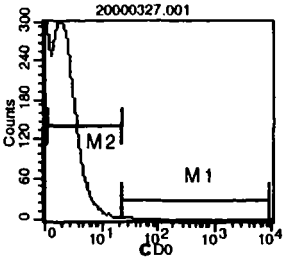
- "Events" = the number of cells falling within R1 and R2 groups,
- "% Gated" = percentage of total events falling within R1 and R2 groups,
- "% Total" = percentage of total events falling within R1 and R2 groups,
- "X Mean" = the mean granularity of R1 and R2,
- "Y Mean" = the mean size of R1 and R2.

The two line plots reflect R1 and R2 groups separately. Parameters described in each **line plot** table are :

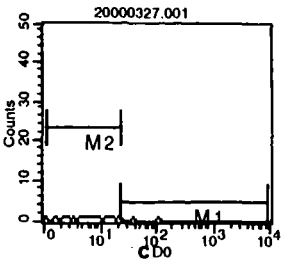
- "Events" = the number of cells falling within M1 and M2 groupings within the particular R grouping,
- "% Gated" = events falling within M1 and M2 expressed as a percentage of the number of events within the particular R grouping,
- "% Total" = events falling within the M1 and M2 regions of each R grouping expressed as a percentage of the total number of events irrespective of the R grouping,
- "Mean" = mean fluorescence of M1 and M2 groups within the particular R grouping.



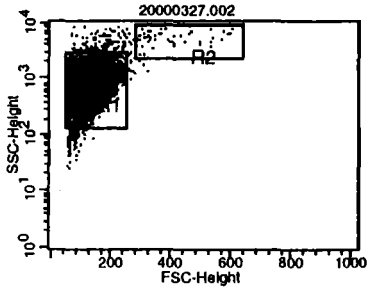
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	40981	98.99	98.99	123.77	577.37
R2	45	0.11	0.11	411.51	5646.67



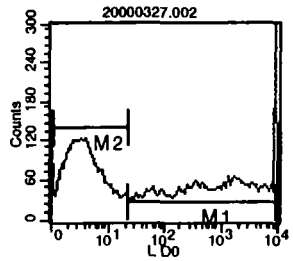
Marker	Events	% Gated	% Total	Mean
All	40981	100.00	98.99	2.27
M1	8	0.02	0.02	26.85
M2	35652	87.00	86.12	2.46



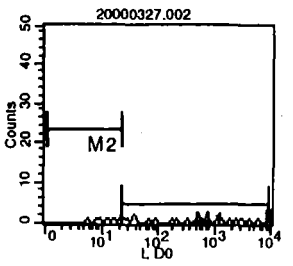
Marker	Events	% Gated	% Total	Mean
All	45	100.00	0.11	10.52
M1	2	4.44	0.00	67.65
M2	41	91.11	0.10	8.19



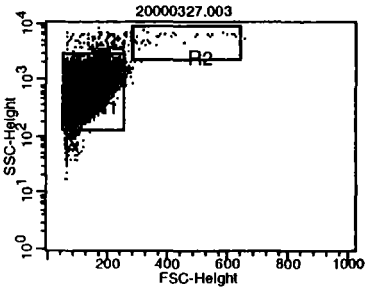
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49632	99.26	99.26	126.88	620.98
R2	49	0.10	0.10	407.43	5494.04



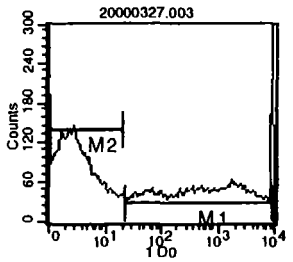
Marker	Events	% Gated	% Total	Mean
All	49632	100.00	99.26	1234.79
M1	27576	55.56	55.15	2218.19
M2	21769	43.86	43.54	5.35



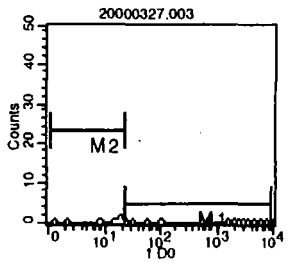
Marker	Events	% Gated	% Total	Mean
All	49	100.00	0.10	1894.66
M1	35	73.47	0.07	2574.12
M2	14	28.57	0.03	13.77



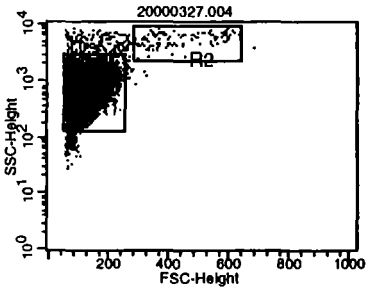
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49346	99.09	99.09	128.01	584.64
R2	31	0.06	0.06	422.19	5710.84



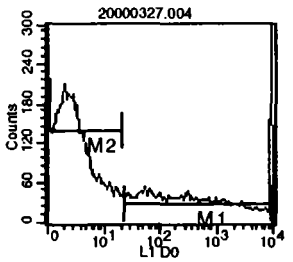
Marker	Events	% Gated	% Total	Mean
All	49346	100.00	99.09	826.35
M1	24177	48.99	48.55	1681.69
M2	24364	49.37	48.92	4.63



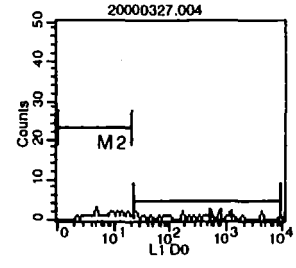
Marker	Events	% Gated	% Total	Mean
All	31	100.00	0.06	1358.23
M1	19	61.29	0.04	2208.46
M2	12	38.71	0.02	12.04



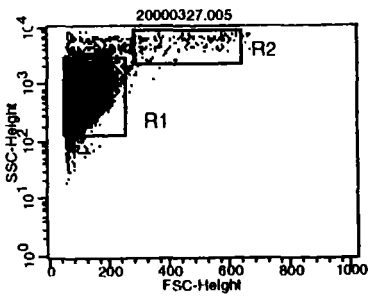
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49505	99.01	99.01	123.87	587.48
R2	111	0.22	0.22	440.40	5504.09



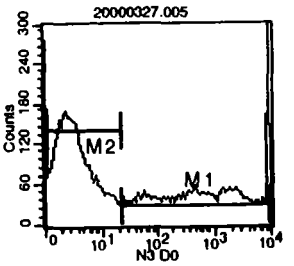
Marker	Events	% Gated	% Total	Mean
All	49505	100.00	99.01	367.45
M1	16306	32.94	32.61	1106.66
M2	32125	64.89	64.25	4.22



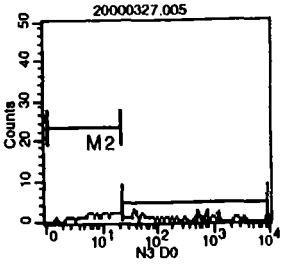
Marker	Events	% Gated	% Total	Mean
All	111	100.00	0.22	301.46
M1	36	32.43	0.07	909.03
M2	71	63.96	0.14	9.42



Region	Events	% Gated	% Total	X Mean	Y Mean
R1	48921	98.53	98.53	126.20	570.79
R2	157	0.32	0.32	444.18	5439.92

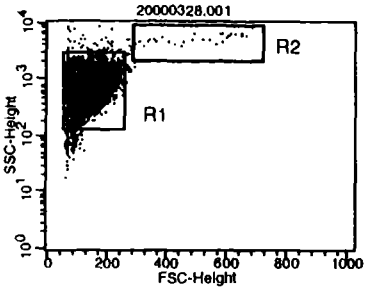


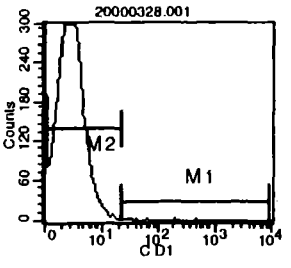
Marker	Events	% Gated	% Total	Mean
All	48921	100.00	98.53	769.68
M1	22188	45.35	44.69	1691.53
M2	26131	53.41	52.63	4.65

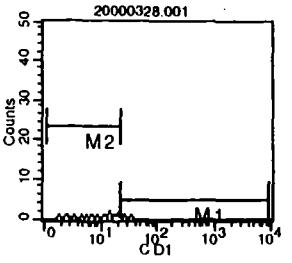


Marker	Events	% Gated	% Total	Mean
All	157	100.00	0.32	202.87
M1	48	30.57	0.10	640.39
M2	109	69.43	0.22	10.20

Day 1



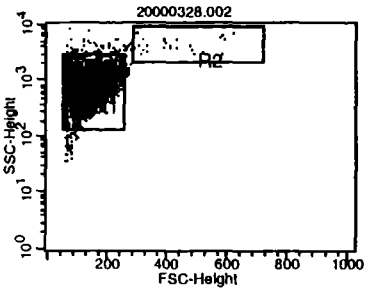


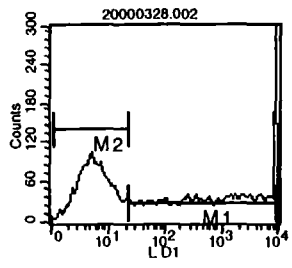


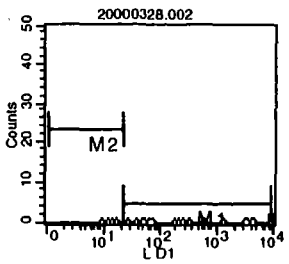
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	41282	99.35	99.35	135.62	593.92
R2	30	0.07	0.07	483.10	5136.82

Marker	Events	% Gated	% Total	Mean
All	41282	100.00	99.35	3.24
M1	31	0.08	0.07	60.38
M2	40270	97.55	96.92	3.25

Marker	Events	% Gated	% Total	Mean
All	30	100.00	0.07	12.06
M1	2	6.67	0.00	30.32
M2	28	93.33	0.07	10.76



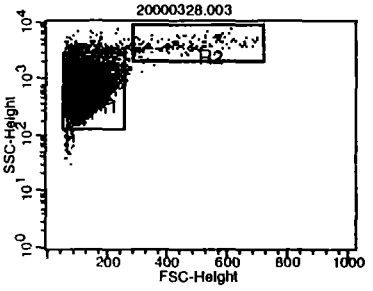


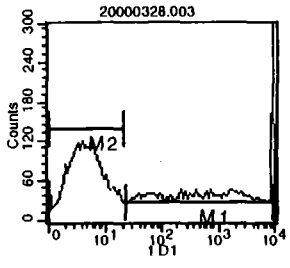


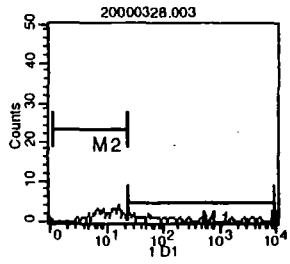
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	32991	99.52	99.52	136.40	656.94
R2	28	0.08	0.08	426.14	4423.00

Marker	Events	% Gated	% Total	Mean
All	32991	100.00	99.52	1512.67
M1	19035	57.70	57.42	2616.61
M2	13957	42.31	42.10	7.02

Marker	Events	% Gated	% Total	Mean
All	28	100.00	0.08	1870.64
M1	23	82.14	0.07	2274.42
M2	5	17.86	0.02	13.28



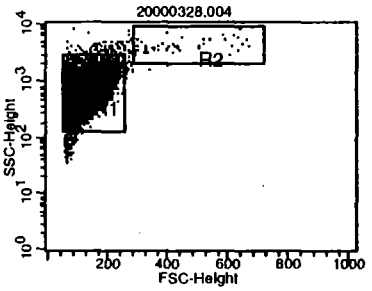


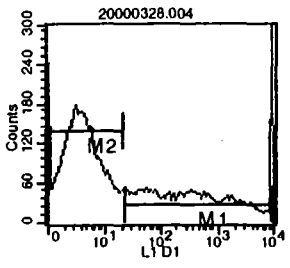


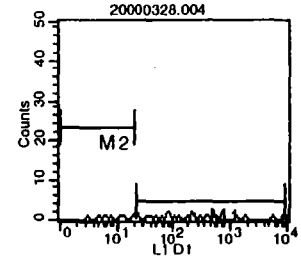
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	39316	98.91	98.91	123.24	623.60
R2	160	0.40	0.40	448.57	4132.77

Marker	Events	% Gated	% Total	Mean
All	39316	100.00	98.91	834.92
M1	19421	49.40	48.86	1684.10
M2	19643	49.96	49.42	5.82

Marker	Events	% Gated	% Total	Mean
All	160	100.00	0.40	383.86
M1	50	31.25	0.13	1204.45
M2	109	68.12	0.27	10.96



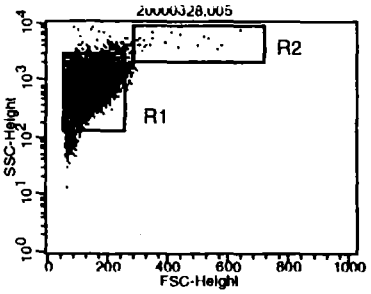


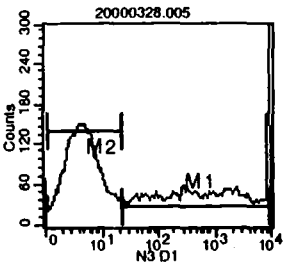


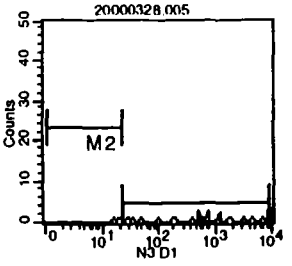
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49672	99.34	99.34	119.75	581.32
R2	51	0.10	0.10	437.41	4321.56

Marker	Events	% Gated	% Total	Mean
All	49672	100.00	99.34	414.58
M1	19439	39.13	38.88	1050.86
M2	29697	59.79	59.39	5.35

Marker	Events	% Gated	% Total	Mean
All	51	100.00	0.10	739.96
M1	33	64.71	0.07	1136.80
M2	16	31.37	0.03	11.31



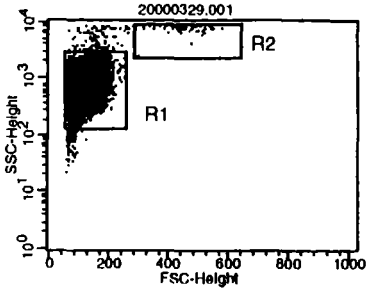


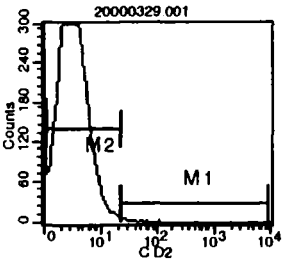


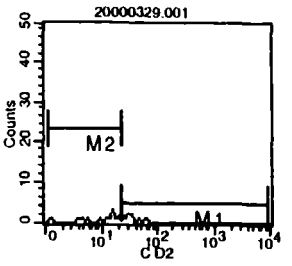
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49644	99.29	99.29	126.60	657.58
R2	29	0.06	0.06	377.34	4707.06

Marker	Events	% Gated	% Total	Mean
All	49644	100.00	99.29	980.39
M1	24880	50.12	49.76	1950.36
M2	24623	49.60	49.25	5.93

Marker	Events	% Gated	% Total	Mean
All	29	100.00	0.06	2412.02
M1	26	89.66	0.05	2688.26
M2	3	10.34	0.01	17.89



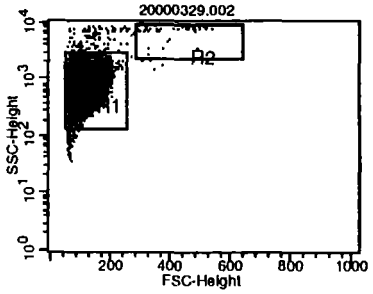


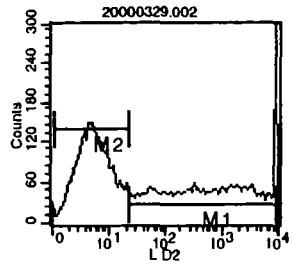


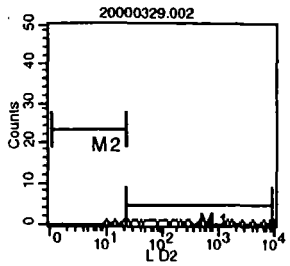
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49675	99.35	99.35	112.29	521.79
R2	53	0.11	0.11	446.94	7414.66

Marker	Events	% Gated	% Total	Mean
All	49675	100.00	99.35	3.72
M1	60	0.12	0.12	30.10
M2	48711	98.06	97.42	3.74

Marker	Events	% Gated	% Total	Mean
All	53	100.00	0.11	22.22
M1	24	45.28	0.05	32.28
M2	29	54.72	0.06	13.90



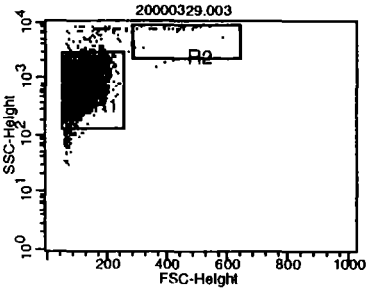


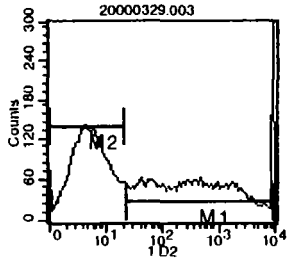


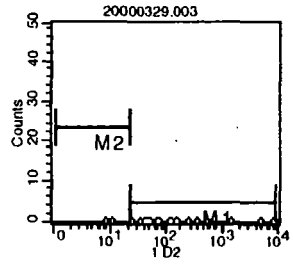
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49744	99.49	99.49	107.64	592.63
R2	39	0.08	0.08	422.72	6907.16

Marker	Events	% Gated	% Total	Mean
All	49744	100.00	99.49	949.16
M1	26432	53.14	52.86	1780.33
M2	23273	46.79	46.55	6.78

Marker	Events	% Gated	% Total	Mean
All	39	100.00	0.08	886.23
M1	35	89.74	0.07	985.61
M2	4	10.26	0.01	16.68



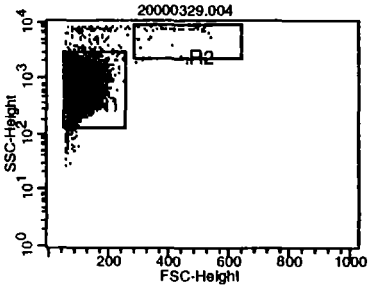


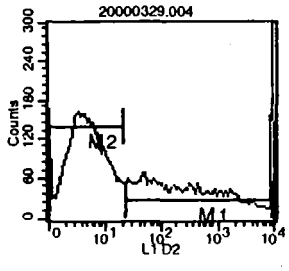


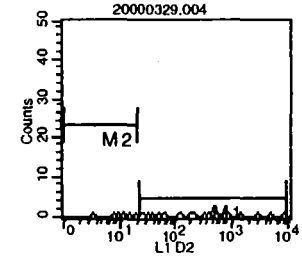
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49780	99.56	99.56	107.96	603.04
R2	26	0.05	0.05	415.58	7391.90

Marker	Events	% Gated	% Total	Mean
All	49780	100.00	99.56	524.09
M1	24835	49.89	49.67	1043.77
M2	24540	49.30	49.08	6.51

Marker	Events	% Gated	% Total	Mean
All	26	100.00	0.05	888.70
M1	24	92.31	0.05	961.94
M2	2	7.69	0.00	9.76



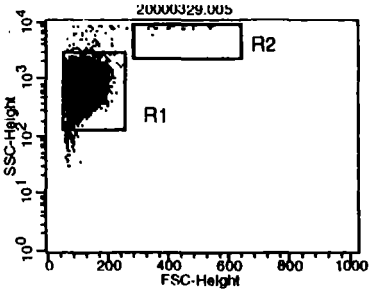


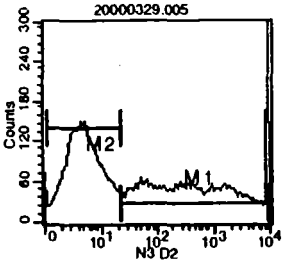


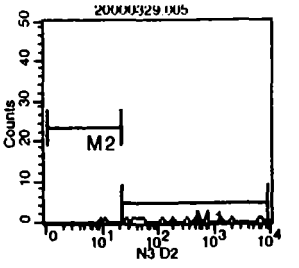
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49733	99.47	99.47	104.20	572.61
R2	38	0.08	0.08	417.39	7024.47

Marker	Events	% Gated	% Total	Mean
All	49733	100.00	99.47	352.32
M1	20428	41.08	40.86	848.81
M2	28822	57.95	57.64	6.07

Marker	Events	% Gated	% Total	Mean
All	38	100.00	0.08	580.71
M1	31	81.58	0.06	709.04
M2	6	15.79	0.01	10.69



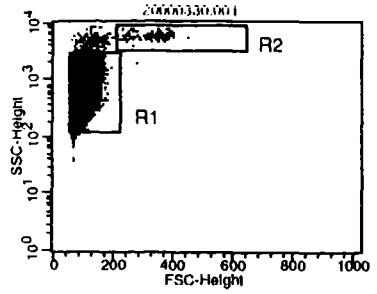


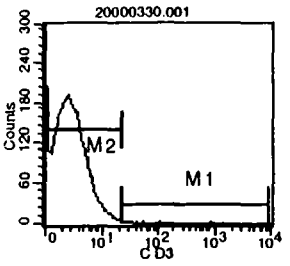


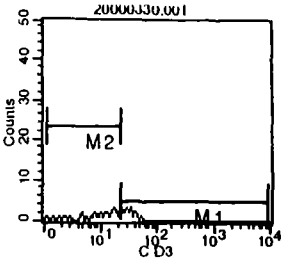
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49805	99.61	99.61	107.67	575.13
R2	20	0.04	0.04	425.00	7709.40

Marker	Events	% Gated	% Total	Mean
All	49805	100.00	99.61	680.15
M1	25237	50.67	50.47	1336.12
M2	24455	49.10	48.91	6.37

Marker	Events	% Gated	% Total	Mean
All	20	100.00	0.04	843.11
M1	18	90.00	0.04	935.70
M2	2	10.00	0.00	9.85



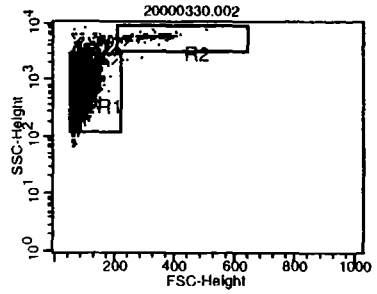


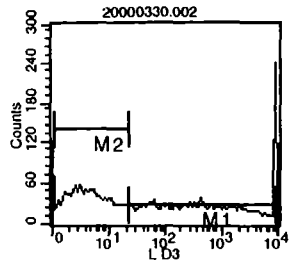


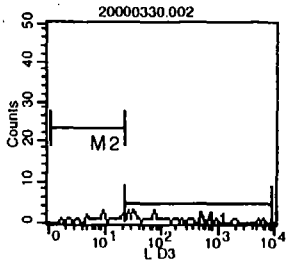
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	28101	98.60	98.60	90.15	536.38
R2	124	0.44	0.44	315.71	3726.33

Marker	Events	% Gated	% Total	Mean
All	28101	100.00	98.60	3.26
M1	19	0.07	0.07	70.61
M2	26324	93.68	92.36	3.38

Marker	Events	% Gated	% Total	Mean
All	124	100.00	0.44	19.88
M1	48	38.71	0.17	32.00
M2	76	61.29	0.27	12.46



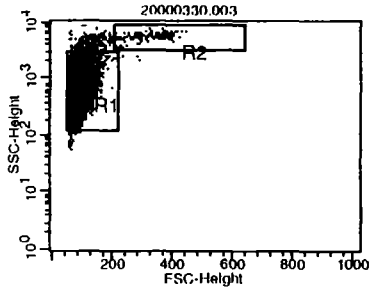


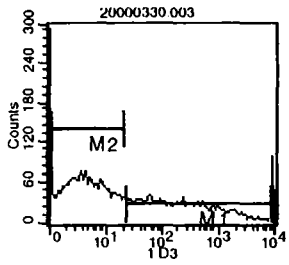


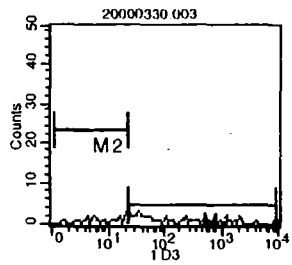
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	20555	99.39	99.39	92.80	583.05
R2	128	0.61	0.61	314.76	5679.77

Marker	Events	% Gated	% Total	Mean
All	20555	100.00	99.39	540.47
M1	10545	51.05	50.21	1070.99
M2	9880	47.83	47.05	6.13

Marker	Events	% Gated	% Total	Mean
All	128	100.00	0.61	214.52
M1	72	56.25	0.35	357.61
M2	56	43.75	0.27	11.55



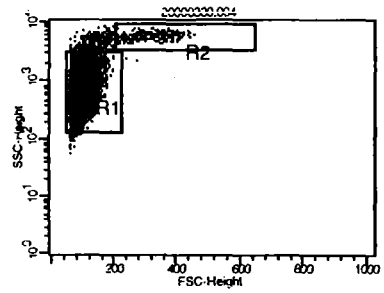


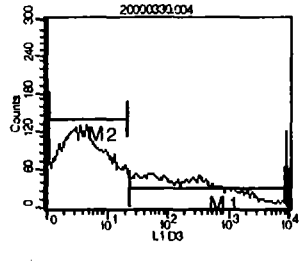


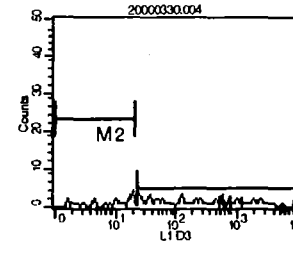
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	25037	99.38	99.38	84.04	604.40
R2	159	0.62	0.62	328.82	5669.66

Marker	Events	% Gated	% Total	Mean
All	25037	100.00	99.38	251.65
M1	10306	41.16	40.42	662.46
M2	14233	56.85	55.82	6.09

Marker	Events	% Gated	% Total	Mean
All	159	100.00	0.62	291.21
M1	107	67.30	0.42	427.42
M2	52	32.70	0.20	10.92



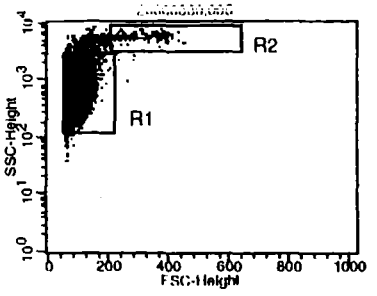


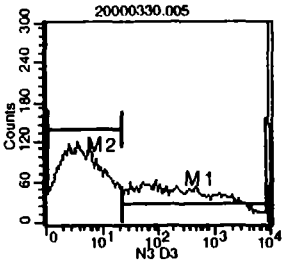


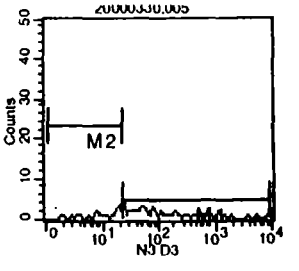
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	42406	98.50	98.50	85.93	577.76
R2	235	0.55	0.55	316.45	5711.87

Marker	Events	% Gated	% Total	Mean
All	42406	100.00	98.50	218.28
M1	15744	37.13	36.57	578.00
M2	25775	60.78	59.87	5.78

Marker	Events	% Gated	% Total	Mean
All	235	100.00	0.55	519.95
M1	172	73.19	0.40	705.81
M2	55	23.40	0.13	11.18



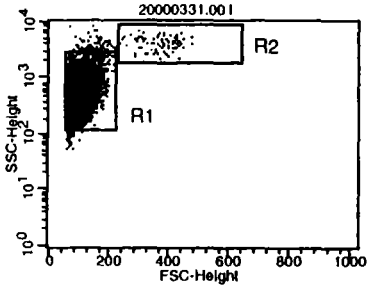




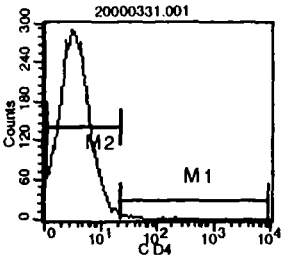
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	45421	98.63	98.63	85.33	556.74
R2	208	0.45	0.15	310.50	5812.22

Marker	Events	% Gated	% Total	Mean
All	45421	100.00	98.63	403.30
M1	20990	46.21	45.58	665.71
M2	23818	52.44	51.72	6.18

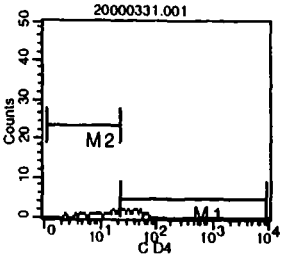
Marker	Events	% Gated	% Total	Mean
All	208	100.00	0.45	237.62
M1	151	72.60	0.33	328.86
M2	57	27.40	0.12	14.02



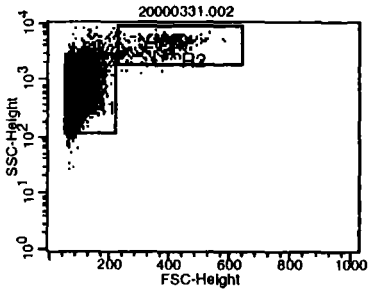
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	40259	99.40	99.40	97.35	513.08
R2	85	0.21	0.21	365.41	4225.74



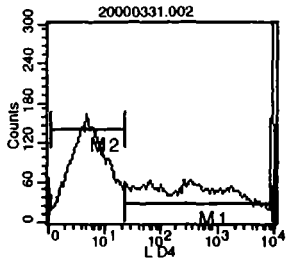
Marker	Events	% Gated	% Total	Mean
All	40259	100.00	99.40	4.05
M1	91	0.23	0.22	32.11
M2	39304	97.63	97.05	4.05



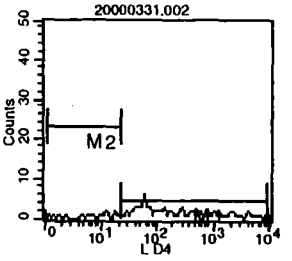
Marker	Events	% Gated	% Total	Mean
All	85	100.00	0.21	27.14
M1	53	62.35	0.13	35.46
M2	33	38.82	0.08	13.66



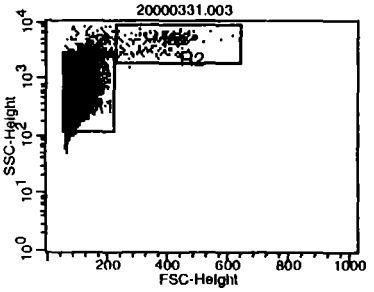
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49368	98.74	98.74	96.80	588.01
R2	346	0.69	0.69	373.60	4317.09



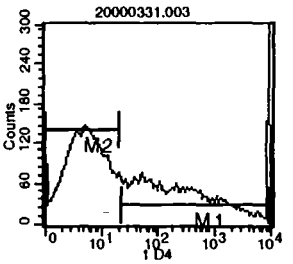
Marker	Events	% Gated	% Total	Mean
All	49368	100.00	98.74	541.90
M1	23700	48.01	47.40	1121.55
M2	25541	51.74	51.08	6.75



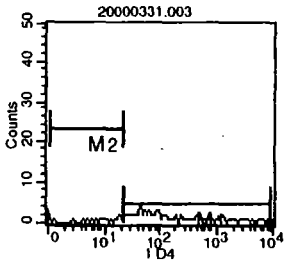
Marker	Events	% Gated	% Total	Mean
All	346	100.00	0.69	506.90
M1	300	86.71	0.60	582.89
M2	43	12.43	0.09	12.00



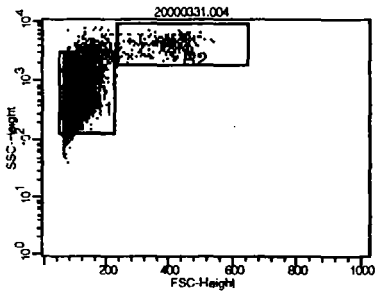
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49456	98.91	98.91	99.48	586.84
R2	243	0.49	0.49	377.00	4577.55



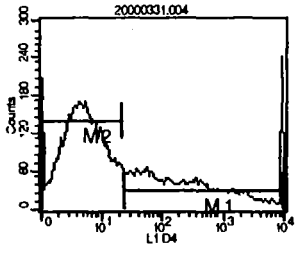
Marker	Events	% Gated	% Total	Mean
All	49456	100.00	98.91	310.59
M1	21112	42.69	42.22	718.34
M2	27768	56.15	55.54	6.67



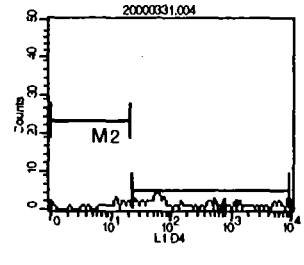
Marker	Events	% Gated	% Total	Mean
All	243	100.00	0.49	467.63
M1	215	88.48	0.43	527.35
M2	25	10.29	0.05	10.01



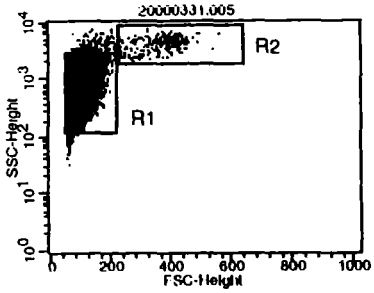
Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49411	98.82	98.82	98.20	547.79
R2	273	0.55	0.55	381.30	4190.57



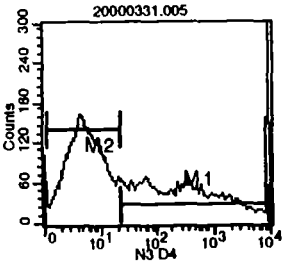
Marker	Events	% Gated	% Total	Mean
All	49411	100.00	98.82	260.41
M1	18159	36.75	36.32	697.43
M2	30634	62.00	61.27	6.31



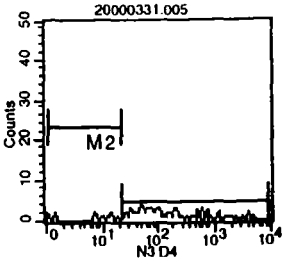
Marker	Events	% Gated	% Total	Mean
All	273	100.00	0.55	538.94
M1	208	76.19	0.42	703.50
M2	59	21.25	0.12	11.91



Region	Events	% Gated	% Total	X Mean	Y Mean
R1	49485	98.97	98.97	95.27	556.85
R2	244	0.49	0.49	370.30	4388.55



Marker	Events	% Gated	% Total	Mean
All	49485	100.00	98.97	380.82
M1	22284	45.03	44.57	837.33
M2	27032	54.63	54.06	6.91



Marker	Events	% Gated	% Total	Mean
All	244	100.00	0.49	208.41
M1	217	88.93	0.43	233.03
M2	24	9.84	0.05	11.66

Appendix D – Sequence Data

The following pages present raw sequence data of calpastatin cDNA's included in pGEX-4T-1 and pEGFP-N3 expression vectors.

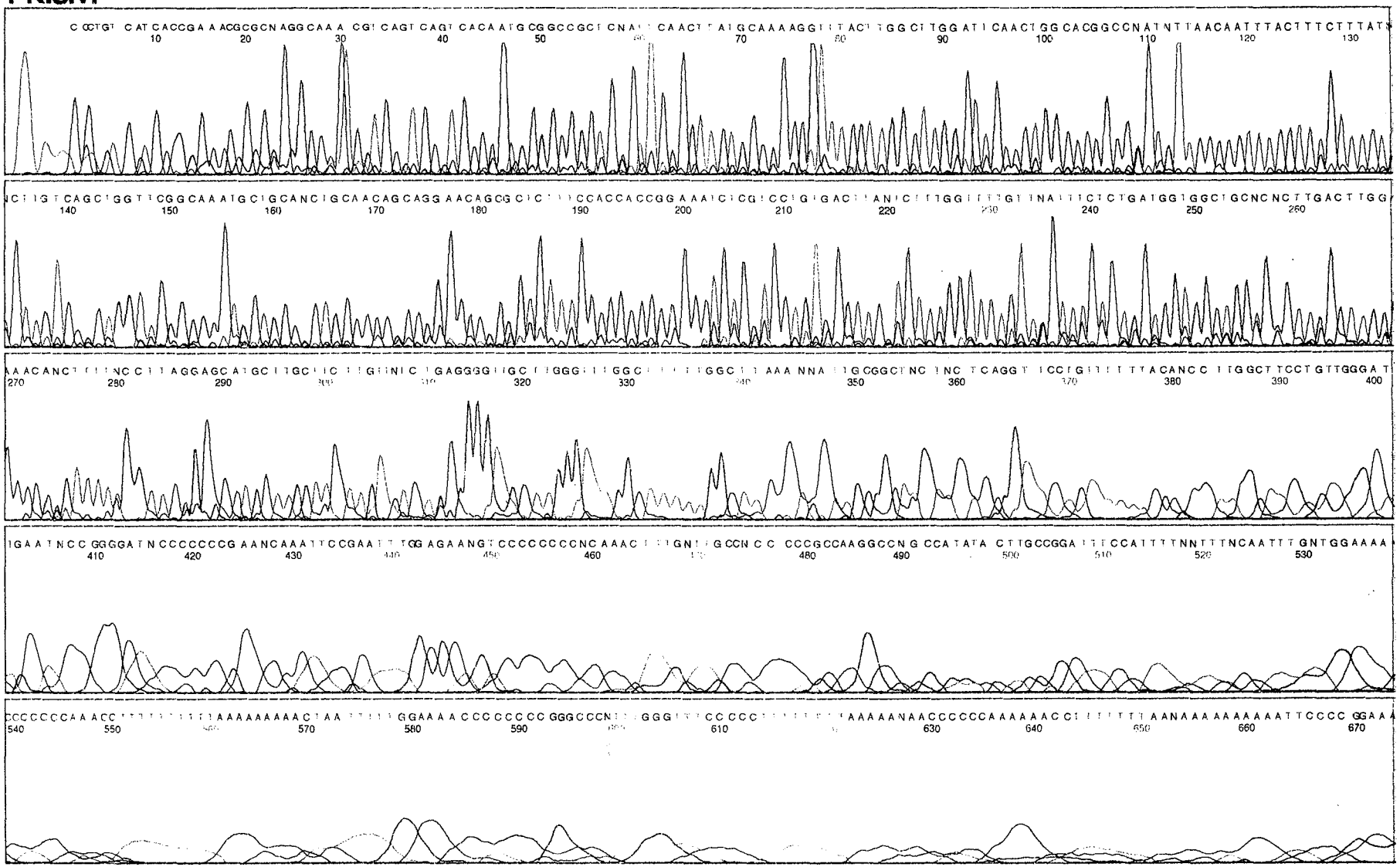
Sequences DLGST$^{3'}$ and DLGST$^{5'}$describe the nucleotide sequence determined by sequencing pGEX-DL plasmid. Similarly, sequences DL1GST$^{3'}$ / DL1GST$^{5'}$ and D1GST$^{3'}$ / D1GST$^{5'}$ describe pGEX-DL1 and pGEX-D1 plasmids respectively.

Sequences DLGFP$^{3'}$ and DLGFP$^{5'}$describe the nucleotide sequence determined by sequencing pEGFP-DL plasmid. Similarly, sequences DL1GFP$^{3'}$ / DL1GFP$^{5'}$ and D1GFP$^{3'}$ / D1GFP$^{5'}$ describe pEGFP-DL1 and pEGFP-D1 plasmids respectively.

PCR primers used in generating these sequences are shown in Table 2.3

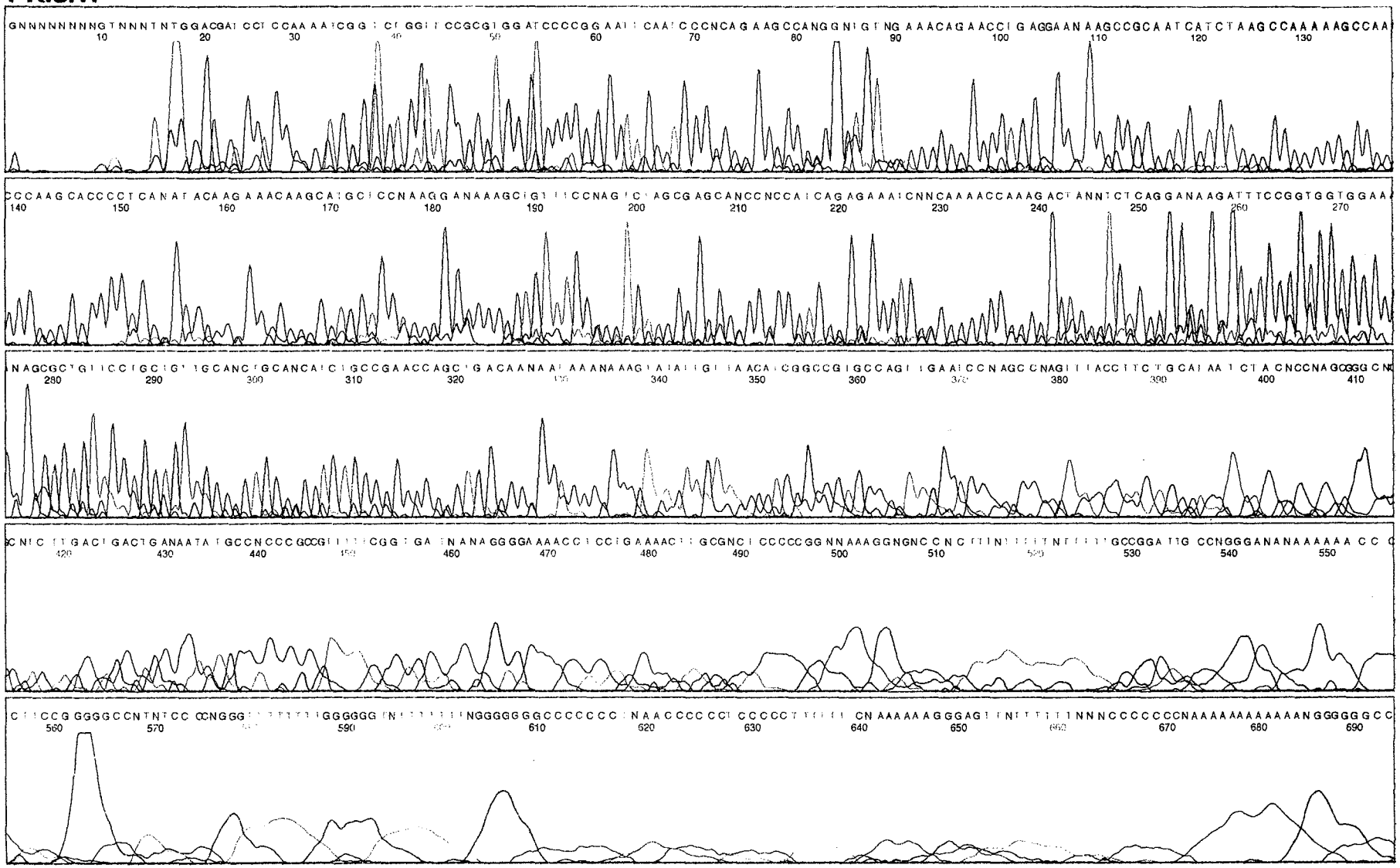
ABI PRISM™ Model Version 2.1.1

pGEX-DL Sequenced with pGEX3' primer



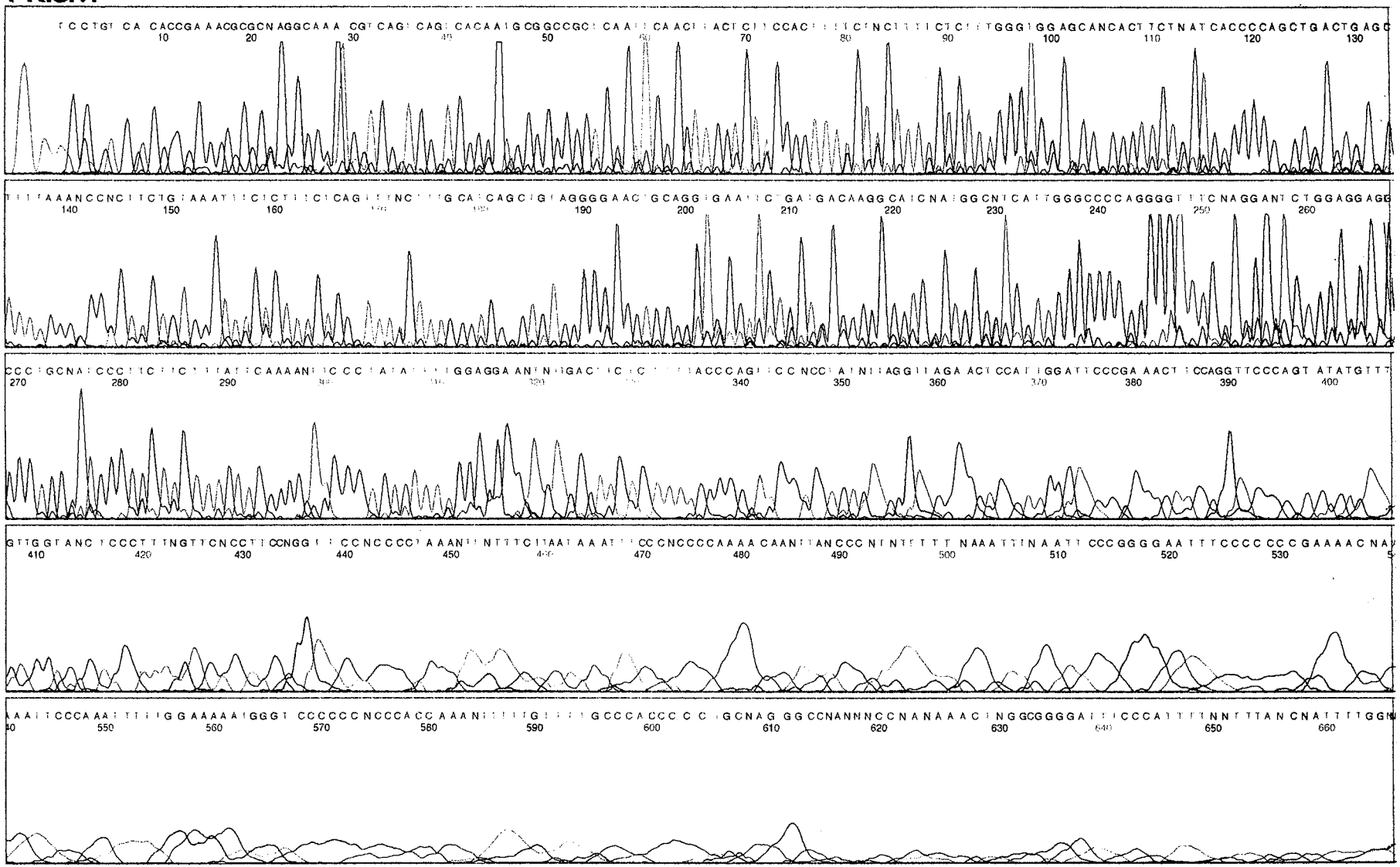
ABI PRISM™ Model Version 2.1.1

pGEX-DL Sequenced with pGEX5' primer



ABI PRISM Version 2.1.1

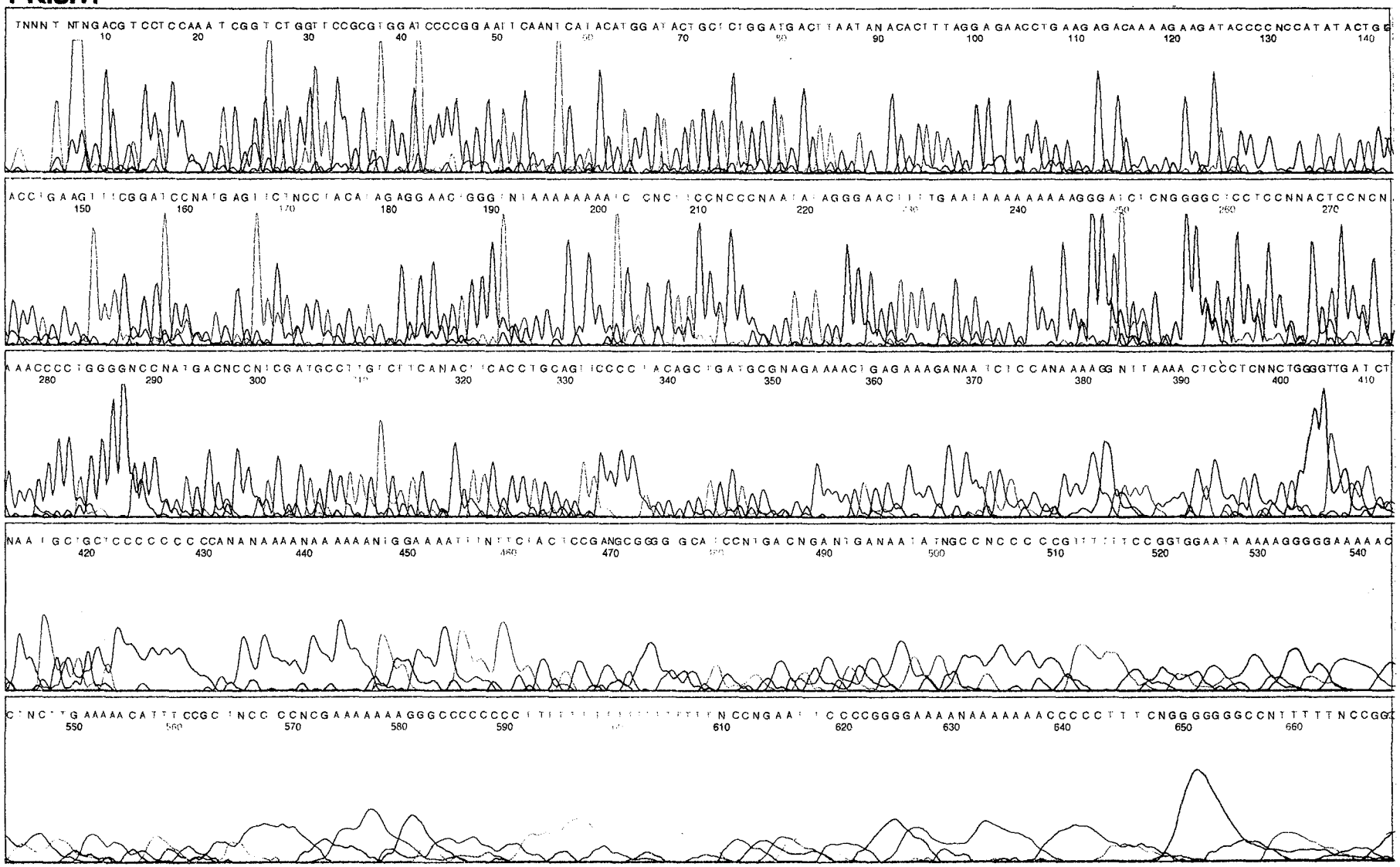
pGEX-D1 Sequenced with pGEX3' primer



ABI PRISM

Model
Version 2.1.1

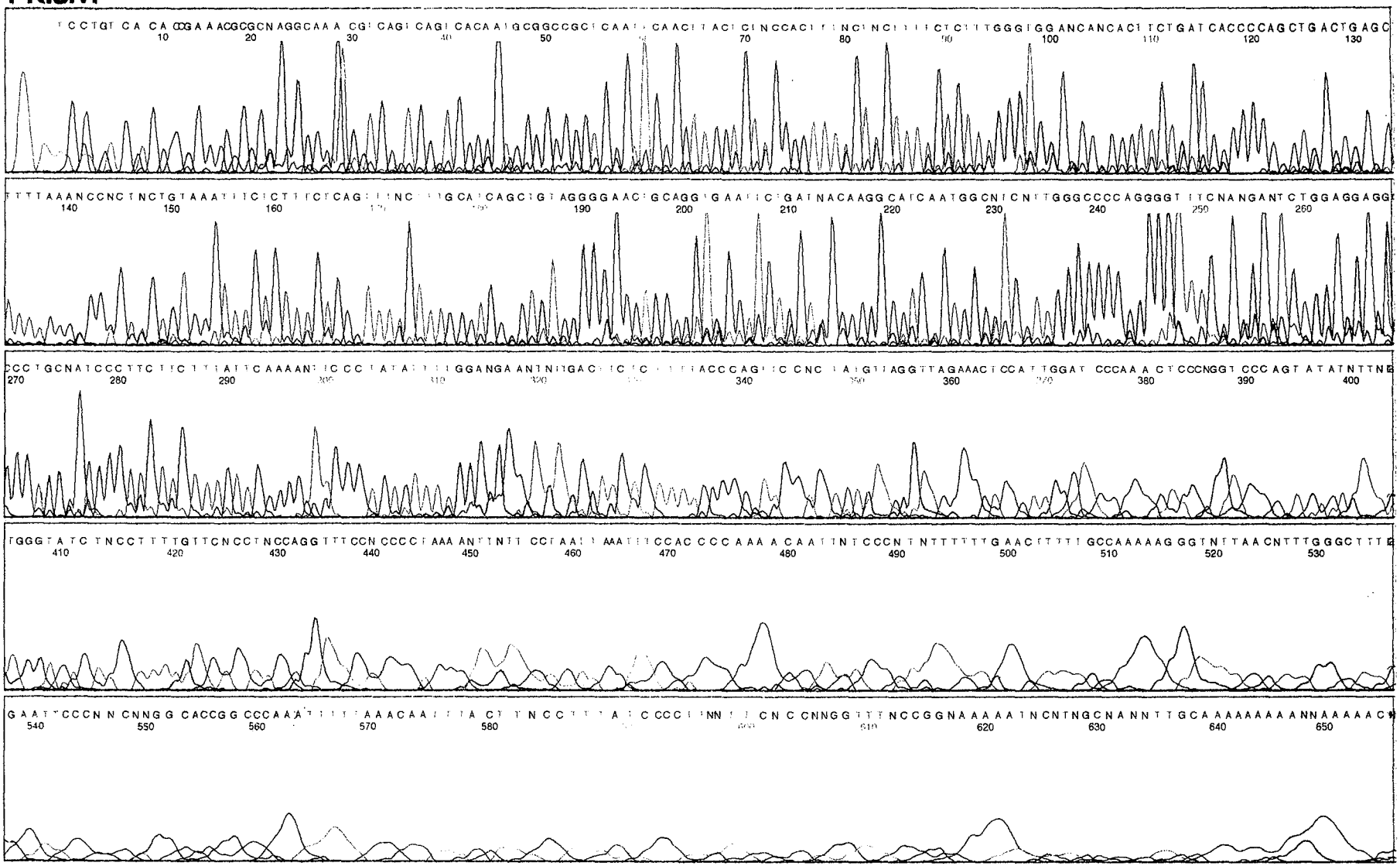
pGEX-D1 Sequenced with pGEX5' primer



ABI PRISM

Model
Version 2.1.1

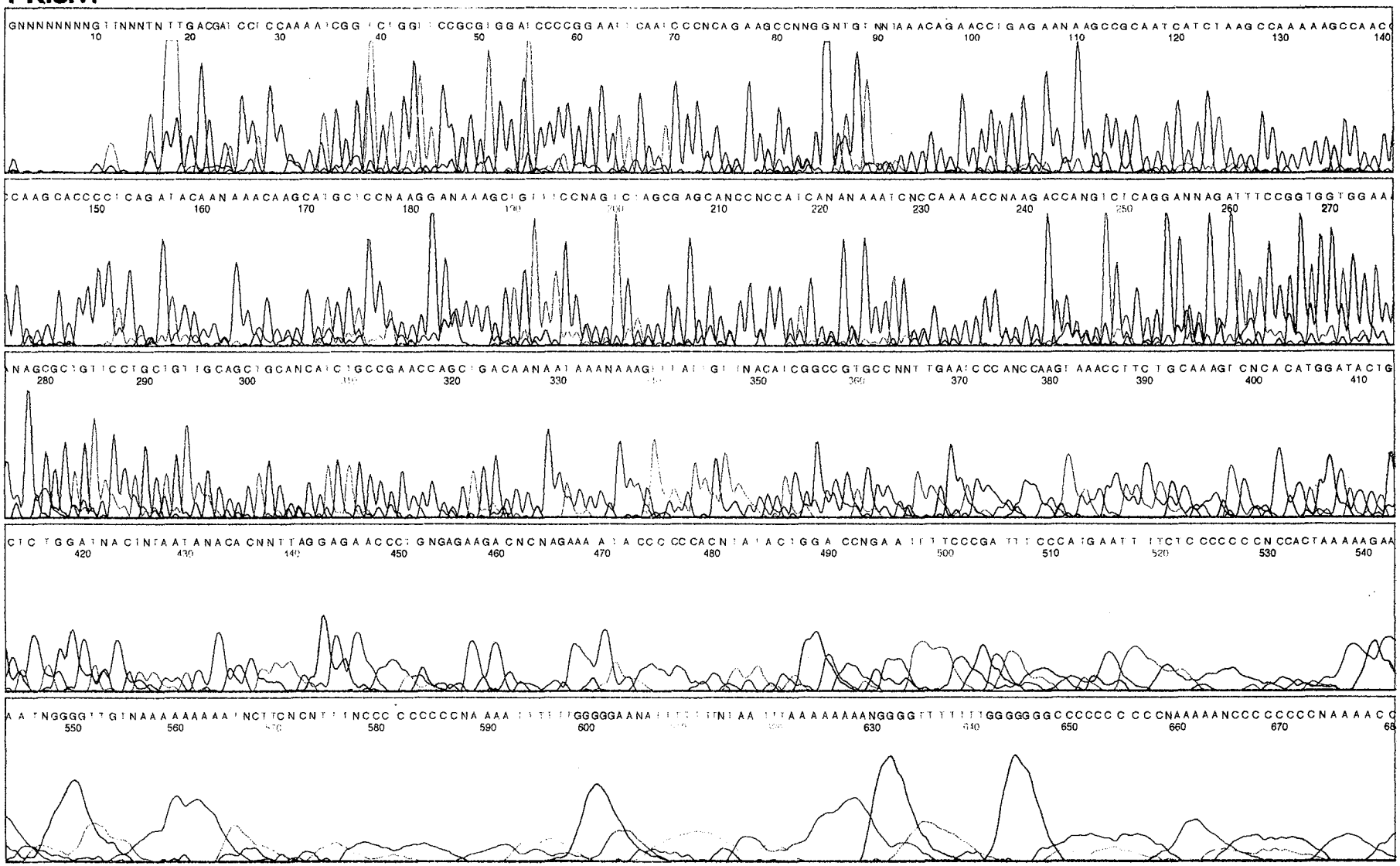
pGEX-DL1 Sequenced with pGEX3' primer



ABI PRISM

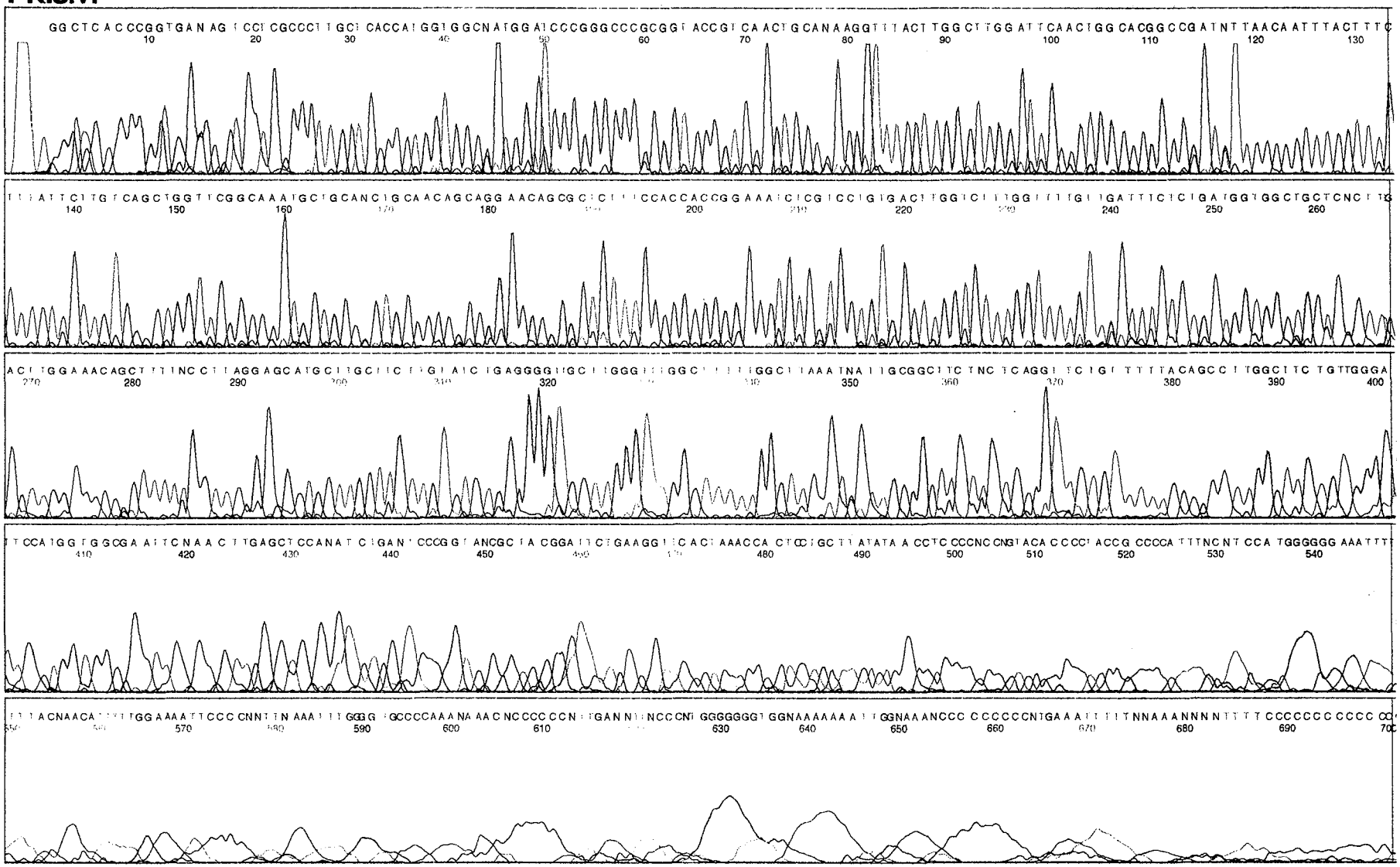
Model
Version 2.1.1

pGEX-DL1 Sequenced with pGEX5' primer



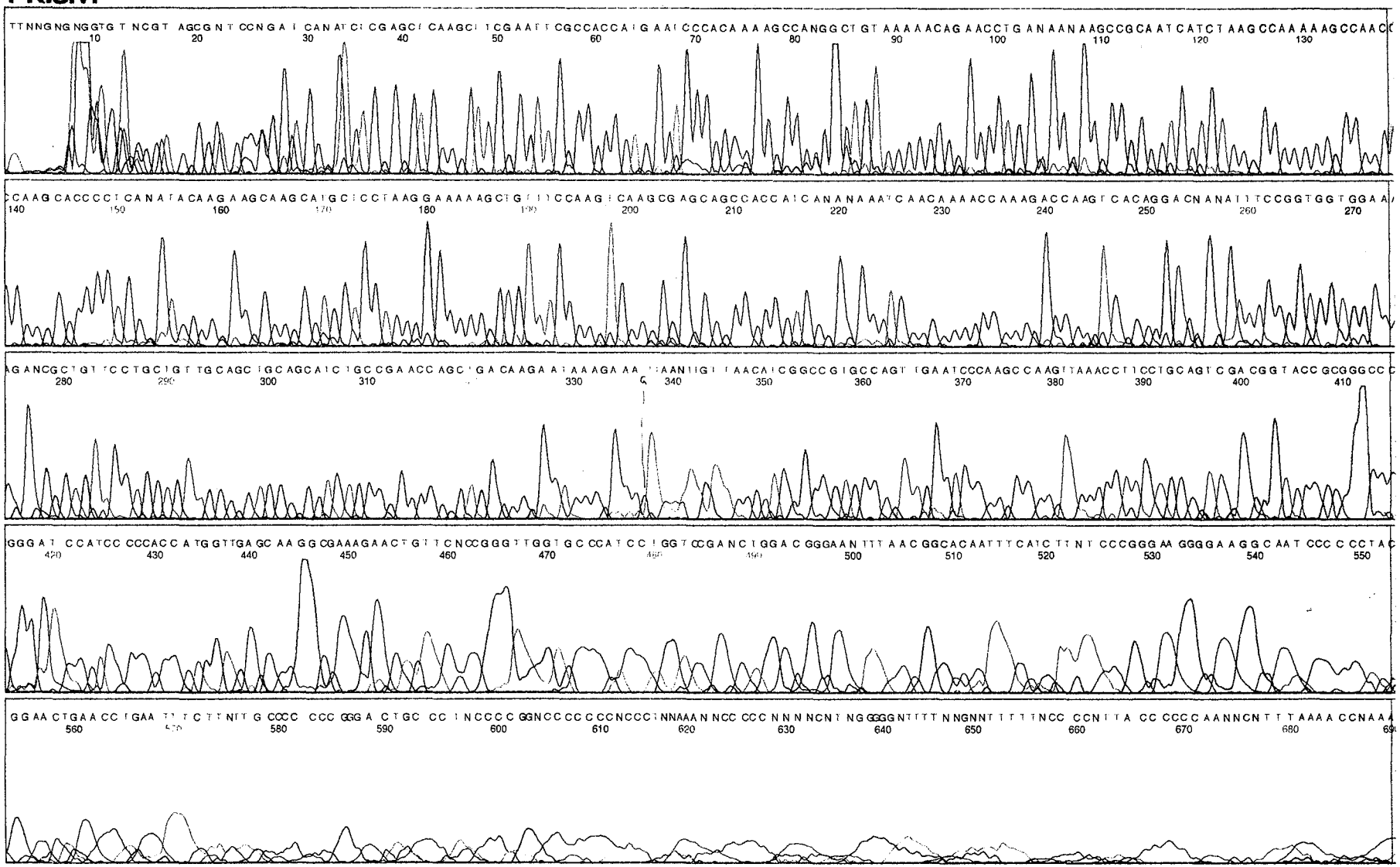
ABI PRISM Model Version 2.1.1

pGFP-DL Sequenced with pGFP3' primer



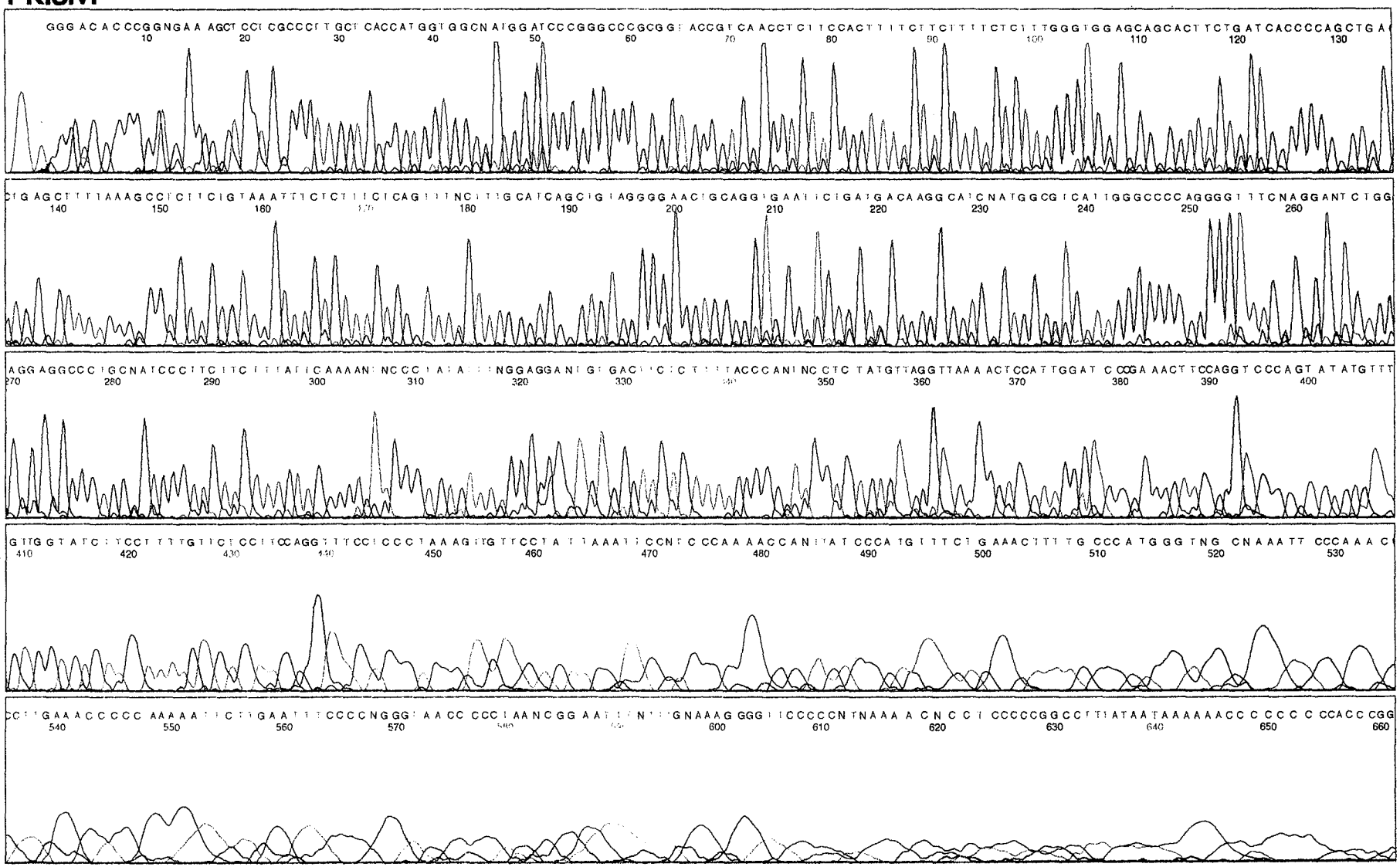
ABI PRISM™ Model Version 2.1.1

pGFP-DL Sequenced with pGFP5' primer



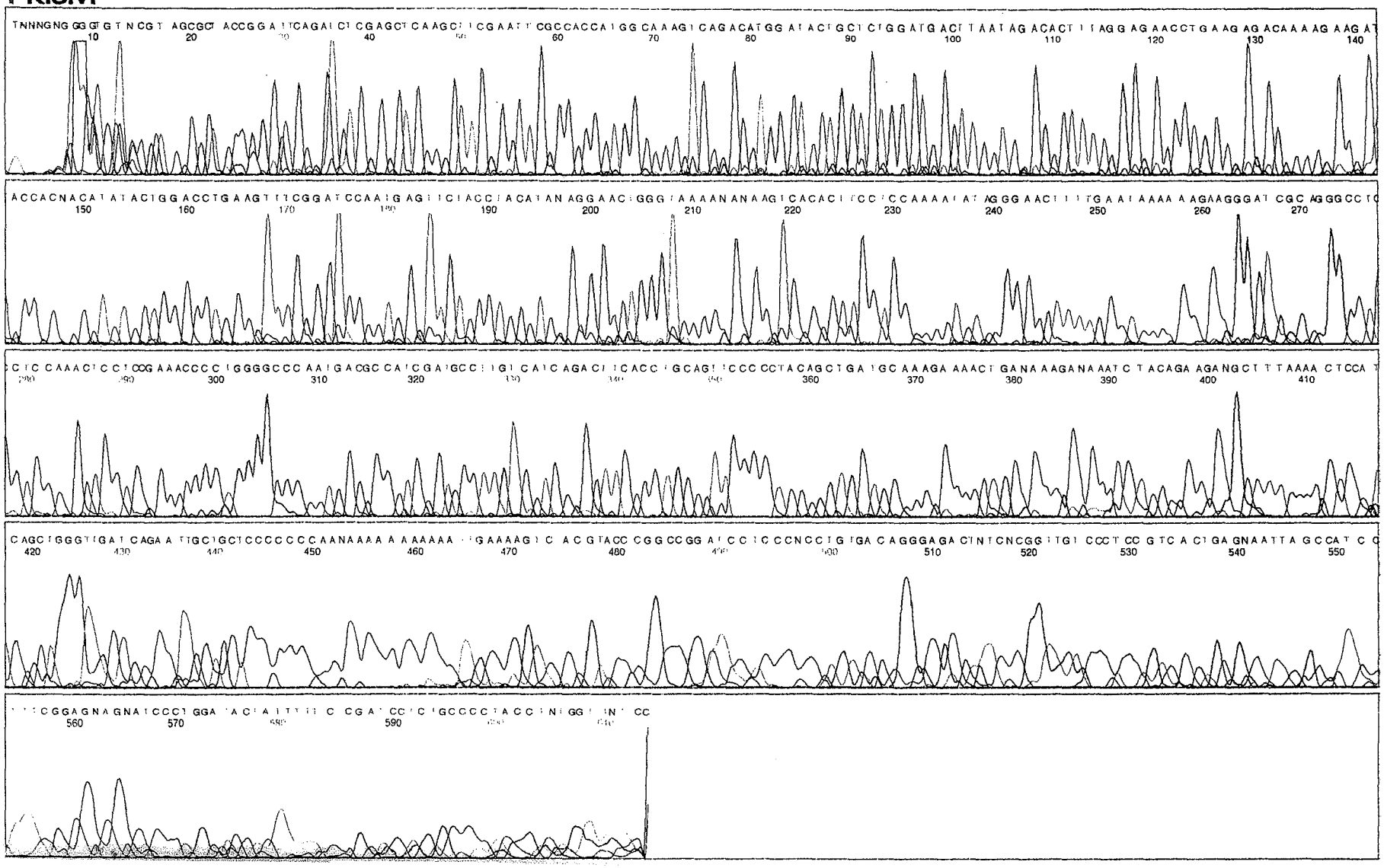
ABI PRISM Model Version 2.1.1

pGFP-D1 Sequenced with pGFP3' primer



ABI PRISM™ Model Version 2.1.1

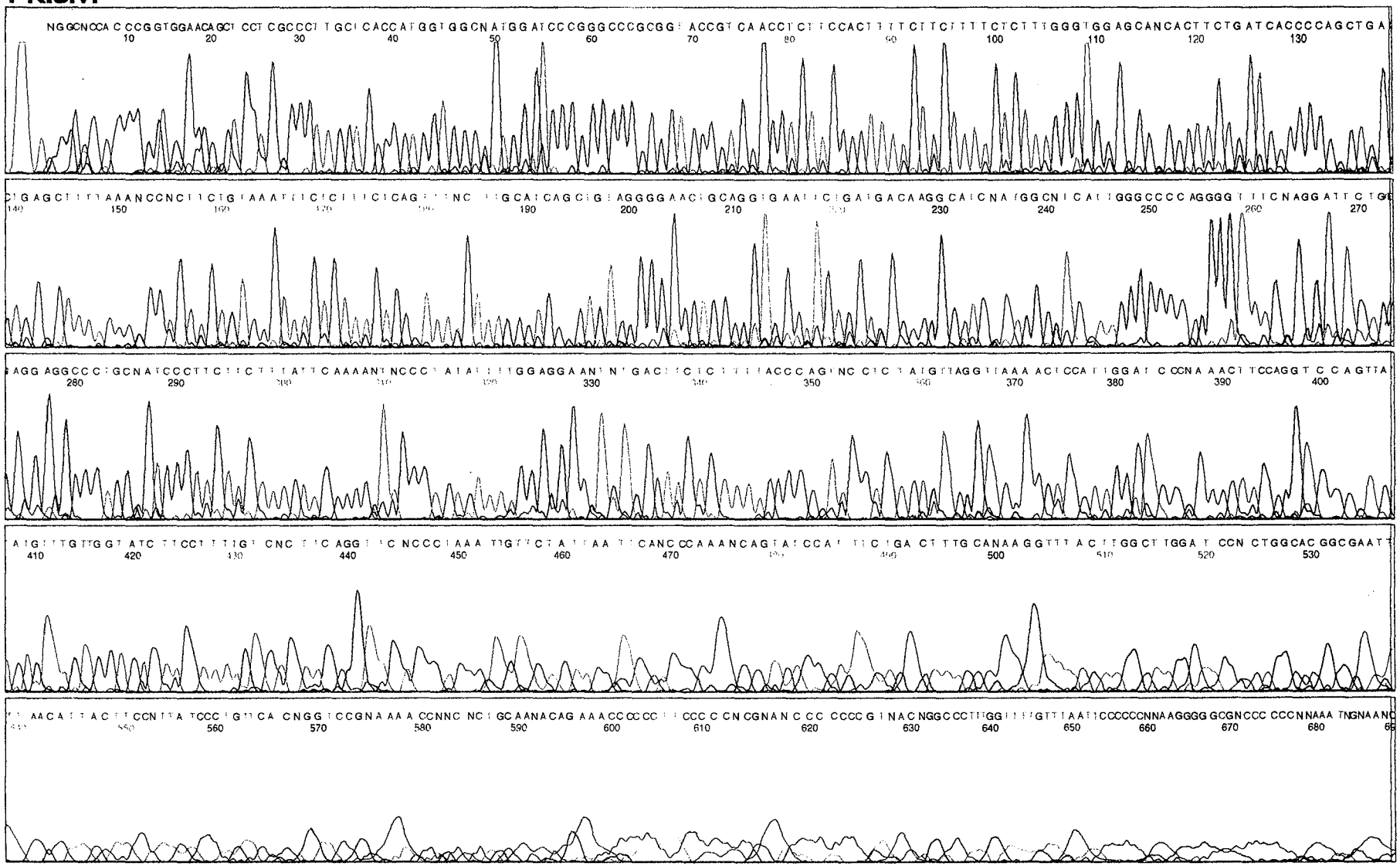
pGFP-D1 Sequenced with pGFP5' primer



ABI PRISM

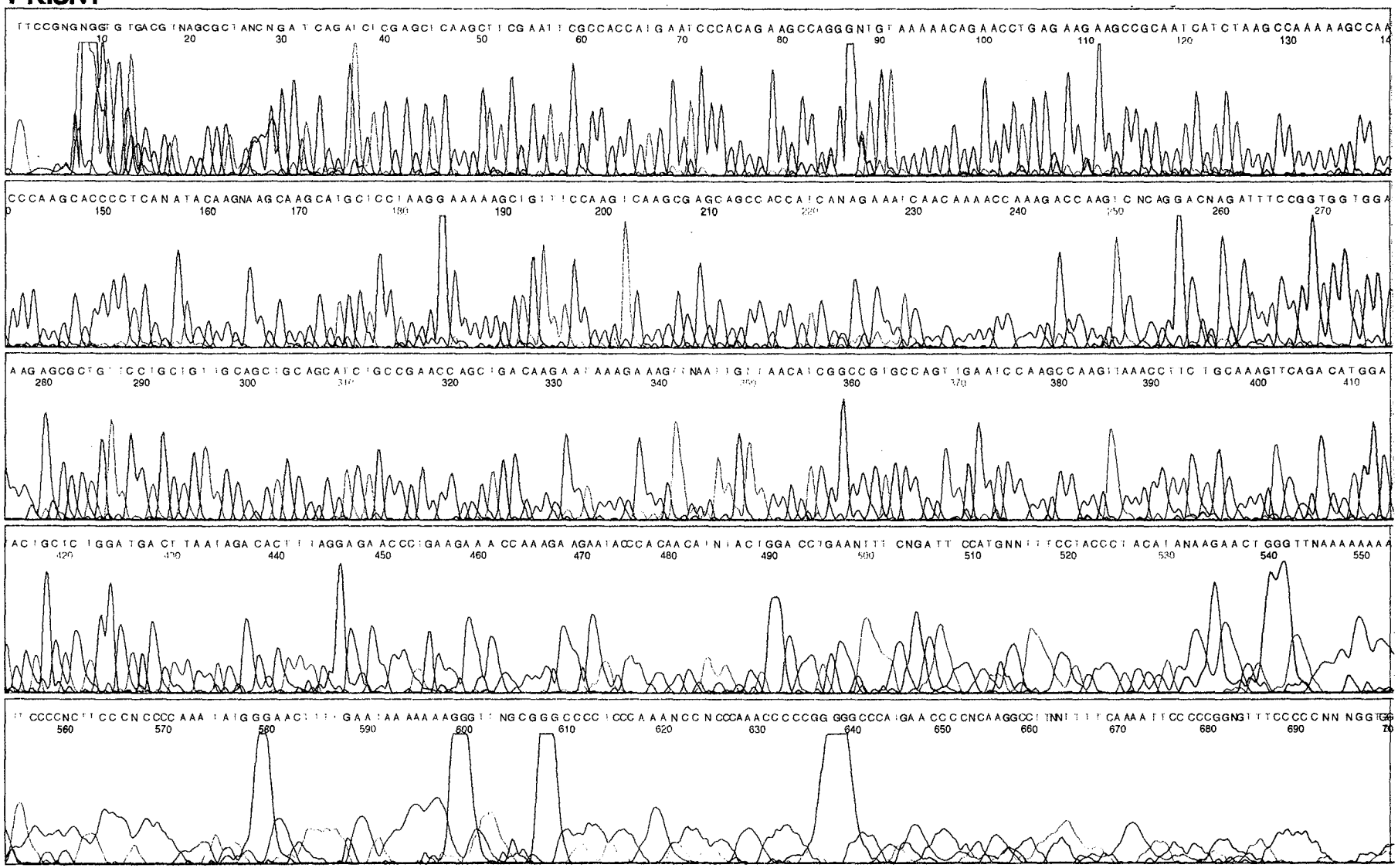
Model
Version 2.1.1

pGFP-DL1 Sequenced with pGFP3' primer



ABI PRISM Model Version 2.1.1

pGFP-DL1 Sequenced with pGFP5' primer



Appendix E – Abbreviations

1°	primary
2°	secondary
3°	tertiary
ABR	Affinity Bioreagents
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
Amp	ampicillin
APB	Amersham Pharmacia Biotech
APS	ammonium persulphate
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
au	arbitrary units
β-MCE	β-mercaptoethanol
BMP	bitmap
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celsius
CaMLD	calmodulin like domain
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
CBND	1-chloro-2,4-Dinitrobenzene
CCS	central consensus sequence
cDNA	complementary DNA
cm	centimetre
DL	ovine calpastatin domain L
D1	ovine calpastatin domain 1
DL1	ovine calpastatin domains L and 1
ddH2O	distilled deionised water
DEAE	diethylaminoethyl
DMEM	dulbecco's modified eagle media
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DSHB	developmental studies hybridoma bank
DTT	dithiothreitol
E64	trans-epoxysuccinyl-L-leucylamido-4-guanidino)butane
E. coli	*Escherichia coli*
EDTA	ethylenediamine-tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N, N',N'-tetraacetic acid
FACS	fluorescent automated cell sorter
FCS	foetal calf serum
FITC	fluoroscein Isothiocyanate
g	gravity
GFP	green fluorescent protein
GSH	reduced glutathione
GST	glutathione-*S*-transferase
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HS	horse serum
IGF-1	insulin-like growth factor 1
IPTG	isopropyl-β-D-thio-galactopyranoside
Kana	kanamycin
kDa	kiloDalton
kg	kilogram
LB	luria bertani
LGMD2A	limb girdle muscular dystrophy type 2A
LP82	lens protein 82
M	molar
ml	millilitre

mg	milligram
mM	milliMolar
mRNA	messenger RNA
NEB	New England Biolab
ng	nanograms
NLS	nuclear localisation Signal
nm	nanometre
OSU	Oregon state university
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	protein denaturing buffer
PIPES	piperazine-N,N'-bis[2-ethanesulphonic acid]
PKA	protein kinase A
PKC	protein kinase C
PKM	protein kinase M
PMSF	phenylmethylsuphonyl Fluoride
PVDF	polyvinylidine difluoride
SDS	sodium dodecyl Sulphate
t	time
TB	terrific broth
TBE	Tris-borate-EDTA
TCA	trichloroacetic acid
TnT	troponin T
TY	tryptone yeast
RNA	ribonucleic acid
Rpm	revolutions per minute
RT	room temperature (20 – 25°C)
TBS	tris buffered saline
TTBS	TBS + Tween 20
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
Tris-HCl	tris[hydroxymethyl] aminomethane hydrochloride
UV	ultraviolet
UVP	Ultraviolet Products
µg	microgram
µl	microlitre
µm	micrometre
µM	microMolar
V	volts
v/v	volume to volume
w/v	weight to volume
X-gal	5-bromo-4-chromo-3-indolyl-β-D-galactosidase

Appendix F – Major suppliers of reagents, consumables and equipment

Affinity Bioreagents Incorporated., Golden, CO. USA.

Antibodies

Anchor NZ Milk Corporation Limited., Auckland, New Zealand.

Non-fat Dry Milk

Amersham Pharmacia Biotech., Uppsala, Sweden.

Antibodies
Bacteria
Plasmid DNA

American Type Culture Collection., Manassas, VA. USA.

Mammalian Cell Line

BDH Laboratories., Poole, Dorset. UK.

Ammonium Acetate
Boric Acid
Bromophenol Blue
$CaCl_2$
Chloroform
Dimethylformamide
DMSO
DPX Mountant
EDTA
Ethanol
Ethidium Bromide
Glacial Acetic Acid
Glass Wool
Glycerol
Glycine
HCl
Isoamylalcohol
Isopropanol
KCl
KH_2PO_4
K_2HPO_4
Methanol
$MgCl_2$
$MgSO_4$
NaCl
NaOH
Phenol
Sodium Acetate
Sucrose
TCA
Triton X-100

Beckman Instruments Incorporated., Palo Alto, CA. USA.

Beckman JA10 rotor
Beckman JA20 rotor
Beckman J2MI centrifuge

Beckton Dickinson., San Jose, CA.USA.

FACS Vantage flow Cytometer

Bio-Rad Laboratories., Hercules, CA. USA.

Acrylamide
Alkaline Phosphatase Conjugate Substrate Kit

APS
BioRad Protein Assay
GelAir Drying System
Microradiance Confocal System
Mini-Protean II
Mini-Sub Cell GT
Nitrocellulose Membrane
TEMED
Transblot Apparatus
UV Transparent Cuvettes

BMG LabTechnologies., Offenburg, Germany.

Fluostar Type 0403

Clontech Laboratories Inc., Palo Alto, CA. USA.

Plasmid DNA

Corning Costar Corporation., Cambridge, MA. USA.

100 mm Sterile Culture Plates

Developmental Studies Hybridoma Bank., Iowa City, IA. USA.

Antibodies

Esco Products Incorporated., Oak Ridge, NJ. USA.

Microscope slides and coverslips

Falcon., Lincoln, NJ. USA.

Centrifuge tubes (15 ml and 50 ml)
Polypropylene tubes (14 ml)
T175 cm Culture Flasks
35 mm Culture Plates

FMC Bioproducts., Rockland, ME. USA.

SeaKem Agarose

Genset., Paris, France.

Oligonucleotides

Germantown (New Zealand) Company., Manukau City, New Zealand.

Agar

Gibco BRL Life Technologies., Gaithersburg, MD. USA.

Bacto-tryptone
DMEM
DNA Molecular Weight Marker (λHindIII)
FCS
Fungizone
Geneticin
HS
Kanamycin
Penicillin-Streptomycin
Trypsin / EDTA
Yeast extract

Heat Systems Ultrasonics Incorporated., Farmingdale, NY. USA.

Sonicator W-225

Heraeus Sepatech GmbH., Osterode, Germany.

Heraeus Biofuge 13 (microfuge)
Heraeus Christ (varifuge)

Hybaid Limited., Ashford, Middlesex. UK.

Hybaid MiniOven MKII

Kinematica., Littau, Switzerland.

Polytron PT3100

Lynnon Biosoft., Vaudreuil, Quebec. Canada.

DNAMAN Version 4.0a

MicroTek International Incorporated., Taiwan.

MicroTek Scan Maker X6el
MicroTek Scan Wizard ver 2.48

Millipore Corporation., Bedford, MA. USA.

Immobilon-P PVDF

Nalgene Nunc International., Rochester NY. USA.

Cryovials (1.5 ml)

New England Biolabs., Beverly, MA. USA,

Bacteria

Olympus., Tokyo, Japan.

Olympus CH2 Light Microscope
Olympus IX-70 Inverted Microscope

Perkin-Elmer Cetus., Norwark, CT. USA.

Perkin Elmer GeneAmp PCR System 2400

Pierce Laboratories., Rockford, IL. USA.

BCA Protein Assay
BSA

Riverlea Dairies., Auckland, New Zealand.

Casein Alacid 730

Roche Molecular Biochemicals., Mannheim, Germany.

Antibodies
CAPS
Dnase-free Rnase
NaN_3
PIPES
Restriction Enzymes
T4 DNA Ligase
TRIS-HCl

Sarstedt., Numbrecht, Germany.

Microfuge tubes (1.5 ml)

Savant., New York, NY. USA.

SpeedVac Concentrator

Schleicher & Schuell., Dassel, Germany.

DEAE Membrane NA-45

Sigma., St. Louis, MO. USA.

AEBSF
Ampicillin
Antibodies
Aprotonin
β-MCE
d-limonene
Freund's Complete Adjuvant
Giemsa Solution
Glutathione Agarose Beads
IPTG
Ketamine HCl
Lysozyme
Paraformaldehyde
PMSF
SDS
X-Gal
Xylazine HCl

Stratagene., La Jolla, CA. USA.

Plasmid DNA

Unicam Limited., Cambridge, UK.

Unicam UV4 Spectrophotometer

Ultra Violet Products., Cambridge, UK.

ImageStore 5000 gel Documentation System
GelBlot Pro Image Analysis System
UV Transilluminator

Qiagen GmbH., Hilden, Germany.

Plasmid Midi Kit
Taq Polymerase and buffers

Zeiss., Oberkochen, West Germany.

Zeiss Standard Microscope with Hg Lamp
Zeiss MC63 Photomicrographic regulator

Appendix G - Publication

Recombinant calpastatin generated in Chapter 3 was used for analytical purposes in the following paper. Conditional upon several recommended changes this paper (Partial Purification and Characterisation of Chinkook Salmon (*Oncorhynchus tshawytscha*) Calpains and an Evaluation of their Role in Postmortem Proteolysis) was accepted for publication on the July 26, 2000, by the Journal of Food Science, Chicago, IL. USA.

Partial Purification and Characterization of Chinook Salmon (*Oncorhynchus tshawytscha*) Calpains and an Evaluation of their Role in Postmortem Proteolysis

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ABSTRACT

The involvement of calpains in the proteolysis of salmon muscle proteins during refrigerated storage was investigated. m-Calpain and μ-calpain were purified and partially purified, respectively. Salmon m-calpain had similar calcium requirements and specific activity against casein as ovine m-calpain. Salmon μ-calpain had similar calcium requirements as ovine μ-calpain, but a lower specific activity against casein than ovine μ-calpain. Autolysis patterns of both calpains differed from those of ovine calpains, and their activities were less than those reported for mammalian muscles. Calpastatin activity was relatively high and comparable to that reported for bovine muscles. Little proteolysis and fiber fragmentation was observed during refrigerated storage. However, proteolysis could be reproduced by incubation of myofibrils with m-calpain.

Key words: calpain, proteolysis, muscle, salmon, *Oncorhynchus tshawytscha*

Introduction

Firmness is an important aspect of fish quality. It deterioriates during postmortem storage. Most of the studies on the deterioration of fish muscle structure during storage have focused on degradation of the connective tissue component of the muscle. Changes in connective tissue have been observed at an ultrastructural level (Hallett and Bremner, 1988; Ando and others 1991) together with an increase in collagen solubility during storage (Sato and others 1997; Aidos and others 1999). In contrast to fish, weakening of the mammalian and avian muscle structure post mortem is considered desirable since it improves tenderness. In the latter case the connective tissue component is little

affected by postmortem storage, but a large number of myofibrillar and cytoskeletal proteins are degraded (Geesink and Koohmaraie 1999a). It is generally accepted that the calcium-dependent proteinases (calpains) are responsible for this degradation (for a review see Koohmaraie 1996). The results of a number of recent studies indicate that some of the changes associated with the action of calpains also occur in fish muscle. These changes include degradation of titin and dystrophin, release of ∝-actinin from the myofibrillar structure and loss of Z-line integrity (Watson and others 1992; Papa and others 1996, 1997). In contrast, little degradation of the calpain substrate desmin was observed during storage of sea bass, brown trout and turbot (Verrez-Bagnis and others 1999).

Relatively little is known about the calpain system in fish muscle. m-Calpain has been purified from carp and tilapia muscle (Sakamoto and others 1985; Wang and others 1993). μ-Calpain has not been purified from fish muscle, but the presence of a Ca^{2+}-dependent caseinolytic peak eluting before m-calpain on an anion-exchange column may indicate its presence in carp muscle (Sakamoto and others 1985). Wang and others (1993), however, did not detect μ-calpain activity in tilapia muscle. The objective of our study was to characterize the calpain system in salmon muscle and to evaluate its role in postmortem proteolysis during refrigerated storage of the muscle.

Materials and Methods

Materials

The experiments were carried out using Chinook salmon (*Oncorhynchus tshawytscha*) from a commercial farm in Canterbury, New Zealand. Sexually immature salmon ranging in weight from 0.3 to 1.5 kg were used. Sexually mature salmon were not used since these are of little commercial value due to a decrease in muscle fat content and an increase in water content. The fish were sacrificed by a blow on the head followed by bleeding. The white dorsal muscle was dissected and used in the various experiments. Ovine μ- and m-calpain were purified from skeletal muscle according to Edmunds and others (1991).

DEAE-Sepharose Fast Flow, Butyl-Sepharose 4 Fast Flow, Mono Q HR 10/10, Phenyl-Sepharose CL-4B, Blue Sepharose CL-6B and the pGEX-4T-1 vector were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Reactive Red 120, glutathione agarose beads and alkaline phosphatase labeled anti mouse IgG were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Monoclonal anti-μ-calpain (clone 9A4H8D3) was purchased from Affinity Bioreagents, Inc. (Golden, Col., U.S.A.). Distilled water and only analytical grade chemicals were used.

Calpain purification

m-Calpain. Muscle (750 g) was homogenized in 3 volumes extraction buffer (100 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.05% 2-mercaptoethanol [MCE], 2 mM phenylmethylsulfonyl fluoride [PMSF]) using a Waring blender (2 x 20 sec, high speed). After centrifugation (17,700 x g_{max}, 1 h, 4 °C), the supernatant was filtered through glass wool and loaded onto a DEAE-Sepharose Fast Flow column (5 x 50 cm, 4 mL/min, 10 mL/fraction). The column was washed with 1 L elution buffer (20 mM Tris/HCl, pH 7.35, 0.5 mM EDTA, 0.05% MCE), and eluted with a gradient of 0 to 125 mM NaCl in 950 mL elution buffer followed by 125 to 500 mM NaCl in 1800 mL elution buffer and 500 mL elution buffer containing 500 mM NaCl. Fractions containing calpain activity were collected (540 mL), and a third of the pooled fractions was loaded on a Reactive Red 120 column (1.5 x 25 cm, 1 mL/min, 10 mL/fraction) equilibrated with elution buffer containing 0.5 M NaCl. The column was washed with 90 mL elution buffer containing 0.5 M NaCl, and eluted with 90 mL elution buffer, pH 8.3. Chromatography on the Reactive Red 120 column was repeated twice for the remaining pooled fractions from the DEAE-Sepharose column. Fractions containing calpain activity were collected and loaded onto a Mono Q HR 10/10 column (2 mL/min, 4 mL/fraction) equilibrated with elution buffer. The column was washed with 25 mL elution buffer and eluted with a linear gradient from 200 to 500 mM NaCl in 140 mL elution buffer followed by 25 mL elution buffer containing 500 mM NaCl. Fractions containing calpain activity were collected and dialyzed against elution buffer.

μ-Calpain. Muscle (250 g) was homogenized in 5 volumes extraction buffer (100 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.05% 2-mercaptoethanol [MCE], 2 mM phenylmethylsulfonyl fluoride [PMSF]). After centrifugation (17,700 x g_{max}, 1 h, 4 °C), the supernatant was filtered through glass wool and loaded onto a DEAE-Sepharose Fast Flow column (2.5 x 40 cm, 2 mL/min, 10 mL/fraction) equilibrated with elution buffer. The column was washed with 300 mL elution buffer and eluted with a gradient of 0 to 1 M NaCl in 700 mL elution buffer followed by 100 mL elution buffer containing 1 M

NaCl. Calpain-containing fractions, as detected by Western blotting, were collected (285 mL), brought to 0.8 M $(NH_4)_2SO_4$ and loaded onto a Butyl-Sepharose 4 Fast Flow column (2.5 x 40 cm, 2 mL/min, 10 mL/fraction). The column was washed with 200 mL elution buffer with 0.8 M $(NH_4)_2SO_4$ and eluted with a gradient of 0.8 to 0 M $(NH_4)_2SO_4$ in 500 mL elution buffer followed by 200 mL elution buffer. Calpain-containing fractions were collected (275 mL) and loaded onto a Mono Q HR 10/10 column (1 mL/min, 5 mL/fraction) equilibrated with elution buffer. The column was washed with 25 mL elution buffer and eluted with a linear gradient from 0 to 1 mM NaCl in 200 mL elution buffer. Calpain-containing fractions (10 mL) were collected and loaded on a Reactive Red 120 column (0.5 x 12 cm, 0.5 mL/min, 2.5 mL/fraction). The column was washed with 30 mL elution buffer containing 0.5 M NaCl, and eluted with 50 mL elution buffer pH 8.3. Calpain containing fractions were collected (67 mL), dialyzed against extraction buffer and concentrated to 3.5 mL using Centriprep 10 concentrators.

Calpain assays

Calpain assays using casein as the substrate were performed according to Koohmaraie (1990). Calpain assays using fluorescein isothiocyanate-labeled casein (FITC-casein) were performed according to Twining (1984). Calpain and calpastatin activity was expressed as described by Koohmaraie (1990). Calpain activity measured using FITC-casein was converted to standard calpain activity units by comparison of activities measured with both methods. Thus, the number of fluorescence units in the FITC-casein assay that corresponded to one unit of activity in the standard casein assay was determined.

Characterization of m-calpain

Calcium-requirement. Salmon m-calpain and ovine m-calpain were dialyzed against elution buffer and the activity against casein in the presence of 0 to 1.6 mM free Ca^{2+} was measured in triplicate.
Autolysis. salmon m-calpain and ovine m-calpain in elution buffer were incubated at 25 °C in the presence of 2 mM free Ca^{2+}. An aliquot was taken after 0, 1, 2, 5 and 10 min of incubation and mixed with an equal volume of 2 X protein denaturing buffer (PDB) [0.12 M Tris/HCl, pH 6.8, 3.4% (w/v) SDS, 10% glycerol, 1.67% (vol/vol) MCE, 0.003% bromophenol blue].
Specific activity. The activity of salmon m-calpain against casein was compared to that of ovine m-calpain using recombinant domain 1 ovine calpastatin. The calpains in elution buffer were incubated with different amounts of the inhibitor and remaining activity was measured in triplicate.

Characterization of μ-calpain

Calcium-requirement. Salmon μ-calpain and ovine μ-calpain were dialyzed against elution buffer and the activity against FITC-casein in the presence of 0 to 0.4 mM free Ca^{2+} was measured in triplicate.
Autolysis. Salmon μ-calpain was incubated at 25 °C with or without 10 units of recombinant domain 1 ovine calpastatin in the presence of 2 mM free Ca^{2+}. An aliquot was taken after 0, 1, 5, 10 and 15 min of incubation and mixed with an equal volume of 2 X PDB.
Specific activity. The activity of salmon μ-calpain against FITC-casein was compared to that of ovine μ-calpain. The relative concentrations of the two calpains were estimated by Western blotting. Western blots were scanned using a Microtek Scanmaker X6 EL (Microtek International Inc., Taiwan). Images were stored using Adobe Photoshop version 4 (Adobe Systems Inc., San Jose, Cal., U.S.A.). Intensity of the bands was quantified using GelBlot-Pro image analysis software (Ultra Violet Products Ltd., Cambridge, UK).

Proteolysis of muscle proteins and activity of the calpain system during refrigerated storage of muscle

Proteolysis of muscle proteins. About 20 g of muscle was dipped in a 1 mM NaN_3 solution and stored for 0, 1, 3, or 7 days in closed 50-mL tubes at 5 °C. After storage, 5 g of the muscle was homogenized in three volumes extraction buffer using an ultra-turrax (IKA, Staufen, Germany). After centrifugation (1,500 x g_{max}, 20 min, 4 °C), an aliquot of the supernatant was mixed with an equal volume 2X PDB and stored at -20 °C until use. The remaining supernatant was discarded and the pellet resuspended in 40 mL elution buffer, centrifuged and the supernatant dicarded. This washing procedure was repeated two more times and an aliquot of the final suspension of washed myofibrils

was mixed with an equal volume of 2X PDB, heated for 3 min in boiling water and stored at -20 °C until use. Degradation of muscle proteins during storage was evaluated by SDS-PAGE. Autolysis of μ-calpain during storage was monitored by Western blotting. Fragmentation of the washed myofibrils was evaluated using a phase contrast microscope (Standard microscope 16; Zeiss, Oberkochen, Germany)

Proteolysis of myofibrillar proteins by m-calpain. Washed myofibrils (5 mg/mL), prepared as described above from at-death muscle, were incubated with m-calpain (0.5 U/mL) in the presence of 5 mM $CaCl_2$ at 25 °C. After 0, 2.5, 5, 10, 15, 30 and 60 min a 200 μL aliquot was mixed with 10 μL EDTA/Tris (200 mM, pH 7). Insoluble material was precipitated using an Eppendorf centrifuge. An aliquot of the supernatant was mixed with an equal volume of 2X PDB, heated for 3 min in boiling water and stored at -20 °C until use. The remaining supernatant was discarded and the pellet was dissolved in PDB buffer, heated for 3 min in boiling water and stored at -20 °C until use. Degradation of muscle proteins was evaluated by SDS-PAGE.

Activity of the calpain system during refrigerated storage of the muscle. Muscle samples (20 g) from 4 salmon were stored for 0 or 2 d at 5 °C. After storage, 10 g samples were homogenized in 5 volumes extraction buffer using an ultra-turrax and centrifuged (27,000 x g_{max}, 30 min, 4 °C). The supernatant was filtered through glasswool and the samples were dialyzed overnight against dialysis buffer (40 mM Tris/HCl, 5 mM EDTA, pH 7.35, 5 °C). After dialysis the samples were centrifuged (27,000 x g_{max}, 30 min, 4 °C), and the supernatants loaded by gravity onto DEAE-Sepharose Fast Flow columns (1.5 x 20 cm) equilibrated with elution buffer. The columns were washed with 150 mL elution buffer, and eluted with a gradient of 0 to 700 mM NaCl in 350 mL elution buffer (2 mL/min, 5 mL/fraction). Calpain and calpastatin activity was localized using FITC-casein as a substrate. Calpastatin activity was tested against ovine m-calpain. Active fractions of the respective components of the calpain system were pooled and the pooled activity determined using FITC-casein.

Preparation of recombinant domain 1 calpastatin

Recombinant domain 1 calpastatin was used to test the specific activity of the calpains. A cDNA fragment corresponding to domain 1 of ovine calpastatin was PCR-amplified using primers containing restriction site overhangs. After restriction digestion, the DNA fragment was subcloned into pGEX-4T-1 vector. Protease-deficient *E. coli* strain BL21 was transformed with the recombinant plasmid, cultured, and recombinant protein expression was induced as described by Liew and others (1997). Cells were harvested by centrifugation, washed twice with ice-cold PBS (0.8% NaCl, 0.02% KCl, 0.1% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.4), and resuspended in one-tenth culture volume of PBS containing 1mM PMSF. The bacterial suspension was sonicated (5 times, 1 min, 50% power) before addition of one-tenth volume of PBS + 10% Triton X-100. The mixture was gently agitated and clarified by centrifugation. Glutathione agarose beads (re-swollen in PBS to a bed volume of 6 mL) were added to the supernatant and mixed overnight at 4°C. Beads were collected by centrifugation, washed 3 times in 10 volumes PBS + 1% Tween 20, and 3 times with PBS alone. One hundred units of thrombin (1 unit ≈ 0.2 NIH units) was combined with 4 mL PBS and gently mixed with the beads at room temperature for 1 h. The supernatant was collected following centrifugation and heated at 95°C for 5 min. Denatured protein was pelleted and the protein concentration in the supernatant measured.

SDS-PAGE, Western blotting and protein determinations

SDS-PAGE was performed according to Laemmli (1970) and Western blotting was performed according to Towbin and others (1979). Protein concentration of samples dissolved in PDB was performed according to Karlsson and others (1994). Protein concentration of myofibrillar suspensions was determined using the biuret method (Gornall and others 1949). Protein concentration of soluble proteins was determined according to Bradford (1976).

Results and Discussion

Calpain purification

Chromatography of the soluble muscle fraction over DEAE-Sepharose revealed the presence of a calpain inhibitor which eluted between 0.11 and 0.21 mM NaCl. Calcium dependent proteolytic

activity eluted between 0.30 and 0.43 mM NaCl. Subsequent chromatograpy of the proteolytic activity resulted in purification of a typical calpain composed of a large subunit of about 80 kDa and a small subunit of about 28 kDa (Table 1, Fig. 1). Attempts to purify the inhibitory activity failed. The salmon calpain inhibitor did not bind to Blue Sepharose CL-6B which has been shown to be very effective in purifying mammalian calpastatin (Geesink and others 1998). Binding of mammalian calpastatin to this medium probably involves domain L (Mellgren 1988). The failure of salmon calpastatin to bind to Blue Sepharose may indicate that it lacks a domain equivalent to mammalian calpastatin domain L. A number of antibodies against mammalian calpains were tested against the purified salmon calpain, but none of them reacted. When these antibodies were tested on total soluble salmon muscle proteins, an antibody against the large subunit of μ-calpain reacted with a band at 80 kDa. Western blotting with this antibody against the different stages of the calpain purification revealed that the protein reacting with this band co-eluted to a certain extent with the purified calpain on DEAE-Sepharose and Reactive Red, but it was separated from the purified calpain after chromatograpy on the Mono Q column. However, no activity, other than that of the purified m-calpain, was detected in the fractions eluting from the Mono-Q. This result could be explained by: (1) the antibody cross-reacting with a protein which was not calpain; (2) the calpain had lost its activity during the purification steps; (3) the calpain did not degrade casein; or (4) the level of calpain activity was below the sensitivity of the standard casein assay. To resolve some of these possibilities several attempts were made to purify the protein using Western blotting as the detection method. A small amount of the protein was eventually partially purified (Table 2, Figures 2A and 2B) and , subsequently, identified as a calpain. This particular calpain eluted between 0.20 and 35 mM NaCl on a DEAE-Sepharose column. The protease did not elute off a phenyl-Sepharose column even if 30% (v/v) ethylene glycol was included in the buffer. This indicates that this particular calpain is more hydrophobic than the mammalian ubiquitous calpains. Furthermore, the protein did not bind completely to the Reactive Red column. During loading and washing a small amount eluted off the column.

Calpain characterization

The calcium requirement of the predominant salmon calpain was slightly lower than that of ovine m-calpain (Fig. 3), but half-maximal activity was close to 0.5 mM Ca^{2+} characterizing it as an m-type calpain.

The autolysis pattern of salmon m-calpain differed from that of ovine m-calpain (Fig. 4). Compared to ovine m-calpain, autolysis of the large subunit of salmon m-calpain proceeded at a faster rate. The small subunit autolyzed to 21 kDa instead of 18 kDa which is typical for mammalian ubiquitous calpains. The autolytic products of calpains, as detected by Western blotting, can be used as indicators of calpain activation in postmortem muscle (Geesink and Koohmaraie 1999a). Unfortunately, none of the available antibodies reacted with salmon m-calpain. This precludes the use of Western blotting as a means to evaluate its possible role in postmortem proteolysis in salmon muscle.

Accurate determination of the specific activity of an enzyme requires a virtually pure enzyme, and that the purified enzyme retains its activity throughout the purification stages. Because we were interested in comparing the specific activity of salmon m-calpain with ovine m-calpain, an alternative approach to determining the specific activity was used. Assuming calpastatin binds both calpains to the same extent, a difference in specific activity would be evident from a difference in inhibition at a given calpastatin concentration. It was found that both calpains were inhibited to a similar extent by recombinant domain 1 calpastatin (Fig. 5), indicating that the specific activity of salmon m-calpain against casein was similar to that of ovine m-calpain.

Using the standard casein assay, the partially purified protein that reacted with the μ-calpain antibody did not express significant proteolytic activity. However, when this protein was incubated with Ca^{2+}, it autolyzed and, because autolysis was inhibited by calpastatin (Fig. 6), the protein is characterized as calpain. Using a more sensitive assay with FITC-labeled casein as a substrate, it was established that this calpain did express caseinolytic activity. The calcium requirement of this calpain was similar to that of ovine μ-calpain (Fig. 7), characterizing it as a μ-type calpain. Interestingly, the autolysis pattern of this calpain also differed from the mammalian form. Autolysis of the large subunit of mammalian μ-calpain proceeds through readily discernable 78 and 76 kDa intermediates. These intermediates were not evident during autolysis of salmon μ-calpain (Fig. 6). The specific activity of salmon μ-calpain was compared to ovine μ-calpain using Western blotting as a means to estimate the relative amounts of the respective enzyme (Fig. 8). Using this method, the specific activity of salmon μ-calpain against casein was estimated to be 25% of the specific activity of ovine μ-calpain.

Evaluation of the role of calpains in proteolysis of salmon muscle proteins

During 7 days of refrigerated storage of the salmon muscle some degradation of muscle proteins occurred as evidenced by the appearance of a 31-kDa breakdown product (Fig. 9). Degradation of troponin-T resulting in protein fragments with a molecular weight of about 30 kDa is the most reported change in myofibrillar proteins during storage of mammalian muscles (for a review see Robson and others 1997). Antibodies against calpain substrates in mammalian muscle (troponin-T, desmin and titin) did not react with these proteins in salmon muscle. As a result, it was not possible to determine whether the 31-kDa band is a degradation product of troponin-T, or whether desmin and titin are also degraded during refrigerated storage of salmon muscle. The slow rate at which the suspected degradation product appears, and the relatively low intensity of the band compared to mammalian muscles (Koohmaraie and others 1991), suggest that relatively little proteolysis occurs during storage of the muscle. In mammalian muscles, postmortem proteolysis results in weakening of the myofibrils. As a result, aged meat yields a higher proportion of smaller fragments than fresh meat (Davey and Gilbert 1969; Moller and others 1973). During storage of salmon muscle no evident increase in fragmentation of the myofibrils upon homogenization was observed (Fig. 10), which supports the suggestion that little proteolysis occurred.

The appearance of the 31-kDa band during storage of salmon muscle could be reproduced by incubating salmon myofibrils with salmon m-calpain (Fig. 11A). During the incubation several degradation products were released from the myofibrils into the soluble fraction (Fig. 11B). Incubation of ovine myofibrils and calpain results in a similar release of soluble degradation products, many of which have been identified as degradation products of titin (Geesink and Koohmaraie 1999b). This similarity suggests that titin is a calpain substrate in salmon muscle.

Western blotting was used to determine whether μ-calpain autolyzed during storage of the muscle (Fig. 12). No obvious decrease in the intensity of the band at 80 kDa was observed and none of the autolysis products shown in Fig. 6 was detected. This result indicates that the enzyme was not activated or that the calpastatin concentration was sufficient to inhibit autolysis to undetectable levels.

Using a shallow gradient we were able to achieve baseline separation between calpastatin, μ-calpain and m-calpain on DEAE-Sepharose Fast Flow. Calpastatin, μ-calpain, and m-calpain eluted between 80 and 190 mM NaCl, 230 and 300 mM NaCl, and 365 and 450 mM NaCl, respectively. The possibility of partial co-elution of the different components of the calpain system was tested by heating the μ-calpain containing fractions and assaying for the presence of the heat-stable calpastatin, and by Westen blotting of the different fractions against μ-calpain (data not shown). The baseline separation between the different components of the calpain system enabled their quantification (Table 3). In accordance with the results of Sakamoto and others (1985) on carp muscle, relatively little m-calpain activity was present in salmon muscle compared to mammalian muscles. μ-Calpain activity was about 100 times lower than the level of the enzyme in mammalian muscles (Koohmaraie and others 1991). Calpastatin levels, however, were relatively high and comparable to those found in beef muscles (Koohmaraie and others 1991). After 2 days of storage the calpastatin activity was higher ($P < 0.05$) than at death. The reason for this is unclear. It may be due to a difference in extraction efficiency. Levels of m-calpain and μ-calpain activity were numerically, but not significantly ($p > 0.05$), lower at 2 days post mortem than at death. This result indicates that the activity of both enzymes is relatively stable during postmortem storage.

The low levels of calpain activity and high calpastatin-to-calpain ratio may explain the low levels of postmortem proteolysis (Fig. 9) and the virtual absence of fragmentation of the muscle fibers during storage (Fig. 10). In mammalian muscles, a comparable, although less extreme, situation is observed with the callipyge sheep. Longissimus muscles from these animals contain about twice the amount of calpastatin found in animals not expressing this genotype. As a result, little postmortem proteolysis and fragmentation of the muscle fibers is observed during postmortem storage of the muscles (Koohmaraie and others 1995; Geesink and Koohmaraie, 1999a).

The results presented here relate to calpain/calpastatin activities and postmortem proteolysis in sexually immature salmon. Compared to sexually immature salmon, the muscle of mature salmon has an inferior texture which has been associated with an increase in muscle proteolysis (Stoknes and Rustad 1995); however, no notable differences were observed in calcium-independent proteolytic activities between muscles from mature and immature salmon. In addition, spawning salmon have a very soft texture and high cathepsin B and L activities (Yamashita and Konagaya 1991). It would, therefore, be of interest to compare calpain and calpastatin activities of mature and immature salmon muscle.

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TABLES

Table 1 - Summary of protein and activity yields at each step of the purification of m-calpain from salmon muscle.

Purification step	Protein conc. (mg/mL)	Activity (units/mg)	Total activity (units)	Purification (fold)
Extract	17.2	0.0071[a]	275[a]	-
DEAE-Seph.	1.33	0.38	275	53.5
Reactive Red	0.177	28.8	240	4056
Mono Q	0.256	64.2	132	9042

[a]Estimates based on activity after DEAE-Sepharose chromatograpy

Table 2 - Summary of protein yields at each step of the purification of μ-calpain from salmon muscle.

Purification step	Protein conc. (mg/mL)	Total protein (mg)
Extract	7.8	9750
DEAE-Seph.	1.88	461
Butyl-Seph.	0.30	83
Mono Q	0.90	9
Reactive Red	0.043	0.15

Table 3 - Calpain and calpastatin activities in salmon muscle at death and after two days storage at 5 °C (mean + standard deviation; n = 4).

Item	Day 0	Day 2
Calpastatin	4.25[a] (0.76)	5.95[b] (0.31)
μ-Calpain	$8.7x10^{-3}$ ($1.0x10^{-3}$)	$5.6x10^{-3}$ ($2.1x10^{-3}$)
m-Calpain	0.24 (0.11)	0.16 (0.10)

[ab]Means with different superscripts differ ($P < 0.05$)

FIGURE CAPTIONS

Figure 1 - Purification of m-calpain from salmon muscle (SDS-PAGE; 12.5% acrylamide). Lanes 1 to 5: Molecular weight marker, soluble extract (25 μg), DEAE-Sepharose Fast Flow (25 μg), Reactive Red (10 μg), Mono-Q (3 μg).

Figure 2 - Partial Purification of μ-calpain from salmon muscle. A. (SDS-PAGE; 12.5% acrylamide) Lanes 1 to 6: Molecular weight marker, soluble extract (25 μg), DEAE-Sepharose Fast Flow (25 μg), butyl-Sepharose (7.5 μg), Mono-Q (7.5 μg), Reactive Red (1.1 μg). B: (Western blot) Lanes 1 to 7: Soluble extract, DEAE-Sepharose Fast Flow, butyl-Sepharose, Mono-Q, Reactive Red, Reactive Red (amido black stain), molecular weight marker (amido black stain).

Figure 3 - Comparison of the Ca^{2+}-requirement of salmon and ovine m-calpain (n = 3; pooled standard deviation = 4.2).

Figure 4 - Autolysis of salmon and ovine m-calpain (SDS-PAGE; 12.5% acrylamide; 3 μg/lane). Lanes 1 to 5: Salmon m-calpain autolyzed for 0, 1, 2, 5 and 10 min at 25 °C. Lanes 6 to 10: Ovine m-calpain autolyzed for 0, 1, 2, 5 and 10 min at 25 °C. Molecular weights are indicated on the left side of the gel.

Figure 5 - Inhibition of salmon and ovine m-calpain by recombinant ovine domain 1 calpastatin.

Figure 6 - Autolysis of salmon μ-calpain in the absence (lanes 2 to 5) and presence (lanes 6 to 9) of recombinant ovine domain 1 calpastatin for 0, 5, 10 and 15 min. at 25 °C (Western blot; 12.5% acrylamide gel; 0.5 μg/lane). Lane 1: Molecular weight marker (amido black stain).

Figure 7 - Comparison of the Ca^{2+}-requirement of salmon and ovine μ-calpain (n = 3; pooled standard deviation = 4.8).

Figure 8 - Comparison of the specific activities of salmon μ-calpain (lane 1) and ovine μ-calpain (lanes 2 to 8) against FITC-casein using Western blotting as a means to estimate the amount of enzyme. Relative activities (%) are indicated below the respective bands.

Figure 9 - SDS-PAGE (12.5% acrylamide; 30 μg/lane) of myofibrillar proteins from salmon muscle after 0, 1, 3 and 7 days postmortem storage at 5 °C (lanes 2 to 4, respectively). The arrowhead at the right side of the gel indicates the 31-kDa degradation product. Lane 1: Molecular weight marker.

Figure 10 - Phase micrograph of myofibrils prepared from salmon muscle after 0, 1, 3 and 7 days postmortem storage at 5 °C.

Figure 11 - SDS-PAGE (12.5% acrylamide) of the insoluble (A; 30 μg/lane) and soluble (B) proteins after incubation of salmon myofibrils with salmon m-calpain for 0, 2.5, 5, 10, 15, 30 and 60 min at 25 °C (Lanes 2 to 8). Lane 1: molecular weight marker. The arrow on the right indicates the 31 kDa degradation product.

Figure 12 - Western blot (7.5% acrylamide gel) against salmon μ-calpain in the soluble muscle fraction (30 μg/lane) after 0, 1, 3 and 7 days postmortem storage of the muscle at 5 °C (Lanes 3 to 6). Lanes 1 and 2 contain molecular weight marker and purified salmon m-calpain (2 μg), respectively.