NOTE TO USERS

DISSERTATION

CHARACTERIZATION OF GENES INVOLVED IN MOLTING AND LIMB REGENERATION IN LAND CRAB, *Gecarcinus lateralis*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HYUN WOO KIM ENTITLED CHARACTERIZATION OF GENES INVOLVED IN MOLTING AND LIMB REGENERATION IN LAND CRAB, *Gecarcinus lateralis* BE ACCEPTED AS FULFILLING IN PART REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF GENES INVOLVED IN MOLTING AND LIMB REGENERATION IN LAND CRAB, *Gecarcinus lateralis*

The claw muscles of decapod crustaceans undergo a programmed atrophy to facilitate withdrawal of the claws at the time of molting. The muscle has four calpain-like proteinase activities (CDPs I, IIa, IIb, and III) that degrade myofibrillar proteins. Three calpain cDNAs were isolated (Gl-CalpM, -B, and -T) using nested polymerase chain reaction (PCR) and 3' and 5' rapid amplication of cDNA ends (RACE) PCR. Gl-CalpM is a homolog of lobster calpain M (Ha-CalpM), as determined by deduced amino acid sequence and estimated mass (~68 kDa). It is expressed at high levels in skeletal muscle and ovary. Gl-CalpB contains all four domains (I, II, II, and IV) of typical calpains and has highest homology to *Drosophila* calpains A and B (Dm-CalpA and B). Based on its estimate mass (~89 kDa) and cross immunoreactivity with a polyclonal antibody raised against Dm-CalpA, the Gl-CalpB appeared to encode CDP IIb, which is a homodimer of a 95-kDa subunit. Gl-CalpT is a homolog of nematode TRA-3 and human calpain 5, which contain a unique "T" domain in place of the N-terminal EF-hand domain IV of typical calpains. Both Gl-CalpB and Gl-CalpT were expressed in a wide variety of tissues at varying levels.

Since muscle atrophy coincides with increased ecdysteroid concentrations in the hemolymph, the molting hormone, 20-hydroxyecdysone (20E), may be involved, either directly or indirectly, in activating calpain-mediated myofibrillar protein degradation. Using nested PCR and RACE, a partial sequence of the ecdysone receptor

(Gl-EcR), a full-length sequence of retinoid receptor (Gl-RXR), and a full-length sequence of an ecdysone early-response gene (Gl-E75) were isolated. Gl-EcR was expressed as a single isoform, while Gl-RXR was expressed as 7 isoforms generated by alternative splicing. As the functional 20E receptor is a heterodimer of EcR and RXR, the different Gl-RXR isoforms may confer different functional properties for tissue responses to the same level of hormone. Gl-E75 has the highest sequence identity to shrimp E75 (Me-E75), although the F domain in Gl-E75 was more similar to that of *Drosophila* E75 (Dm-E75). Quantitative real-time PCR showed that expression of EcR and Gl-CalpT was highly correlated, suggesting that CalpT is directly regulated by the ecdysone receptor.

cGMP is a second messenger inhibiting ecdysteroidogesis in the molting gland (Y-organ) by molt-inhibiting hormone (MIH), suggesting that MIH action is mediated by a nitric oxide (NO)-sensitive guanylyl cyclase. The potential role of a NO signal transduction pathway was investigated. Using various PCR techniques, a full-length cDNA encoding land crab NO synthase (NOS) was isolated. The Gl-NOS cDNA encoded a protein containing 1199 amino acids with an estimated mass of ~136 kDa. RT-PCR showed that NOS was expressed in Y-organ, eyestalk ganglion, thoracic ganglion, ovary, testis, and gill. This is the first report of NOS in non-neuronal tissues, which suggests that NOS is involved in regulating ecdysteroid metabolism in the Y-organ and other tissues.

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CHAPTER ONE

INTRODUCTION

Regulation of molting in decapod crustaceans

Like other arthropods, crustaceans grow by periodic shedding their old exoskeleton, which is defined as molting or ecdysis. Both before (premolt or proecdysis) and after (postmolt or metecdysis) ecdysis there are major metabolic events specifically associated with growth, including degradation of the old exoskeleton and synthesis of the new exoskeleton, formation and dissolution of gastroliths in some species, atrophy and restoration of skeletal muscle in the claws, and regeneration of lost appendages (Skinner, 1985). Drach (1939) divided the molting cycle into five stages (A to E) based on hardness of specific regions of the exoskeleton. By observing sequential changes in the epidermis of the tropical land crab (*Gecarcinus lateralis*), Skinner (1962) further refined the molting cycle at the cellular level. Stage A is the period immediately after ecdysis (Stage E) and lasts one or two days. In this stage the gastroliths are completely dissolved and epidermal cells decrease in size. In Stage B the endocuticle layers of the exoskeleton are formed and muscle in the claw grows. Through stage C the exoskeleton hardens further by calcification and thickening of the endocuticle layer. The end of metecdysis (stages A through C_3) is marked by the formation of the membranous layer of the exoskeleton (Carlisle and Dohrn, 1953). Stage C_4 is intermolt or anecdysis, which is the longest period in the molting cycle and lasts about 10 or 11 months in adult land crabs. Premolt (proecdysis) usually begins about 2 months before ecdysis and is subdivided into 5 substages (from D_0 to D_4) by structural changes in the integument (epidermis and exoskeleton). Proecdysis is triggered by increasing concentrations of molting hormone,

20-hydroxyecdysone (20E) and other ecdysteroids in the hemolymph. During stage D_0 gastroliths, calcareous deposits in the lining of the stomach, are formed and the claw muscles atrophy. The first sign of proecdysis in integumentary issues is apolysis, which is the separation of epidermis from the old exoskeleton in stage D_1 (Jenkin, 1966). During stage D_2 the epidermis increases in thickness and secretes a new epicuticle and exocuticle under the old exoskeleton. Hemolymph ecdysteroid concentrations increase through stage D_2 and reach a peak in stage D_3. In stage D_4 the hemolymph turns to pink from astaxanthin, a carotenoid that is reabsorbed from the exocuticle of old exoskeleton. Changes in the concentration of astaxanthin in the hemolymph have been used as an index of an animal's progress through proecdysis (Skinner, 1985). Degradation of the old exoskeleton continues up until ecdysis. Epidermal cells begin to decrease in size just prior to ecdysis and continue to decrease until they reach a size comparable to that of anecdysisal animals.

Although the molting cycle varies according to environmental influences, it becomes progressively longer as animals age. Molting cycles in larvae are usually a few days or weeks. The first seven molt cycles of the amphipod *Traskorchestia traskiana* is as short as a few days (Soyez and Kleinholz, 1977). By the sixth growing season, lobsters (*Homarus americanus*) molt once annually (Hughes and Matthiessen, 1962). Some species enter a terminal anecdysis at the puberty molt and no longer molt under unstressed conditions (Skinner, 1985). The effect of temperature has been reported for various crustaceans (Chang and Bruce, 1980; Hughes et al., 1972). In lobsters, the molting cycle is shortened by increasing the temperature to 22-24 °C. Low temperature delays molting in the fiddler crab, *Uca pugnax* (Passano, 1960). The effect of light

appears to be variable in different species (Weis, 1976; Stephens, 1955). Although environmental signals may affect molt cycle it is likely that internal signals determine an animal's response to external factors (Skinner, 1985).

Like all other arthropods the internal signal controlling the molting cycle is the steroid hormone 20E (Hoffmann and. Porchet, 1984). Although a variety of ecdysteroids are present, 20E is the dominant ecdysteroid in the hemolymph in most species. Ecdysteroids are synthesized and secreted from the molting glands, or Y-organs, a pair of epitheliod glands located in the cephalothorax (Lachaise et al., 1993). The Y-organs are small, compact structures that are no more than a few millimeters in diameter even in large species, such as the crab *Cancer magister* (Hoffman, 1967). Cells of the Y-organ are of a single type and have many mitochondria and extensive smooth endoplasmic reticulum, which are the distinctive characteristics of steroid-secreting cells (Buchholz and Adelung, 1980). Like other arthropods, crustaceans are unable to synthesize cholesterol and the rate of ecdysteroid synthesis depends on the availability of cholesterol and its access to enzymes through short-lived carrier proteins, the activity of steroidogenic enzymes, and the availability of enzyme cofactors, mainly NADPH for cytochrome P450 monooxygenases (Baghdassarian et al., 1996).

The Y-organs are regulated negatively by a neuropeptide, molt-inhibiting hormone (MIH). MIH is produced and released from the X-organ/sinus gland complex in the eyestalk ganglia and inhibits ecdysteroid synthesis in the Y-organs. The existence of MIH was suggested by the observation that eyestalk ablation leads to rapid increase in hemolymph ecdysteroid titer and induces molting (Passano, 1953). Injection of eyestalk extracts into eyestalkless animals at specific stages of the molting cycle lengthens the

duration of the cycle (Soyez and Kleinholz, 1977; Ranga, 1965). However, regulation of ecdysteroidogenesis in the Y-organs is more complex. For example, eyestalk ablation does not shorten the molting cycle in females undergoing vitellogenesis (Lachaise et al., 1992) and some species show continuous fluctuation of ecdysteroid titer in molt cycle without eyestalks (Chang, 1985; Hopkins, 1983). In addition, as crustacean hyperglycemic hormone (CHH), gonad-inhibiting hormone (GIH), and mandibular organ-inhibiting hormone (MOIH) are also expressed in the X-organ, eyestalk ablation may cause imbalances in other physiological processes. Thus, regulation of the molting cycle is not simply a suppression of Y-organ by MIH. It involves a complex interaction between reproduction, growth, and environmental factors.

MIH sequences have been characterized in several decapod crustaceans. *In vivo* assays were employed in the isolation of an 8.7-kDa peptide from the sinus glands of *Homarus americanus* (Chang et al., 1987). Once the amino acid sequence of MIH was determined (Webster, 1991; Chung et al., 1996), the complete cDNA sequence was reported (Klein et al., 1993; Lee et al., 1995). Recently, a genomic DNA study of *Cancer parurus* showed that at least two copies of MIH genes exist (Lu et al., 2000). The MIH genes of *Cancer feriatus* and *C. pagurus* consist of three exons and two introns, and span approximately 4.3 kb (Chan et al., 1998; Lu et al., 2000). The 5' flanking regions of MIH genomic DNA contains several potential binding sites for known transcription factors, such as cAMP response element binding (CREB) protein, putative Cf1/USP, and Broad Complex Z2 (Chan et al.,1998; Lu et al., 2000). The brachyuran MIHs share high sequence similarity. They encode a 113 amino acid prohormone (proMIH) composed of a 35 residue signal peptide and a 78 amino acid mature peptide (Watson et al., 2001). MIH

contains six cysteine residues, which are characteristic of the crustacean hyperglycemic hormone (CHH) peptide family (Keller, 1992). A structural study suggests that the α-helices near the N-terminus and C-terminus are essential for MIH function (Katayama et al., 2003).

MIH mRNA is detected in eyestalk neural ganglia (Umphrey et al., 1998). It is expressed at lower levels in brain, fertilized eggs, embryos, and larvae (Chan et al., 1998). Recently, a MIH polyclonal antibody raised to a unique amino acid sequence of MIH (Lee and Watson, 2002) showed strong immunoreactivity in secondary limb regenerates, but not in primary limb regenerates in the land crab (*Gecarcinus leteralis*) (K.J. Lee, personal communication). These findings of nontraditional sites of MIH expression suggest a possible alternative mode of action, such as a neuromodulator or neurotransmitter (Watson et al., 2001).

The signal transduction pathway of MIH is poorly understood. cDNAs encoding high affinity MIH receptors in Y-organ membrane have yet to be characterized. Both cAMP and cGMP have been implicated as second messengers, although cGMP appears to be the main messenger regulating Y-organ ecdysteroidogesis. MIH induces a transient 2 fold increase of cAMP and longer 60-fold increase in cGMP in Y-organs (Saïdi et al., 1994). A signaling pathway involving cGMP dependent kinase (PKG) is involved in inhibiting the Y-organs (Baghdassarian et al., 1996). Substrates of either the PKG or PKA are unknown. However, in the Y-organs of the crayfish (*Orconectes limosus*), a 95-kDa-phosphoprotein increased during premolt and became the dominant phosphoprotein at stage D_1, while a 68 kDa (pp68) and a 17 kDa phosphoprotein decreased (Böcking and Sedlmeier, 1994). None of those proteins have been identified, but it strongly suggests

that protein phosphorylation/dephosphorylation is involved in regulating ecdysteroidogenesis.

Molt-induced claw muscle atrophy

Claw muscle atrophy enables successful withdrawal of the claws from the old exoskeleton at ecdysis (Mykles and Skinner, 1990). Molting and muscle atrophy and restoration was first reported in the land crab (*Gecarcinus lateralis*) by Skinner (1966). The mass of the claw muscle decreases about 40% during proecdysis and is restored and grows larger to fill the enlarged space provided by the new exoskeleton after successful exuviation (Skinner, 1966). This reduction is not from cell death, since the DNA content is unchanged (Skinner, 1966). The reduction in fiber volume is proportional to the reduction in muscle mass (Mykles and Skinner, 1981). These data suggest that the total number of fibers remains constant as myofilaments are degraded. Thin filaments are preferentially degraded compared with thick filaments (11 thin filaments for each thick filament). Therefore, there is a decrease in the thin-to-thick myofilament ratio from about 9:1 to 6:1 during proecdysis (Mykles and Skinner, 1981; Ismail and Mykles, 1992). This may help to retain contractile function and accelerate restoration of fibers to the intermolt condition by maintaining sarcomeric structure (Mykles, 1999). The atrophy is specific to the claw muscle and does not occur in thoracic muscle and leg muscle (Mykles and Skinner, 1982). In fiddler crab, the closer muscle in the major claw, which contains mostly slow-twitch or S_1 fibers, undergoes a greater atrophy than that in the minor claw, which contains slow-tonic or S_2 fibers (Ismail and Mykles, 1992). These data suggest that fiber types differ in sensitivity to ecdysteroids (Mykles, 1999).

Calpains are Ca^{2+}-dependent proteases that play a major role in molt-induced muscle atrophy. They can degrade all the myofibrillar proteins to acid-soluble products *in vitro* (Mykles and Skinner, 1982; Mykles and Skinner, 1986). Elevated intracellular Ca^{2+} stimulates protein degradation in claw muscles maintained *in vitro* and proteolysis is reduced 30% to 53% by calpain inhibitors, such as Ep-475 or leupeptin (Mykles, 1990). Total calpain activity is increased about two-fold in atrophic muscle (Mykles and Skinner, 1982). Crustacean muscle contains four calpains (CDP I, CDP IIa, CDP IIb and CDP III; native masses 310, 125, 195 and 59 kDa, respectively; Mykles and Skinner, 1986). All require millimolar Ca^{2+} for full activity *in vitro* and are inhibited by cysteine proteinase inhibitors (Mykles and Skinner, 1986). CDP I is the least efficient of the four calpains in degrading myofibrillar proteins (Mattson and Mykles, 1993). CDP IIb is a homodimer of a 95-kDa subunit and a homolog of Dm-CalpA by immunological analysis (Beyette et al., 1993). CDP IIa is a homodimer of a 60-kDa subunit. In western blots it reacts with an antibody directed to a sequence in the active site of mammalian calpains, but not with polyclonal antibodies raised against mammalian μ- and m- calpains or Dm-calpA (Beyette et al., 1997). CDP IIa and CDP IIb are the most effective in degrading myofibrillar proteins, such as myosin heavy chain and light chain, actin, and some tropomyosins and troponins (Mattson and Mykles, 1993). CDP III (59 kDa) was first cloned and characterized in lobster (Ha-CalpM) (Yu and Mykles, 2003). It lacks a calmodulin-like domain in the C-terminus that is characteristic of typical calpains.

The ubiquitin/proteasome-dependent proteolytic system is also activated during atrophy. Polyubiquitin mRNA levels increase five-fold, ubiquitin-protein conjugates increase 8-fold, and proteasome subunits increase two-fold in atrophic muscle of the land

crab (Shean and Mykles, 1995). The heat-activated proteasome hydrolyzes myosin, troponins, and tropomyosins *in vitro* (Mykles and Haire, 1991). However, the heat-activated proteasome does not degrade myofibrillar proteins to acid-soluble products (Mykles and Haire, 1991), suggesting that calpains and /or other proteases are necessary for complete hydrolysis of the proteolytic fragments. In addition, inhibitors that completely block calpain activity inhibit proteolysis no more than 53% in organ culture experiments (Mykles, 1990), which suggests that about half of protein degradation is mediated by other proteinases. At higher Ca^{2+} concentration, EST, a calpain inhibitor, suppresses proteolysis only 35%. The 26S proteasome complex, which is composed of a 20S proteasome and two PA700 regulatory complexes, is induced by elevated Ca^{2+}.

The proposed proteolytic pathways involved in myofibrillar protein degradation are summarized in Fig.1-1. The primary pathway is mediated by calpains, which hydrolyze all myofibrillar proteins to acid-soluble products. The proteasome may be involved in limited cleavages of myofilaments to large fragments and these large fragments can be further broken down to peptides or amino acids either by calpains or other proteases (Mykles, 1997).

Atrophy is coincident with increasing concentration of ecdysteroids in the hemolymph, suggesting molt-induced claw muscle atrophy is induced by activation of an ecdysteroid receptor. Recently, ecdysteroid receptors have been isolated and characterized from crustaceans (Durica and Hopkins, 1996; El Haj et al., 1997). Ecdysteroids, like vertebrate steroid hormones, regulate ecdysteroid response gene transcription after binding to a nuclear receptor called the ecdysteroid receptor (EcR). Usually, EcR itself cannot bind to the hormone; it heterodimerizes with a vertebrate retinoid receptor (RXR)

homolog (ultraspiracle, or USP, in insects) to form a functional receptor (Yao et al., 1993). Heterodimeric receptors (EcR/USP) bind a specific response element motif in the promoter regions of early response genes, such as E74 or E75. These early response gene products are usually transcription factors that activate an ecdysteroid cascade reaction (Huet et al., 1995).

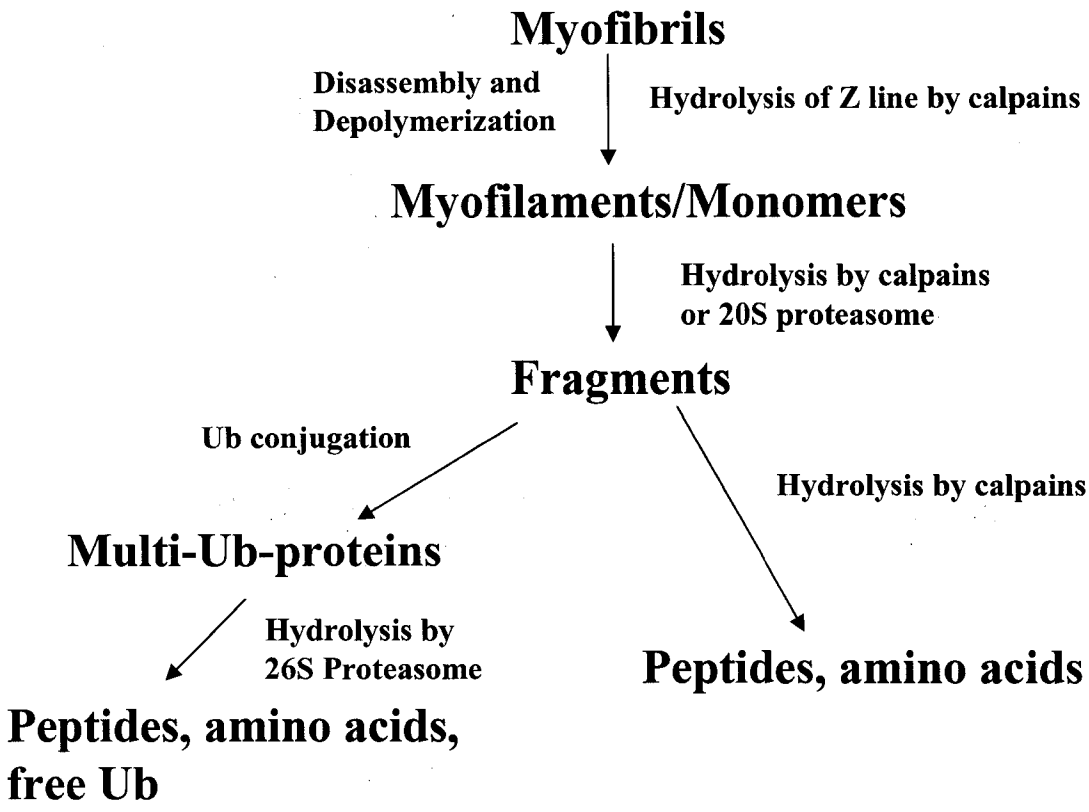
cDNAs encoding EcR (UpEcR) and RXR (UpRXR) were cloned from fiddler crab (*U. pugilator*) (Chung et al., 1998). Both the DNA binding domain (DBD or domain C) and ligand binding domain (LBD or domain E) of UpEcR is closely related to those domains in insect homologs. UpRXR shares greatest similarity to insect USPs in the DBD, while its LBD shares greater amino acid similarity to vertebrate RXR (Chung et al., 1998). Alternative splicing produces different isoforms. Insect EcRs are expressed in two or three A/B domain isoforms. Only one A/B domain isoform has been identified in crustacean EcR, but several alternatively spliced isoforms around the LBD are expressed (Durica et al., 2002). The N-terminal A/B domain is involved in regulation. It contains the ligand-independent transactivation function (AF-1); each isoform, generated by alternative splicing or different promoters, differs in transcriptional regulation or cell specificity. The insect RXR homolog USP has isoforms with different N-terminal A/B regions (Jindra et al.,1997; Kapitskaya et al., 1996). Isoform expression level varies with different physiological stages. The crustacean RXR homolog, UpRXR, has two splicing isoforms in the hinge region, while only one A/B domain isoform has been identified (Durica et al., 2002). One UpRXR isoform has a five amino acid insertion within the "T" box, which is a conserved domain near the DBD and is required for DNA binding (Wilson et al., 1992). The other isoform has a deleted 33 amino acid sequence in the

flexible loop region connecting the LBD and DBD. This isoform may be important for interacting with EcR or other transactivators.

A cDNA encoding another nuclear receptor family protein, MeE75, has been cloned from a shrimp, *Metapenaeus ensis* (Chan, 1998). E75s in insects have two or three isoforms in the A/B domain and the expression pattern of each isoform is varies during the molting cycle and ecdysteroid levels (Segraves and Hogness, 1990; Jindra et al., 1994). MeE75 has three isoforms in the A/B domain (75A, 75C, and 75D). MeE75A is constitutively expressed, while MeE75C is expressed at high levels at the transition from early to late premolt.

My graduate work has focused on isolating and characterizing three calpain cDNAs involved molt-induced muscle atrophy and genes that regulate molting. In chapter two three calpain mRNAs and their expression in different tissues are characterized. Chapter three describes the cloning of three ecdysteroid-related genes (ecdysone receptor, EcR; retinoid X receptor, RXR; and ecdysone early response protein, E75) and their and calpain expression in response to eyestalk ablation and limb regeneration. Chapter four reports the cloning of the first nitric oxide synthase (NOS) gene in a crustacean and discusses its possible role in regulating ecdysteroidogenesis in the Y-organ. Chapter five is a summary and outlines future directions of the research. An appendix is included that contains a full-length sequence of molt inhibiting hormone (MIH); three partial clones of guanlylyl cyclases (GCs); a partial sequence of elongation factor 2 (EF2), which was used as a positive control for RT-PCR; and ATF-4/cryptocephal transcription factor, which is involved in ecdysone-regulated events during *Drosophila* molting and metamorphosis.

Figure 1-1. Proteolytic pathways involved in molt-induced muscle atrophy. Calpains hydrolyze the Z-line to release myofilaments, which are subsequently degraded by calpains and the proteasome. Abbreviation : Ub, Ubiquitin.



CHAPTER TWO

ISOLATION AND STRUCTURAL ANALYSIS OF THREE CALPAINS FROM LAND CRAB (*GECARCINUS LATERALIS*)

Abstract

Crustacean muscle has four calpain-like proteinase activities (CDP I, IIa, IIb, and III) that are involved in molt-induced claw muscle atrophy. Using various PCR techniques, three full-length calpain cDNAs (Gl-CalpM, B, and T) were isolated from limb regenerates of land crab, *Gecarcinus lateralis*. Gl-CalpM appeared to be a homolog of Ha-CalpM from lobster based on deduced amino acid sequence, mass (~68 kDa), and structural organization, as it lacks the EF hand domain IV. It was expressed at high levels in skeletal muscles, integument, and ovary. Evolutionarily, Gl-CalpM and Ha-CalpM apparently diverged from an arthropod calpain, Gl-CalpB (~89 kDa), which can be grouped with Dm-CalpA and B from *Drosophila.* Based on its estimated mass and cross immunoreactivity with polyclonal antibody raised to 70-kDa protein from Dm-CalpA, Gl-CalpB is likely to encode CDP IIb, which is a homodimer of a 95-kDa subunit. It was expressed at varying levels in all tissues examined. Gl-CalpT was a homolog of TRA-3 in the nematode, *Caenorhabditis elegans*. It contained a unique "T" domain in place of an EF-hand domain IV. It was expressed in most tissues.

Introduction

Since the first calpain was purified from skeletal muscle by Ishiura (1978), the number of calpains has grown to include a large family of structurally related intracellular Ca^{2+} dependent cysteine proteinases containing a papain-related catalytic domain (Reverter et al., 2002). In human, 15 calpain genes have been characterized so far (Sorimachi and Suzuki, 2001). Calpains from various non-vertebrate species such as yeast (Futai et al., 1999), fungus (Denison et al., 1995), nematode (Sokol and Kuwabara, 2000; Syntichaki et al., 2002), *Drosophila* (Jekely and Friedrich, 1999; Spadoni et al., 2003), and lobster (Yu and Mykles, 2003) have been reported.

Typical calpains are organized into 4 domains. Domain I is a NH_2-terminal domain that varies in length and sequence between different calpain genes. Autolysis of the N-terminus is associated with activation of some calpains (Hata et al., 2001; Zimmerman and Schlaepfer, 1991), but not in a lobster calpain (Beyette and Mykles, 1997). Domain II is a cysteine protease domain that has a catalytic triad (Cys, His, Asn) required for activity. However, in some calpains the catalytic residues are mutated, such as in mammalian calpain 6 (Lys, His or Tyr, Asn), *Drosophila melanogaster* CALPC (Arg, Ile, Asp), and *Trypanosoma brucei* calpain-like protein (Ser, Tyr, Asn), which suggests that calpains have functions other than protease activity (Goll et al., 2003). Domain II also contains two highly-conserved Ca^{2+}-binding regions involved in enzyme activation (Moldoveanu et al., 2002). Domain III contains a C2-like region that mediates Ca^{2+} dependent phospholipid binding and activation (Tompa et al., 2001; Hosfield et al., 1999; Strobl et al., 2000). Domain IV is a calmodulin-like domain containing five EF-hand motifs, the first three of which (EF-1, EF-2 and EF-3) bind Ca^{2+} with varying affinities.

The fifth EF-hand (EF-5) mediates dimerization with a 28-kDa regulatory subunit in vertebrate heterodimeric calpains.

Atypical calpains lack a C-terminal calmodulin-like domain (Goll et al., 2003). Truncated calpains, such as mammalian nCl-2' and *Drosophila* Dm-CalpA' lack domain IV (Sorimachi et al., 1993; Theopold et al., 1995). In other calpains domain IV is replaced by a different sequence, such as domain T in mammalian calpain 5 and nematode TRA-3 or the "SOL" domain in mammalian SOLH and *Drosophila* SOL. SOL and SOLH have Zn-finger domains in the N-terminal region, suggesting that they are DNA-binding proteins.

The biochemical properties of crustacean calpains have been extensively studied in lobster muscle, which contains four calpains (CDP I, CDP IIa, CDP IIb and CDP III; native masses 310, 125, 195, and 59 kDa, respectively; Mykles and Skinner, 1986). All require millimolar Ca^{2+} for full activity *in vitro* and are inhibited by calpain inhibitors (Mykles and Skinner, 1986). CDP I has not been well characterized and it is the least efficient of the four calpains in degrading myofibrillar proteins (Mattson and Mykles, 1993). CDP IIb is a homodimer of a 95-kDa subunit and related to Dm-CalpA, as determined by immunological analysis (Beyette et al., 1993). In western blots CDP IIa reacts with an antibody directed to a peptide sequence in the active site of mammalian calpains, but not with polyclonal antibodies raised against mammalian μ- and m-calpains or Dm-CalpA (Beyette et al., 1997). These results indicate that CDP IIa differs in structure from conventional calpains. CDP IIa and CDP IIb are the most effective in degrading myofibrillar proteins, such as myosin heavy and light chains, actin, tropomyosin, and troponin (Mattson and Mykles, 1993). A cDNA encoding CDP III was

cloned and characterized in lobster (Ha-CalpM; Yu and Mykles, 2003). It lacks a calmodulin-like domain in the C-terminus that is characteristic of typical calpains. Ha-CalpM is highly expressed in skeletal muscle, but its mRNA and protein levels do not change significantly over the molting cycle. It may be involved in restructuring and/or maintaining contractile structures in crustacean skeletal muscle (Yu and Mykles, 2003).

Here we report the cloning a cDNAs encoding three calpain genes (Gl-CalpM, Gl-CalpB and Gl-CalpT) isolated from the land crab, *Gecarcinus lateralis*. The domain organization of the deduced sequences of the three calpains was compared to that of calpains from other species. The expression of the three calpains in nine tissues was quantified by real-time RT-PCR.

Materials and Methods

Animals

Adult land crabs, *Gecarcinus lateralis*, were collected from San Miguel Reserve near Fajardo, Puerto Rico. They were kept in covered plastic cages with aspen bedding moistened with tap water and maintained at 27 °C, 50% humidity, and a 12 h dark/12 h light cycle. They were fed cat chow, carrots, and lettuce twice a week. All eight walking legs were autotomized in some animals to induce limb regeneration.

Cloning of calpains

Partial calpain cDNAs were initially obtained by nested RT-PCR using degenerate primers directed to highly conserved sequences in a wide variety of calpain proteins in the GenBank database (http://www.ncbi.nlm.nih.gov), including those from *C. elegans*

(NP502751), fruitfly (NP477047, AAD04331), rat (NP058813), and human (AAH08751). Conserved sequences were identified by aligning proteins using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html). Two sets of degenerate primers were designed to anneal to DNA sequences encoding LG(N/D/E)CW(L/F), EKA(Y/F)AK, or (G/R)HAY(T/S)(V/I) in the protease domain: CPN Forward 1, (G/A)II (C/T)T(A/G/T/C) GG(A/G/T/C) (G/A)A(A/G/T/C) TG(C/T) TGG; CPN; Forward 2 GA(G/A) AA(G/A) GC(A/G/T/C) (C/T)A(C/T) GC(A/G/T/C) AA(G/A); CPN Reverse 1, IA(C/T) (A/G/T/C)(G/C)(A/T) (G/A)TA (A/G/T/C)GC (G/A)TG IC); and CPN Reverse 2, (C/T)TT (A/G/T/C)GC (G/A)T(G/A) (A/G/T/C)GC (C/T)TT (C/T)TC. All the primers were synthesized and purified by Integrated DNA Technologies, Inc. (Des Moines, Iowa, USA).

Total RNA was isolated from primary limb regenerates using a Qiagen RNeasy mini kit. cDNA was synthesized according to the manufacturer's protocol using the SuperScript II RNase H-reverse transcriptase first strand synthesis system (Invitrogen, Inc.). Briefly, 12 μl of a mixture containing 1 μl oligo (dT)12-18 (500 μg/ml), 1 μg total RNA, and 1 μl 10 mM dNTPs was heated to 65 °C for 5 min and chilled on ice for 1 min. 5X First-Strand Buffer (4 μl), 2 μl 0.1 M DTT, and 1 μl RNaseOUT recombinant ribonuclease inhibitor (40 units/μl) were added and the mixture was incubated at 42 °C for 2 min. The reaction was initiated by the addition of 1 μl (200 units) of SuperScript II and incubated at 42 °C for 50 min. The reaction was inactivated by heating at 70 °C for 15 min. PCR was performed using an ABI 9600 thermal cycler (Perkin-Elmer, Inc.). The reactions contained 3 μl cDNA mixture, 3 μl 10X Takara EX Taq buffer, 2 μl 250 μM dNTPs, 1 μl CPN F1 primer, 1 μl CPN R1 primer, 0.2 μl Takara EX Taq DNA

polymerase (5 units/ μl), and 18.8 μl PCR grade deionized water. Initial denaturation (95 °C for 5 min) was followed by 35 amplifying cycles (95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec) and final extension at 72 °C for 7 min. For the second PCR reaction, 0.2 μl of the first PCR reaction was used, in conjunction with one of two primer pairs (CPN F1/CPNR2 and CPN F2/CPNR1), other reaction components and PCR conditions were same as those in first reaction.

The PCR products were separated with agarose (1.2%) gel electrophoresis and stained with ethidium bromide. The PCR products were purified from the gel slices using the Qiaquick Gel Extraction kit (Qiagen, Inc.), ligated into TA cloning vector with TOPO TA Cloning Kit (Invitrogen, Inc.), and transformed into One Shot TOP 10 *E. coli* strain (Invitrogen, Inc.). Transformants were first selected by blue-white colony selection on LB agar plates containing 50 μg/ml ampicillin (Sigma-Aldrich, Inc.) and subjected to PCR with T7 and M13-reverse vector primers to verify sizes of inserts. Plasmids were purified using the Qiagen spin mini prep kit and sequenced using T7 and M13-reverse vector primers (Davis Sequencing, Inc.). If needed, gene-specific primers were used to obtain the complete sequences of both strands.

RACE (Rapid Amplification of cDNA Ends) was used to obtain full-length mRNA sequences. For the 3' sequence, the Invitrogen 3' RACE System was used. Briefly, first-strand cDNA synthesis reactions contained 200 ng limb regenerate poly (A^+) RNA and adaptor primer (5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3'). First-round PCR on 20 ng cDNA included a universal amplification primer (5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3') and gene-specific forward primers (Table 2-1) at the following conditions: denaturation at 96 °C for 5 min, 35

amplification cycles (96 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 2 min), and extension at 72 °C for 10 min. Nested PCR on 30 μl of each reaction was conducted with gene-specific primers (Table 2-1) and abridged universal amplification primer (5'-GGCCACGCGTCGACTAGTAC-3' using the same conditions as the first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

SMART™ RACE cDNA Amplification Kit (BD Biosciences, Inc.) was used to obtain the 5' sequences of the three calpains. The first-strand cDNA synthesis reaction contained 3 μl poly(A+) RNA (50 ng), 1 μl 10 mM 5' CDS primer [5'-(T)25N-1N-3'], and 1 μl 10 mM SMART II A oligo (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') was incubated at 68 °C for 2 min. After chilling the reaction for 2 min on ice, 2 μl 5X First-Strand buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; and 30 mM $MgCl_2$), 1 μl 20 mM DTT, 1 μl 10 mM dNTPs , and 1 μl PowerScript Reverse Transcriptase were added. The reaction was covered with 20 μl paraffin oil and incubated at 42 °C for 1.5 h in a ABI 9600 thermal cycler (Perkin-Elmer, Inc.). The reaction mixture was diluted 10-fold with autoclaved distilled water and used for first-round PCR with 10X Universal Primer A Mix (0.4 mM 5'-CTAATACGACT CACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and 2 mM 5'-CTAATACGACTCACTATAGGGC-3') and gene-specific reverse primers (Table 2-1) under the following conditions: denaturation at 96 °C for 5 min, 35 amplification cycles (96 °C for 30 sec, 65 °C for 15 sec, 72 °C for 3 min) and extension at 72 °C for 10 min. Second-round PCR was conducted using nested gene-specific primers (Table 2-1) and nested universal primer A (10 mM, 5'-AAGCAGTGGTATCAACGCAGAGT-3').

The PCR conditions were the same as those used for first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Purified products were sequenced to confirm identities. Continuous sequences of three calpains were obtained by PCR using primer pairs to the start and stop codons of each sequence.

Calpain mRNA expression by RT-PCR

Total RNA was isolated from tissues (claw muscle, leg muscle, limb regenerate, thoracic muscle, gill, heart, hind gut, thoracic ganglion, X-organ, and Y-organ) using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Inc.). Total RNA (1 μg) was DNase-treated and reverse-transcribed using Superscript II RNase H-reverse transcriptase first strand synthesis system (Invitrogen, Inc.). All PCR reactions were performed in a Perkin Elmer 9600 GeneAmp thermal cycler using TaKaRa Ex Taq HotStart polymerase (Takara Inc.) and 2 μl of the first strand cDNA mixture as template. The PCR conditions were an initial denaturation at 95 °C for 4 min, 35 polymerization cycles (denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec), and final extension at 72 °C for 2 min. PCR reactions were analyzed by separating some or all of the 20 μl reaction volume on 2% agarose gels. Primers (Table 2-2) were designed using IDT BioTools program (http://biotools.idtdna.com/gateway).

Transcript levels were quantified by real-time PCR using a Cepheid Smart Cycler instrument and sequence-specific primers (Table 2-2). Elongation factor 2 (EF2), which was constitutively expressed, served as an internal control. The identities of each PCR product was confirmed with regular PCR. The PCR products were ligated into TOPO 2.1 vector using TOPO TA Cloning kit (Invitrogen Inc.,USA). Standard curves were

generated using serial dilutions (10 fg to10 ng) of plasmids containing either Gl-CalpM, Gl-CalpB, Gl-CalpT, or Gl-EF2 inserts (Yu and Mykles, 2003; Medler and Mykles, 2003). Reaction mixtures contained 2 µl LightCycler FastStart Reaction Mix (Roche;10 X buffer, Fast Taq DNA polymerase and dNTPs), 2 µl 25 mM $MgCl_2$, 2 µl cDNA template, 1 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl) and 12 µl PCR grade water. The PCR conditions were denaturation at 96 °C for 5 min, 40 polymerization cycles (96 °C for 20 sec, 65 °C for 15 sec and 72 °C for 30 sec), and melting curve detection (60 °C +0.2/sec). PCR products were evaluated by melting temperature analysis and separation on 2% agarose ethidium bromide-stained gels.

DNA molecular weights were calculated by the Schepartz lab biopolymer calculator (http://paris.chem.yale.edu/extinct.html) and copy numbers were calculated with the equation:

Copy number = (weight of the plasmid (g)/molecular weight of the plasmid (g/mol)) x 6.25 x 10^{23} (number/mol)

From the equation above 1 ng plasmid is equal to 4.6 x 10^8 in copy number when $\log^{-2}$ from the standard curve. In the standard curve the -Log value was converted into actual copy number using following equation:

Log copy number = $\text{Log}(10^{(-\log \text{ value at a critical cycle number})} \text{ x } (4.6 \text{ x } 10^8))/10^{-2})$

The Statview Program (SAS Institute, Inc.) was used for statistical analysis and each graph was made using the SigmaPlot program (SPSS, Inc.).

Results

Cloning of three calpain cDNAs

Nested PCR with degenerate primers generated products of the expected size from cDNA synthesized from primary limb regenerate mRNA. After cloning into a plasmid vector, 10 different clones were selected for sequencing. The calpain-like sequences encoding a part of the protease domain fell into 4 categories: two were similar to lobster CalpM (accession number: AY124009) and *Drosophila* CalpA (accession number NP477047) and CalpB (accession number NP524016); a third was similar to human Capn5 (accession number: CAA71584) and *C. elegans* TRA-3 (accession number NP502751); and the fourth was similar to *C. elegans* clp-1 (accession number NP741237). 3' and 5' RACE using nested sequence-specific primers yielded full-length sequences of three of the four initial sequences, designated Gl-CalpB, Gl-CalpM and Gl-CalpT (*G. lateralis* calpains B, M, and T). Expression of each calpain was confirmed by RT-PCR. However, the clp-1 like sequence was not amplified and not characterized further; from its amino acid sequence identity (83%), it may have resulted from contamination of the limb regenerate mRNA with mRNA from a parasitic nematode that occurs in some individuals. The full ORFs were confirmed by PCR using specific primer sets containing the start codon and stop codon sequences for each calpain.

Nucleotide and deduced amino acid sequences of Gl-CalpM are shown in Fig. 2-1. The ORF of the Gl-CalpM (1677 bp) encodes a protein of 558 amino acids with predicted mass of 65.23 kDa. The deduced amino acid sequence was most similar to that of Ha-CalpM (66% identity) and *Drosophila* calpain A (48% identity). It has conserved catalytic (domain II) and C2-like (domain III) domains, but lacks a C-terminal calmodulin-like

domain (domain IV) (Fig. 2-4). Comparison of the lobster and land crab sequences showed that Gl-CalpM lacks two acidic insertions found in Ha-CalpM: a 10 aspartate insertion in the acidic loop region of domain III and a DDSDD insertion near the end of domain II. A minor, alternatively-spliced variant of Gl-CalpM was obtained with 3' RACE; it is truncated 958 bp from the start codon, which results in the absence of the catalytic asparagine in domain II (Fig. 2-1).

Gl-CalpB cDNA (2740 bp) encoded a protein of 754 amino acids with a predicted mass of 88.89 kDa (Fig. 2-2). The deduced amino acid sequence is 61% identical to *Drosophila* calpain B, (accession number NP524016), 51% identical to Dm-CalpA (accession number NP477047) and 50% identical to human calpain 3 or p94 (accession number NP-775110.1). In addition to domains II and III, Gl-CalpB has a calmodulin-like domain (domain IV) containing 5 EF hand motifs (Fig. 2-6).

Gl-CalpT cDNA (2639 bp) encoded a protein of 639 amino acids with a predicted mass of 74.56 kDa (Fig.2-3). The amino acid sequence is 47% identical to human Capn5 (accession number O15484) and 41% identical to TRA-3 from *Caenorhabditis elegans* (accession number AAB60256). Gl-CalpT resembles other calpains from domain I to domain III, but domain IV is replaced with a T domain found in Capn5 and TRA-3 (Fig. 2-5)

Calpain structure analysis

Multiple amino sequence alignment showed high similarity in domains II and III in calpains from nematode (TRA-3), arthropods (Gl-CalpM, Gl-CalpB, Gl-CalpT, Dm-CalpA and Dm-CalpB) and mammals (calpain1 and calpain3) (Fig.2-4). All have

conserved catalytic triad (C, H, N) and two non-EF hand Ca^{2+} binding sites in domain II. A C2-like acidic sequence in domain III was shared between all calpains. The major difference between the two CalpM sequences was that Ha-CalpM had two acidic amino acid insertions, one (DDSDD) near to end of domain II and the other (DDDDDDDDDDRG) in the C2 acidic loop region, that Gl-CalpM did not. Unlike a muscle-specific mammalian Capn3, calpains from arthropods and nematode lacked an insertion sequence, IS1, in domain II. Domain IV, when present, was well conserved from arthropods to mammals (Fig. 2-6), although Dm-CalpA has an insertion sequence between EF-1 and EF-2 not found in any other calpain. The sequence of the T domain of Gl-CalpT was similar to that of other "T domain" calpains (Fig. 2-5).

To study the sequence relationships of the three land crab calpains, a phylogenetic analysis was done based on the deduced amino acid sequences of domains II and III of calpains from arthropods, nematode, and mammals (Fig. 2-10). TRA-3, Capn5, and Gl-CalpT clustered together as a distinct group; the T domains in these calpains have no sequence similarity with any other calpains and indicate that they have an independent origin from other calpains (31). Interestingly, the *Drosophila* genome had no TRA-3 homolog. Mammalian Capn1 and Capn3 were grouped together and were more closely related to calpains with an EF hand domain. Crustacean specific Ha-CalpM and Gl-CalpM are diverged from arthropod calpains such as Gl-CalpB and Dm-CalpA and B. Dm-calpains shows that they were recently diverged into Dm-CalpA and B.

Calpain mRNA expression

RT-PCR showed that most tissues expressed the three calpains, although the levels

of expression varied between tissues (Fig. 2-8). The levels in nine tissues were quantified by real-time PCR (Fig. 2-9). Gl-CalpM and CalpB mRNAs were expressed about 100-fold higher than Gl-CalpT in claw muscle and about 20-fold higher in thoracic muscle. There was not a significant difference in expression level between Gl-CalpB and CalpT both in thoracic and claw muscle. Although level of expression varies overall expression pattern between thoracic and claw muscle was similar. However, heart had a different expression pattern. Gl-CalpB was expressed about 5-fold higher than Gl-CalpM and about 2.6-fold higher than Gl-CalpT; there was no significant difference between Gl-CalpM and T. Gl-CalpB was more highly expressed than Gl-CalpM and Gl-CalpT in gill, digestive gland, and testis. Interestingly, testis and ovary differed significantly in expression pattern. Gl-CalpM was expressed in testis about 4.4-fold higher than Gl-CalpB and about10.5-fold higher than Gl-CalpT, while Gl-CalpB in ovary was expressed about 100-fold higher than Gl-CalpM and about 32-fold higher than Gl-CalpT. There was no significant difference in expression of the three calpains in integument.

Discussion

Compared with the work on mammalian calpains, very little is known about the properties and diversity of invertebrate calpains. However, completion of genome projects on *D. melanogaster* and *C. elegans* have accelerated our understanding of invertebrate calpain genes. *Drosophila* has four calpain genes (Dm-CalpA, B, C, and SOL). Both Dm-CalpA and B resemble typical calpains containing all four domains and are active Ca^{2+}-dependent proteases *in vitro* (Jekely and Friedrich, 1999). Dm-CalpC was initially identified by the genome project as CG3692 and predicted to have 9 transmembrane

domains in the N-terminal region. However, a recent study showed that Dm-CalpC actually has much shorter N-terminal domain that lacks membrane-spanning segments; it is catalytically inactive because all three catalytic amino acid residues are mutated (Spadoni et al., 2003). SOL (Small Optic Lobe) has a zinc-finger motif in the N-terminal region and the "SOL" domain in place of the calmodulin-like domain in C-terminal region. It has the conserved catalytic triad and is required for the development of the optic lobes (Delaney et al., 1991).

It is likely that all three crustacean calpains have Ca^{2+}-dependent protease activity. The deduced amino acid sequences of the protease domain are highly conserved, including the three amino acid residues essential for catalytic activity. In addition, the three calpains contain two well-conserved non-EF hand Ca^{2+} binding sites in domain II and a C2-like Ca^{2+}/phospholipids-binding site in domain III (Fig 2-4). In the absence of Ca^{2+} the catalytic residues are misaligned and the substrate binding cleft is disrupted; binding of Ca^{2+} to domains II and III activates the enzyme by driving the realignment of the active site residues (Strobl et al., 2000; Moldoveanu et al., 2002). The interaction of residues R104 and E333 in domain II of mammalian calpain provides cooperativity between the two Ca^{2+} binding sites (Moldoveanu et al., 2003); these two residues are conserved in the crustacean genes.

Gl-CalpB appears to be the only typical calpain in crustaceans, as it is the only land crab calpain with the C-terminal EF-hand domain IV (Fig. 2-4). It is expressed in all tissues and therefore likely has a housekeeping function. Sequence alignment analysis of domains II and III and its estimated mass (89 kDa) indicates that Gl-CalpB is a member of an arthropod calpain gene family. The Gl-CalpB amino acid sequence has the highest

sequence identity with Dm-CalpB and Dm-CalpA, although Dm-CalpA has an insertion sequence in domain IV and Dm-CalpB has a longer domain I sequence (Jekely and Friedrich, 1999). Gl-CalpB has no insertion sequence in domain IV, like Dm-CalpB, and a domain I length similar to that in Dm-CalpA.

Gl-CalpM from land crab has the highest structural similarity with Ha-CalpM, the first calpain cloned from a crustacean (Yu and Mykles, 2003). Both are truncated forms with similar estimated masses (~66 kDa). Unlike other truncated forms, crustacean CalpM does not appear to be produced by alternate splicing of a typical calpain gene. Dm-CalpA', for example, is an alternatively-spliced transcript of Dm-CalpA (Theopold et al., 1995). In mammals, nCL-2', which lacks domains III and IV, is produced by alternative splicing of the nCL-2 gene (Sorimachi et al., 1993). We were not successful in obtaining longer cDNAs containing the domain IV region using 3' RACE PCR with various sequence-specific primer sets. The absence of a longer mRNA is supported by immunoblot results using an antibody raised against a 28-amino acid N-terminal sequence of Ha-CalpM; only two proteins with masses of 62 and 68 kDa were detected (Yu and Mykles, 2003). A truncated alternately-spliced isoform of Gl-CalpM, Gl-CalpM', was isolated by 3' RACE. Gl-CalpM' is likely to be catalytically inactive, since the alternative transcript has a stop codon inserted before the Asn residue of the catalytic triad. As it is expressed at very low levels, its function, if any, is unknown.

Ha-CalpM was first identified as a muscle-specific calpain, because it is expressed at the highest levels in lobster skeletal muscles (Yu and Mykles, 2003). It might first appear that the tissue expression pattern of Gl-CalpM deviates significantly from that of Ha-CalpM. However, the apparent differences are much less, when one considers the

fiber-type compositions of the muscles. Real-time PCR shows that the levels of Ha-CalpM are about 4-fold higher in fast muscle than in slow muscle (Yu and Mykles, 2003). The claw closer and thoracic muscles in the land crab are composed of only slow fibers; no fast-type muscles have yet been identified in land crab. If one excludes the fast muscles, the ratios of Gl-CalpM expression in slow muscle in relationship with that in other tissues are similar to those of Ha-CalpM. Gl-CalpM was also highly expressed in ovary and integument. Unfortunately, the lobster ovary and integument were not analyzed for Ha-CalpM expression (Yu and Mykles, 2003).

The Gl-CalpT is a member of the "T domain" calpain family. Its amino acid sequence has the highest sequence identity with human Capn5 and nematode TRA-3. TRA-3 was first isolated as a sex determinant of the soma and germ line in hermaphrodites of *C. elegans* (Barnes and Hodgkin, 1996). TRA-2A is a substrate for TRA-3 and cleavage of TRA-2A by TRA-3 generates a peptide that has feminizing activity (Sokol and Kuwabara, 2000). Two mammalian calpains, Capn5 and Capn6, have the same structure as Gl-CalpT and TRA-3. However, the catalytic triad C, H, N in Capn6 is replaced by either K, H, N in human or K, Y, N in mouse, which suggests that Capn6 is catalytically inactive (Dear et al., 1997; Matena et al., 1998). Interestingly, the *Drosophila* genome apparently lacks a CalpT-like gene.

The tissue expression of CalpT in adult land crabs suggests that it has functions other than sex determination. Real-time PCR data showed that Gl-CalT is highly expressed in hind gut, heart, digestive gland, ovary, and integument. Human Capn5 is highly expressed in the colon, small intestine, and testis, while tissue specificity of the TRA-3 has not been reported in *C. elegans* (Mugita et al., 1997). In contrast, Gl-CalpT

was expressed highly in the hind gut and ovary, but its expression in testis was 17 times lower than in ovary (Fig. 2-9).

It is not known how the cDNAs encoding Gl-CalpB and Gl-CalpT correspond with three of the four calpain-like proteases characterized biochemically (Mykles and Skinner, 1986). Ha-CalpM encodes CDP III, based on similar masses and chromatography properties (Yu and Mykles, 2003). Gl-CalpB may encode CDP IIb, as the subunit mass (95 kDa) of the purified lobster CDP IIb (Mykles and Skinner, 1986) is similar to the estimated mass (89 kDa) of the deduced Gl-CalpB amino acid sequence. In addition, CDP IIb and the Dm-CalpA 95-kDa gene product share immunological properties. Polyclonal antibodies raised against lobster CDP IIb and Dm-CalpA protein cross-react, while a polyclonal antibody raised against a conserved 20-amino acid sequence around the cysteine residue in the active site of mammalian μ- and m-calpains (GATRTDICQGALGDCWLLAA) does not react with either Gl-CalpB or Dm-CalpA (Beyette et al., 1997). Analysis of this same sequence in Gl-CalpB (GATRFDVKQGELGDCWLLAA), indicates that three residues (Thr, Ile, and Ala) in mammalian calpains are replaced by Phe, Lys and Glu in Gl-CalpB. The replacement of two uncharged residues with two charged residues may explain why the antibody did not react with lobster CDP IIb.

The identity of Gl-CalpT with CDP I or IIa is less certain. The polyclonal antibody raised against the 20-residue mammalian active site sequence reacts with a 60-kDa protein in immunoblots of a partially-purified preparation of lobster CDP IIa. However, the deduced amino acid sequence of Gl-CalpT has an estimated mass of 74 kDa, significantly greater than the 60 kDa estimated from immunoblots. CDP I is not well

characterized. Its large native mass (310 kDa) suggests it is multimer of the proteins encoded by CalpB, CalpT, and/or an additional unidentified calpain gene. CalpM can be excluded, as the Ha-CalpM antibody did not react with any proteins in CDP I fractions eluting from a gel filtration column (Yu and Mykles, 2003). Further work is required to reconcile the CalpB and CalpT cDNAs with the calpain activities.

Table 2-1. Primers used in 3' and 5' RACE for cloning land crab calpains

NAME	SEQUENCE	TM(°C)	DESCRIPTION
cCalpM R1	5'-CCGTTGGACATGATGCTTTCTGG-3'	59.9	Gl-CalpM 5'RACE first round reverse primer
cCalpM R2	5'-TCCACCATACTCTCGTTGATGTTC-3'	58.0	Gl-CalpM 5'RACE nested reverse primer
cCalpM F1	5'-GGCGGGAACATCAACGAGAG-3'	59.0	Gl-CalpM 3'RACE first round forward primer
cCalpM F2	5-CTCGCTCATAGGTTGTGCCATCG-3'	61.2	Gl-CalpM 3'RACE nested forward primer
cCalpB R1	5'-CAGTAAAGTCCTCCATTGCCTCACAG-3'	60.9	Gl-CalpB 5'RACE first round reverse primer
cCalpB R2	5'-TTGGTCACTAGGCACTATTTGATAGAAG -3'	58.2	Gl-CalpB 5'RACE nested reverse primer
cCalpB F1	5'-GCTATGATGGATGTGGATCGCTC-3'	58.8	Gl-CalpB 3'RACE first round forward primer
cCalpB F2	5'-GATGACTTCATCATGTGCTCTGTG-3'	57.4	Gl-CalpB 3'RACE nested forward primer
cCalpT R1	5'-GAAGGTGAGGATGGTGCGTCTCAAGA -3'	63.9	Gl-CalpT 5'RACE first round reverse primer
cCalpT R2	5'-TATGTTGATTTTGCGGCAAGCGGTGATTC -3'	64.0	Gl-CalpT 5'RACE nested reverse primer
cCalpT F1	5'-GACGATTGGAGGCTATGTGGATGAC-3'	60.7	Gl-CalpT 3'RACE first round forward primer
cCalpT F2	5'-CTCTCTGATGTGCTGTGCCATAACTCC-3'	62.6	Gl-CalpT 3'RACE nested forward primer

Table 2-2. Primers used for quantifying calpain and EF2 mRNAs by real time PCR.

Name	Sequence	TM(°C)	Description
RcEF2 F1	5'-TTC TAT GCC TTT GGC CGT GTC TTC TC-3'	62.6	elongation factor 2 forward primer
RcEF2 R1	5'-TGA TGG TGC CCG TCT TAA CCA GAT AC-3'	62.1	elongation factor 2 reverse primer
RcCalpM F1	5'-GGC GGC TGC AGG AAT TAC ATT AAC AC-3'	62.2	Calpain M forward primer
RcCalpM R1	5-'TGT ACC TGA AGA AAT CGA CGT CCA GC-3'	62.2	Calpain M reverse primer
RcCalpB F1	5'-GTT CAA CTT TGA GGG CTT CAG CAA GG-3'	62.2	Calpain B forward primer
RcCalpB R1	5'-GAT AAC CAG CAG AGT TGA GGG CTT GA-3'	62.3	Calpain B reverse primer
RcCalpT F1	5'-TCT CTG ATG TGC TGT GCC ATA ACT CC-3'	62.3	Calpain T forward primer
RcCalpT R1	5'-TGA TGC AGA GAC CTG TGA CCA TTC TG-3'	62.2	Calpain T reverse primer

Figure 2-1. The complete sequence of Calpain M (Gl-CalpM) cDNA from land crab. The sequence (2531 bp) contained a full open reading frame (220 – 1896 bp) encoding a protein of 558 amino acids with predicted mass of 65.23 kDa. Locations of degenerate primer sites for initial nested RT-PCR are underlined. Three amino acid residues comprising the conserved catalytic triad (C, H, N) are in bold. Asterisk indicates the stop codon. The poly(A) signal is in bold and underlined. Arrow indicates the alternative splicing site that produces a truncated isoform.

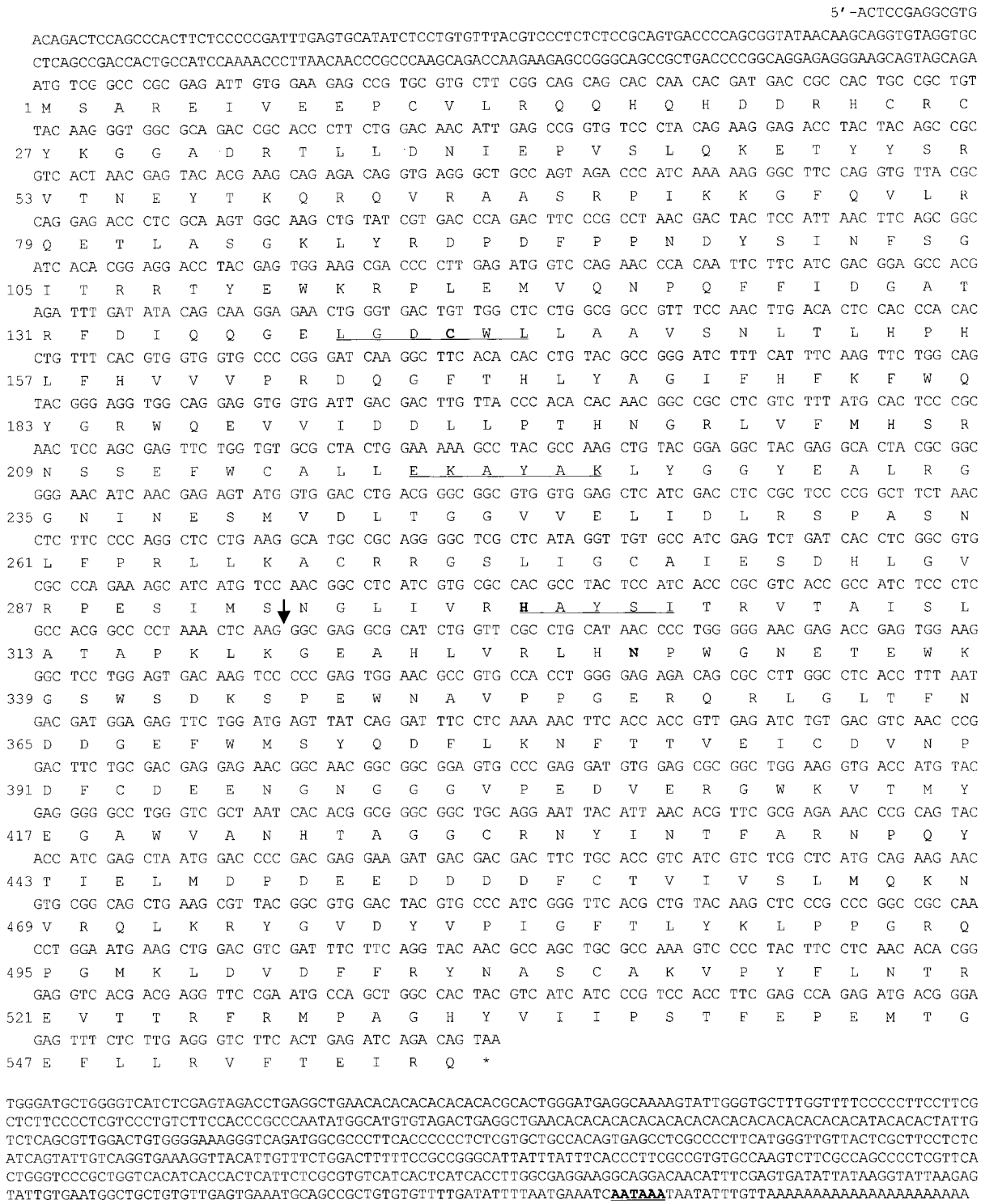


Figure 2-2. The complete sequence of Calpain B (Gl-CalpB) cDNA from land crab. The sequence (2740 bp) contained a full open reading frame (167 – 2431 bp) encoding a protein of 754 amino acids with predicted mass of 88.89 kDa. Locations of degenerate primer sites for initial nested RT-PCR are underlined. Three amino acid residues comprising the conserved catalytic triad (C, H, N) are in bold. Asterisk indicates the stop codon. The poly(A) signal is in bold and underlined.

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AGTTCTTCCTCAACTACTGCGCGTTCAAGTAATAAACCGCTGAAACCGAACACCAGAGACGCTTCGTTAATAACTGGAGTGTTTGCCCAAAGAAGACAACAACG
CCCCTGCACAAACGAACGCCAAGTAACCCCTTGAACACAGACGAGACAGCCAAACCCCGACG
   ATG GAC GAA GAG GAG GCG CAG TAC AAC GAA GAG GAG GAG CAG GAG GAG GAG AAC GAA GAG GGA GAG GAA GAG GAG GTC
 1 M   D   E   E   E   A   Q   Y   N   E   E   E   E   Q   E   E   E   N   E   E   G   E   E   E   E   V
   GAT GAG TCA TAC GAT CGT GTA GAC AAT CCC ATT GGA GAC GAT ACT ATA GAA AAG ACA CTC TAT GAC GAT GAG GGA AAT
27 D   E   S   Y   D   R   V   D   N   P   I   G   D   D   T   I   E   K   T   L   Y   D   D   E   G   N
   GAG ATG TTC TTC GTT TTC GAT AAG TTC TAC ATG TTT GGG GAG CGT GGA TCA GGC CTT CGT CCC CGC GGC CAG GTG CAG
53 E   M   F   F   V   F   D   K   F   Y   M   F   G   E   R   G   S   G   L   R   P   R   G   Q   V   Q
   GAT TTC TAT GAG CTA CGG CAA CAG TGT CTT GAT AAT GGC ACA CTC TTC GAG GAC CCT GAC TTC CCG GCT GAA GAC ACC
79 D   F   Y   E   L   R   Q   Q   C   L   D   N   G   T   L   F   E   D   P   D   F   P   A   E   D   T
   TCC ATC TTC TTC TCT CGC AGC CCA CCT AAG CCT TTC GAG TGG AAG AGG CCT CAT GAA ATC ACA GAT GAG CCA CAA CTT
105 S   I   F   F   S   R   S   P   P   K   P   F   E   W   K   R   P   H   E   I   T   D   E   P   Q   L
   TTT ATT GAT GGA GCC ACT CGC TTT GAT GTC AAG CAA GGA GAG CTG GGT GAC TGT TGG CTG CTG GCT GCT GTG GCA AAC
131 F   I   D   G   A   T   R   F   D   V   K   Q   G   E   L   G   D   C   W   L   L   A   A   V   A   N
   CTA ACC CTC AAC CGT CGC CTC TTC TAT CAA ATA GTG CCT AGT GAC CAA GGC TTT GGA GAT AAC TAT GCT GGC ATC TTC
157 L   T   L   N   R   R   L   F   Y   Q   I   V   P   S   D   Q   G   F   G   D   N   Y   A   G   I   F
   CAC TTC AGG TTT TGG CAG TAT GGC CGC TGG GTA GAT GTT GTG GTG GAT GAC CGA CTT CCC ACC TTC TAT GGC CGA CTG
183 H   F   R   F   W   Q   Y   G   R   W   V   D   V   V   V   D   D   R   L   P   T   F   Y   G   R   L
   GTC TTC ATG CAC TCA GAG GAG AAG AAT GAG TTC TGG TCT GCC CTA GCA GAG AAG GCA TAT GCC AAG TTG CAT GGA TCA
209 V   F   M   H   S   E   E   K   N   E   F   W   S   A   L   A   E   K   A   Y   A   K   L   H   G   S
   TAT GAG GCA CTG AAG GGA GGC ACC ACC TGT GAG GCA ATG GAG GAC TTT ACT GGC GGT GTG TCT GAG ATC TAT GAC CTT
235 Y   E   A   L   K   G   G   T   T   C   E   A   M   E   D   F   T   G   G   V   S   E   I   Y   D   L
   ACT AAG GCT CCC CCG AAC CTG TTC AAC ATC ATG CTG AAG GCC TAC CAG AGA GGC TCC CTG ATG GGC TGC TCT ATT GAT
261 T   K   A   P   P   N   L   F   N   I   M   L   K   A   Y   Q   R   G   S   L   M   G   C   S   I   D
   CCA GAC CCC AAT GTG GTG GAG GCA CGC TGT GAC AAT GGT CTG ATT CGT GGT CAT GCA TAC TCC ATC ACC CGC ATC AAG
287 P   D   P   N   V   V   E   A   R   C   D   N   G   L   I   R   G   H   A   Y   S   I   T   R   I   K
   TAC TGT GAC ATT GAG ACA CCT AGG GTT TCT GGT AAG ATC CCC CTG GTC CGC ATC CGA AAT CCT TGG GGC AAT GAG GCT
313 Y   C   D   I   E   T   P   R   V   S   G   K   I   P   L   V   R   I   R   N   P   W   G   N   E   A
   GAG TGG GTT GGC TCT TGG AGT GAC AAA AGT CCT GAG TGG CAG TTC ATT CCT CCT GAG GAG AAA GAA GAG ATG GGC CTC
339 E   W   V   G   S   W   S   D   K   S   P   E   W   Q   F   I   P   P   E   E   K   E   E   M   G   L
   ACC TTT GAA CAT GAT GGA GAG TTC TGG ATG TCC TTC AAG GAC TTC CTT ACC AAT TTC ACC ATG CTG GAG ATG ACC AAC
365 T   F   E   H   D   G   E   F   W   M   S   F   K   D   F   L   T   N   F   T   M   L   E   M   T   N
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417 A   W   I   R   G   S   T   A   G   G   C   R   N   F   L   D   S   F   W   H   N   P   Q   Y   R   I
   ACC CTG AGT GAG GTG GAT GAT GAT GAT GAT GAC AAC AAA TGC ACA GTG ATT GTG GCA CTG ATG CAG AAG AAT CGC CGT
443 T   L   S   E   V   D   D   D   D   D   D   N   K   C   T   V   I   V   A   L   M   Q   K   N   R   R
   TCA CAA AGG AAG CTT GGC CTG GAG TGC CTT ACT ATT GGT TTT GCA ATT TAT CAT TTG CGA GAC CCA GAC AGT GTT CCT
469 S   Q   R   K   L   G   L   E   C   L   T   I   G   F   A   I   Y   H   L   R   D   P   D   S   V   P
   CGG CCA CTG GAC CTG AAC TTC TTC AAG TAC TCA GCA TCA GTT GCT CGC TCC CCA TCT TTC ATC AAC ATG AGG GAG GTT
495 R   P   L   D   L   N   F   F   K   Y   S   A   S   V   A   R   S   P   S   F   I   N   M   R   E   V
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521 S   C   R   F   K   L   P   P   G   T   Y   C   I   V   P   S   T   F   E   P   N   E   D   G   E   F
   ATT TTG AGA GTC TTC TCT GAA AAA GCT AAT GAG ATG GAA GAA AAT GAT GAA GAT GTA GGC TTC GGA CAG GTG GAT GAC
547 I   L   R   V   F   S   E   K   A   N   E   M   E   E   N   D   E   D   V   G   F   G   Q   V   D   D
   AGG GTC CGA CCT GAG GAT GAT GCA CAG GAA GTG GAG GCT GAT GAG AGG ATC AAC GCC TTC TTC AGG AAA GTG GCT GGC
573 R   V   R   P   E   D   D   A   Q   E   V   E   A   D   E   R   I   N   A   F   F   R   K   V   A   G
   GAT GAC CTG GAG ATT GAC TGG AAG GAG CTG CAG GAT GTC CTC AAC TTT GCC CTG AAA AGA GAG TTC AAC TTT GAG GGC
599 D   D   L   E   I   D   W   K   E   L   Q   D   V   L   N   F   A   L   K   R   E   F   N   F   E   G
   TTC AGC AAG GAT GTA TGT CGC AGC ATG ATT GCT ATG ATG GAT GTG GAT CGC TCA GGA AAG CTG GGC CTC CAA GAA TTC
625 F   S   K   D   V   C   R   S   M   I   A   M   M   D   V   D   R   S   G   K   L   G   L   Q   E   F
   CTG CAG TTG TGG ATG GAT ATC AGA GTG TGG AAG AAT GCC TTC AAG CTA TAT GAC AAG GAC AGC TCT GGC CAA CTG TGT
651 L   Q   L   W   M   D   I   R   V   W   K   N   A   F   K   L   Y   D   K   D   S   S   G   Q   L   C
   TCA TTT GAA CTG CGT CAA GCC CTC AAC TCT GCT GGT TAT CGC CTC AAC AAC CAT ATT TGT GAC GCC CTC ATG CTC CGC
677 S   F   E   L   R   Q   A   L   N   S   A   G   Y   R   L   N   N   H   I   C   D   A   L   M   L   R
   TAT GGG GAT CGG GAT GGC AAA GTG TCA TTT GAT GAC TTC ATC ATG TGC TCT GTG AAG CTG AAA ACC ATG ATG GAA ATC
703 Y   G   D   R   D   G   K   V   S   F   D   D   F   I   M   C   S   V   K   L   K   T   M   M   E   I
   TTC CAG GAG AGG GAC CCC GAC AGG ACT ACG AAG GCC ACC TTC AGC CTT GAG GAG TGG GTA GAA AAC ACT ATG TAC TCA
729 F   Q   E   R   D   P   D   R   T   T   K   A   T   F   S   L   E   E   W   V   E   N   T   M   Y   S
   TAG
    *

AACTACATCAGCTTATTTACTGTTGAGCTAAATTTAATTAGCTTTACTATAATGAAAGAATCTTTATAATTATGTATGGTGATTTTTTGGGAGTTTTGCCTAATT
GCATATTCAGACTTGTGTTGTGAAATGATGATGTCTTTTAATGCCAAATTTAAGGGCCCATTCTGTTAGGCTTATCATTGAACTGTGCTCTTACATTTTAGCCAT
GGTCTAAGTAAAATAATTACTGGATCAGGCTAATGTTGAAAATGCAGTGTTTCACTATACAAACCCGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 2-3. The complete sequence of Calpain T (Gl-CalpT) cDNA from land crab. The sequence (2639 bp) contained a full open reading frame (304 – 2223 bp) encoding a protein of 639 amino acids with predicted mass of 74.56 kDa. Locations of degenerate primer sites for initial nested RT-PCR are underlined. Three amino acid residues comprising the conserved catalytic triad (C, H, N) are in bold. Asterisk indicates the stop codon. The poly(A) signal is in bold and underlined.

```
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   GAGAGCAAGGCAACACCACACACCCTCGAAAGGACAGGCAACAAGCAGGTGGAAGTGTGGAGTTGTGAGGATAATGTACAGGTAGCTTAAACTTGTTCACA
  ATG GGT CTG TTC AGC TCC ACC AAG AAC TTC CGC GGC CAA GAC TAC GCC AAA CTG AAG AAG GAT TGT CTC CAT AGA GGA
1 M  G  L  F  S  S  T  K  N  F  R  G  Q  D  Y  A  K  L  K  K  D  C  L  H  R  G
  GAA AAG TTC AGT GAT CCC AAG TTC CCT CCA CGT GAC TCC TCA TTG TAC TTC TCA AAG CAG CCA CCT GGA GTT GTC ACC
27 E  K  F  S  D  P  K  F  P  P  R  D  S  S  L  Y  F  S  K  Q  P  P  G  V  V  T
  TGG AAG AGG CCT CAT GAA ATA TTA GAC AAG CCT CAG CTC TTC ATT GAG GGT GCA AGT GCC AAA GAT GTG ACT CAA GGT
53 W  K  R  P  H  E  I  L  D  K  P  Q  L  F  I  E  G  A  S  A  K  D  V  T  Q  G
  CAG CTA GGC AAC TGC TGG TTT GTT GCA GCA TGT GCC ACT CTT GCT GGT GTC AAG GAA CTC TGG CAC AAG GTC ATC CCA
79 Q  L  G  N  C  W  F  V  A  A  C  A  T  L  A  G  V  K  E  L  W  H  K  V  I  P
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105 D  Y  K  D  Q  E  Y  G  D  L  H  P  G  I  F  H  F  R  F  W  R  F  G  E  W  V
  GAG GTG GTG GTG GAT GAC CTG CTT CCC ACC ATT GAG GGG CAG CTT ATC TTC ACT CAC TCT AAG GAG AGA GGA GAA TTC
131 E  V  V  V  D  D  L  L  P  T  I  E  G  Q  L  I  F  T  H  S  K  E  R  G  E  F
  TGG TGT GCC TTA CTG GAG AAA GCT TAT GCC AAA CTT TAT GGC TCA TAT GAA GCA CTT GAG GGA GGT AAT CTC AGT GAT
157 W  C  A  L  L  E  K  A  Y  A  K  L  Y  G  S  Y  E  A  L  E  G  G  N  L  S  D
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183 A  L  V  D  L  T  S  G  V  S  A  H  L  D  L  T  I  G  G  Y  V  D  D  F  E  K
  CGG AAG CAA CTA TTC AAA ATG ATG TCC AAG GAA ATG AAT GAA CAC TCT CTG ATG TGC TGT GCC ATA ACT CCA CAC AGC
209 R  K  Q  L  F  K  M  M  S  K  E  M  N  E  H  S  L  M  C  C  A  I  T  P  H  S
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235 H  E  E  A  E  M  R  T  N  V  G  L  V  K  G  H  A  Y  G  I  T  A  C  R  K  I
  AAC ATA GGA GAT ACA GGC CTC TTC TCC ATC TTC AAG GGC GCC CAG AAG GTG AGG ATG GTG CGT CTC AAG AAC CCT TGG
261 N  I  G  D  T  G  L  F  S  I  F  K  G  A  Q  K  V  R  M  V  R  L  K  N  P  W
  GGA GAG AAA GAG TGG AAT GGG GCC TTC AGT GAT GGA TCA CCA GAA TGG TCA CAG GTC TCT GCA TCA GAG CGA CAG AAG
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313 L  G  L  T  F  E  D  D  G  E  F  W  M  T  F  E  D  F  L  E  H  F  T  D  L  S
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469 V  I  I  P  T  T  F  K  P  D  E  T  G  E  F  L  L  R  I  F  T  S  K  D  P  D
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495 A  K  E  P  I  R  D  Q  P  K  S  P  W  Y  S  C  F  K  K  A  V  M  V  T  T  I
  ACT GTG AAG TGT GCC AGT AAC TTG GAG AAA CAA TCT GCA TTT GGA GGT GAT GCT GAT GCA TAT TGC ATC ATA AAG TGC
521 T  V  K  C  A  S  N  L  E  K  Q  S  A  F  G  G  D  A  D  A  Y  C  I  I  K  C
  GGG GGA GAA ACT GTT CGG ACA CCA GTG AAA GGA CAG CAA TCC TAT ATG ACA CCA CAG CCA TTT TAC CGT GCT AAG CCT
547 G  G  E  T  V  R  T  P  V  K  G  Q  Q  S  Y  M  T  P  Q  P  F  Y  R  A  K  P
  GAA CAG CCC ATT GTG ATT GAA ATC TGG AAC AGC AAC ATG TTA GTG GAT GGC TTC ATT GGG CGA GCT GAG GTG ACT GCA
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  CCT ATC AAT CCA AAC TAT ACC CAG GTT CAA CTT TCA CTG TAT GGC AGA CGC AAG GAG AAA ACT GTA GAG AAA CAA GGG
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625 H  L  L  V  Q  V  Y  S  D  D  D  L  T  S  I  *
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  GCAAGTGCAAACCATTTTCCGATTTTAACTATAAAAGGTATCCAAGACATTAAAAACAAGGTTAAATAAAGCAATACTGTAAAAAAAAAAAAAAAAAA-3'
```

Figure 2-4. Comparison of the deduced amino acid sequences in domains II and III of calpains from arthropods, nematode, and human. Amino acid sequences were aligned using ClustalW (see Materials and Methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical between all the sequences are highlighted in black. Asterisks indicate residues of the catalytic triad (C, H, N). Open inverted triangles indicate conserved residues involved in Ca^{2+} binding. Roman numerals indicate boundaries between domains. Arrows indicate locations of two acidic amino acid expansions found in lobster Ha-CalpM, but not in Gl-CalpM; one (DDSDD) is positioned near to end of domain II and the other (DDDDDDDDDDRG) is located in the acidic loop region in domain III. A unique insertion sequence in Calpain 3 (IS1) is boxed. The end of a truncated Gl-CalpM isoform (Gl-CalpM') produced by alternative splicing is indicated by arrow. Accession numbers: Calpain 5, JC5772; TRA-3, NP502751; Dm-CalpA, NP477047; Dm-CalpB, NP524016; Calpain 1, AAH08751; Calpain 3, NP058813; and Ha-CalpM, AAM88579.

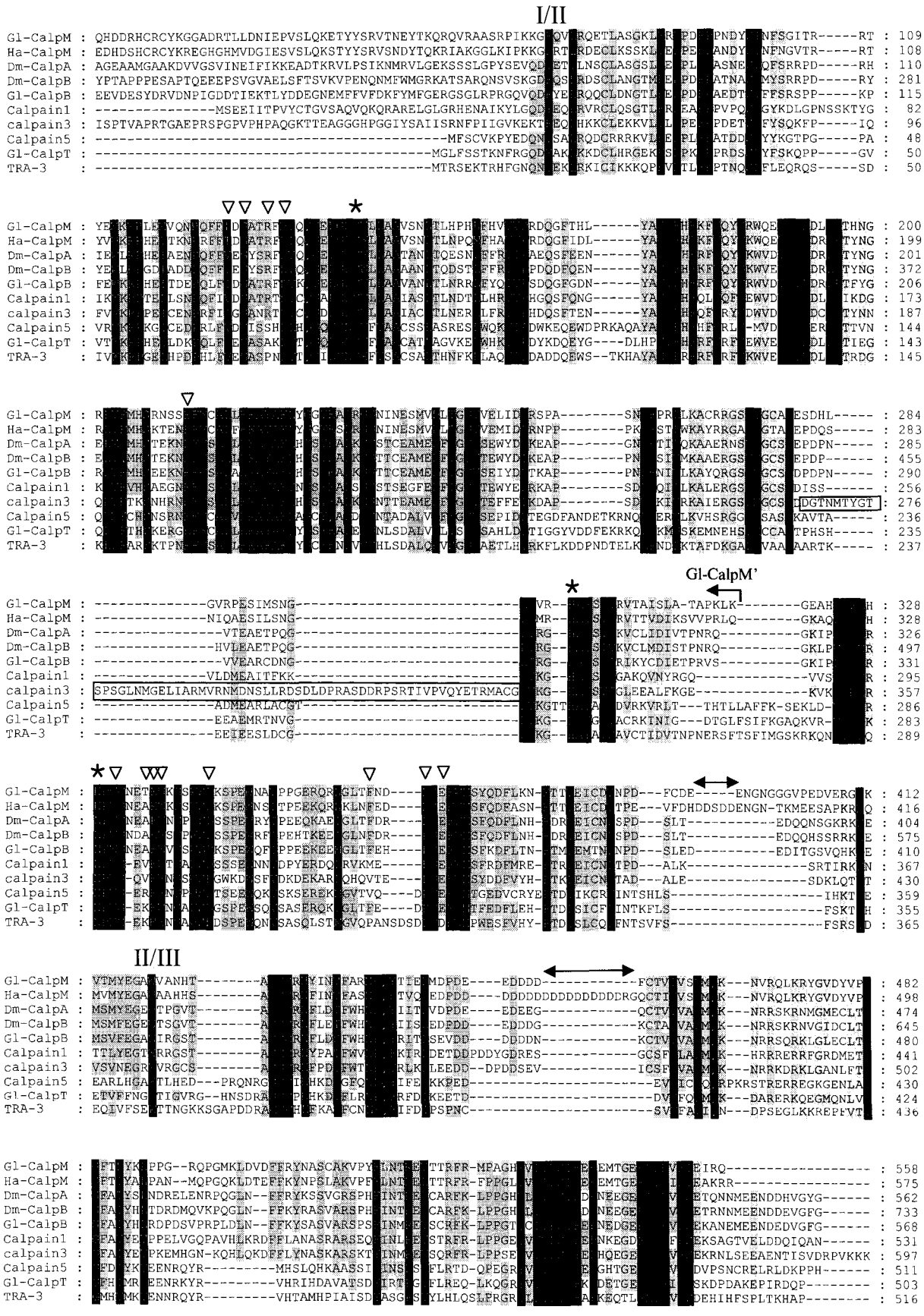


Figure 2-5. Comparison of amino acid sequences of the domain T in Calpain T cDNAs from land crab (Gl-CalpT), nematode (TRA-3), and human (calpain 5). Deduced amino acid sequences were aligned using ClustalW (see Materials and Methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical between all the sequences are highlighted in black. Accession numbers: Calpain 5, JC5772 and TRA-3, NP502751.

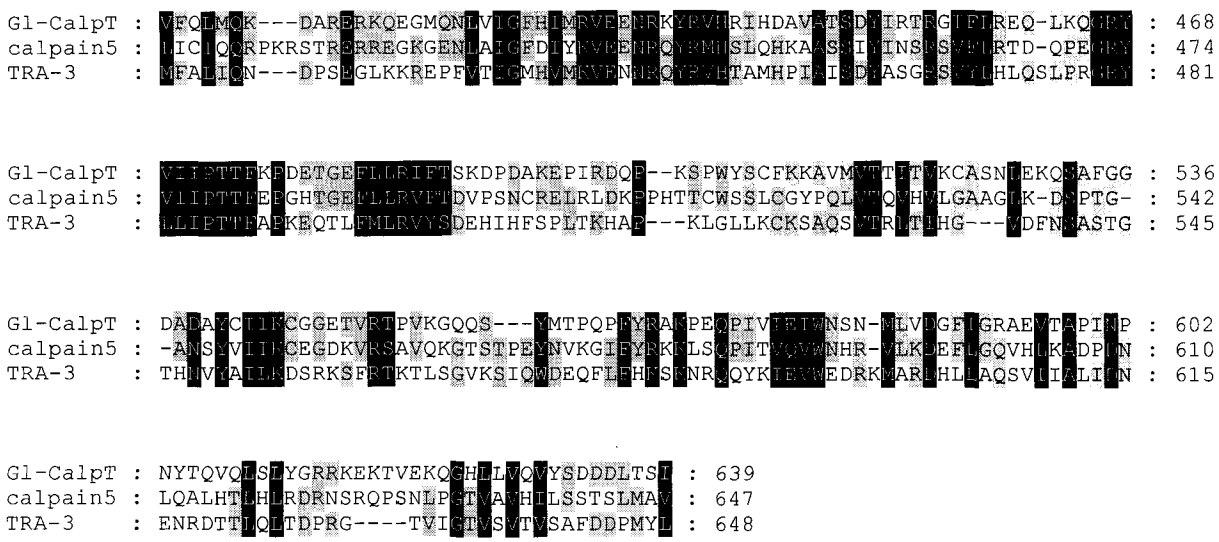


Figure 2-6. Comparison of amino acid sequences in domain IV of calpains from arthropods and human. Deduced amino acid sequences were aligned using ClustalW (see Materials and Methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical between all the sequences are highlighted in black. Domain IV is calmodulin-like domain that contains five EF hand motifs (arrows). Accession numbers: Dm-CalpA, NP477047; Dm-CalpB, NP524016; Calpain 1, AAH08751; and Calpain 3, NP058813.

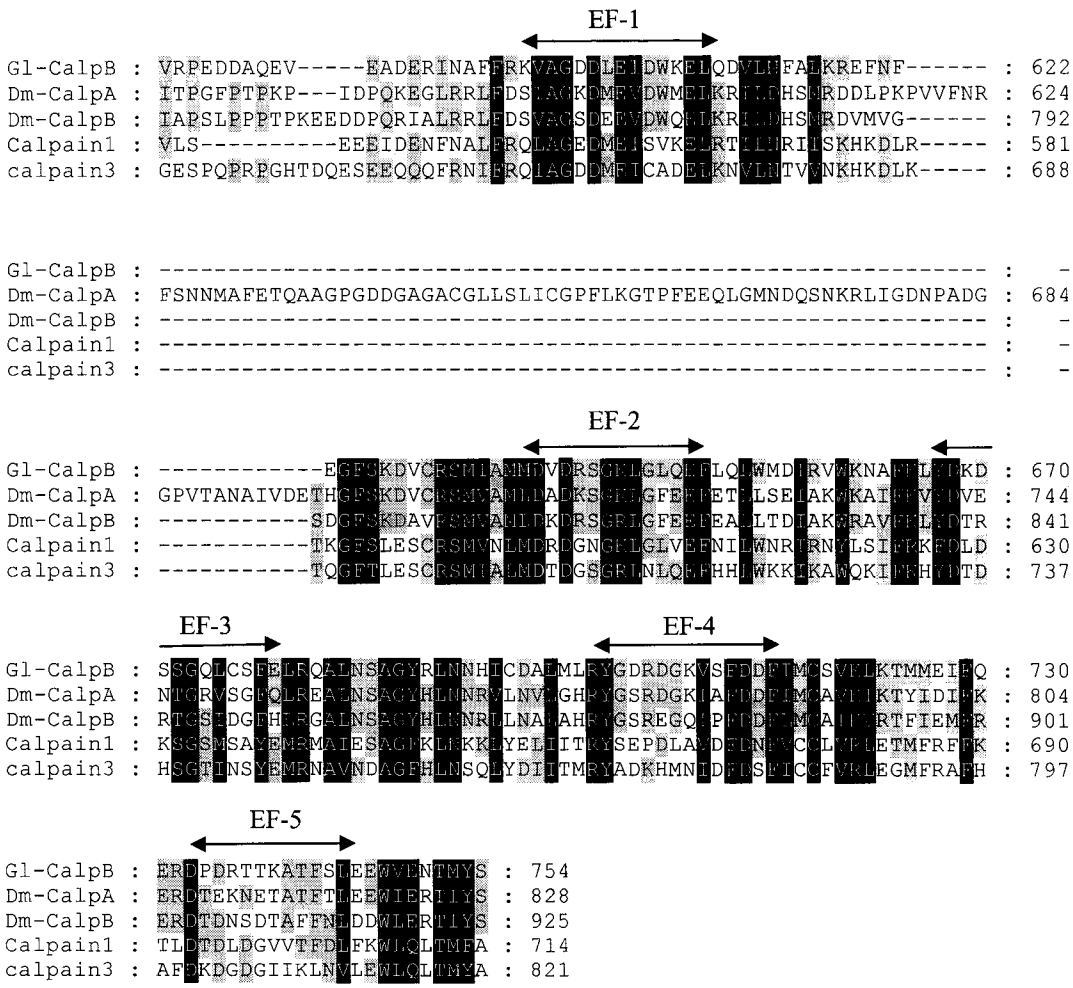


Figure 2-7. Comparison of domain organization in calpains from arthropods, nematode, and mammals. Calpains from land crab (Gl-CalpM, -CalpB and -CalpT), *Drosophila melanogaster* (Dm-CalpA and CalpB), *Caenorhabditis elegans* (TRA-3), and human (Capn5 or nCL-3) are depicted. All calpains share conserved proteolytic (domain II) and C2-like (domain III) domains. The N-terminal domain I varies in sequence and length between different calpains. Calpains differ in the N-terminal region. "Typical" calpains have a calmodulin-like domain (domain IV) containing five EF-hand motifs. "Atypical" calpains either lack a N-terminal domain or have domain IV replaced with a T domain. Numbers indicate amino acid residues from the N terminus.

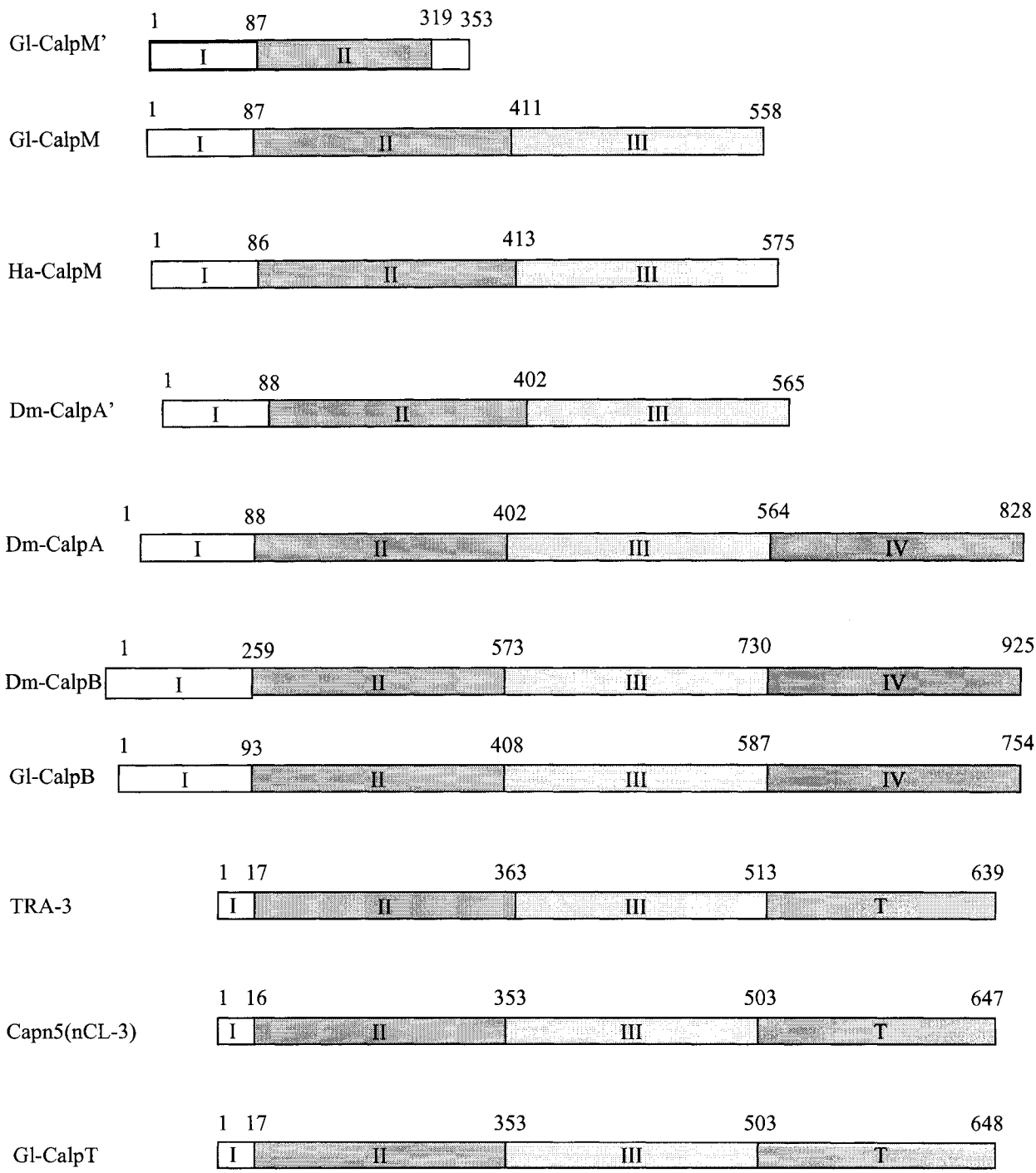


Figure 2-8. Tissue expression of land crab calpain mRNAs using RT-PCR. Total RNA from each tissue was DNase-treated, reverse-transcribed, and PCR-amplified using primers specific for Calpains M, B, and T. Shown is a reversed image of an ethidium bromide-stained agarose gel of PCR products. Lane 1, claw muscle; 2, leg muscle; 3, limb regenerate; 4. thoracic muscle; 5, gill; 6. heart; 7, hind gut; 8. thoracic ganglion; 9, X-organ; and 10, Y-organ. M, DNA size markers.

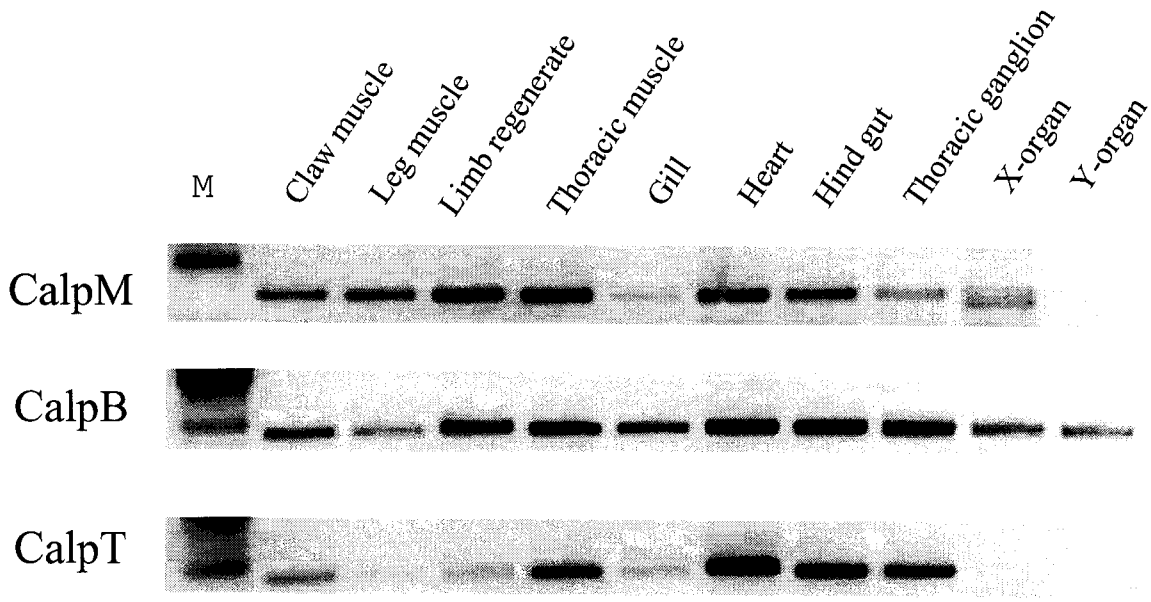


Figure 2-9. Quantification of calpain mRNAs in tissues from intermolt land crabs using real-time PCR. Transcript levels, expressed as Log copy number, were determined in nine tissues. The elongation factor 2 (EF2) mRNA served as an internal standard to optimize the PCR reactions. P-values between means for each calpain within each tissue are given at the top of each graph (see Materials and Methods for statistical analysis). There were three expression patterns. In skeletal muscle, CalpM and CalpB were expressed at similar levels, which were expressed at least an order of magnitude higher than CalpT. In heart, gill, thoracic ganglion, digestive gland, and testis, CalpB was expressed at higher levels than CalpM and CalpT. In ovary and integument, CalpM was expressed at higher levels than CalpB, which was expressed at higher levels than CalpT.

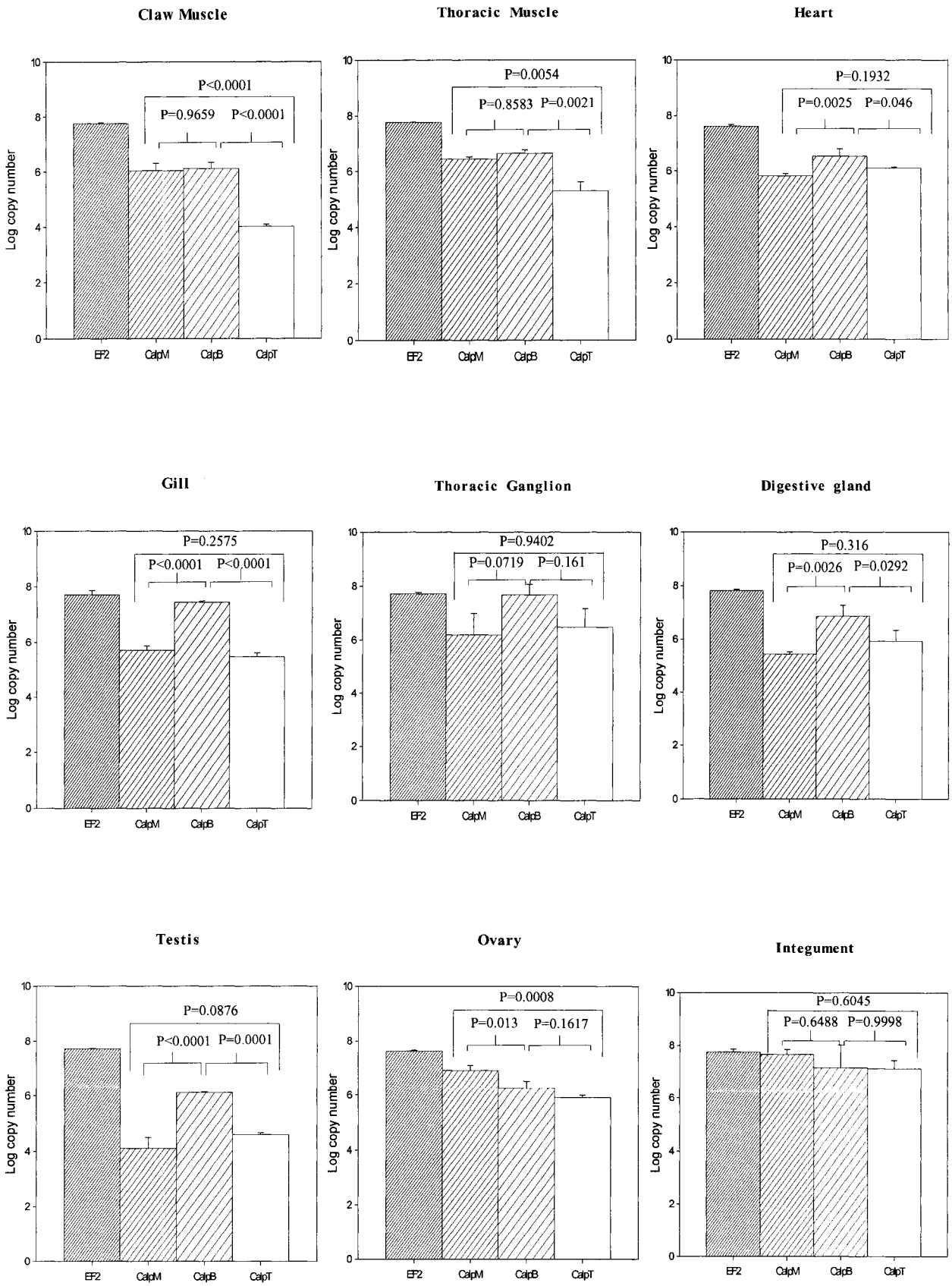
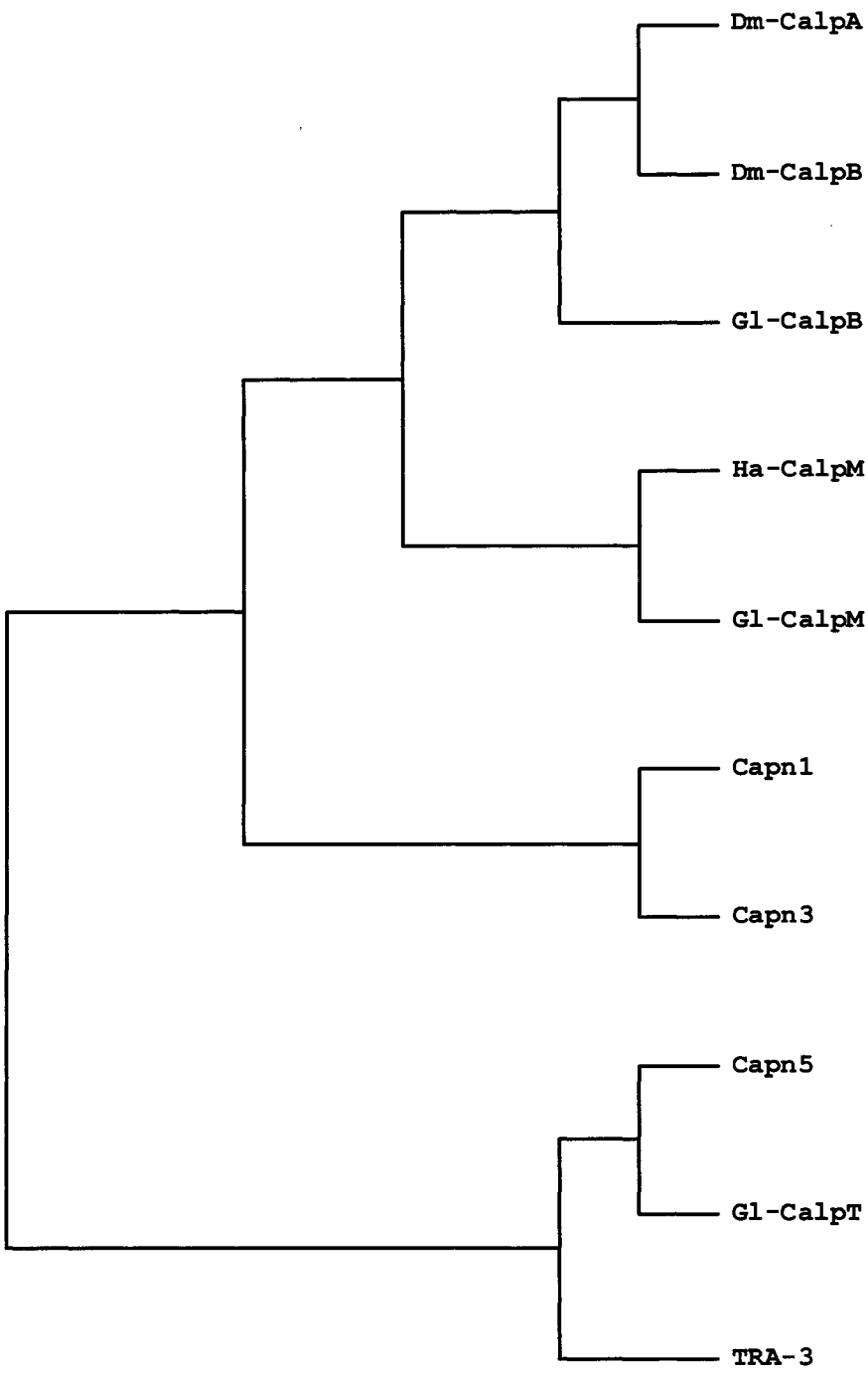


Figure 2-10. Phylogenetic relationships of selected calpains from arthropods, nematode, and mammals. The deduced amino acid sequences of the proteolytic domain (domain II) in land crab (Gl-CalpM, -CalpB and -CalpT), lobster (Ha-CalpM), fruit fly (Dm-CalpA and -CalpB), nematode (TRA-3), and human (Calp5) calpains were analyzed using ClustalW and Treeview software (see Materials and Methods). The calpains group into four subfamilies: T-like calpains (TRA-3, Gl-CalpT, and Capn5), mammalian "typical" calpains (Capn1 and Capn3), crustacean M calpains (Gl-CalpM and Ha-CalpM), and arthropod A/B calpains (Gl-CalpB, Dm-CalpA, and Dm-CalpB).



CHAPTER THREE

ECDYSTEROID RELATED GENES AND CALPAIN EXPRESSION

Abstract

Increasing levels of the molting steroid hormone, 20-hydroxyecdysone (20E), may induce molt-induced claw muscle atrophy. Using PCR with degenerate primer sets partial cDNA sequences encoding ecdysone receptor (Gl-EcR; 905 bp) and full length cDNAs encoding retinoid receptor (Gl-RXR;1515 bp) and E75 ecdysone response gene (Gl-E75; 3258 bp) were cloned from the land crab (*Gecarcinus lateralis*). The deduced amino acid sequence of Gl-EcR had 93% identity with UpEcR from fiddler crab (*Uca pugilator*) and 66% identity with LmEcR from locust (*Locusta migratoria*). Gl-RXR had 95% identity with UpRXR and occurred as 7 alternatively-spliced isoforms. A Gl-RXR isoform, which lacked a 5 amino acid (GAVEG) insertion, was expressed preferentially in claw muscle. Two truncated Gl-RXR isoforms without the E domain were also expressed highly in both claw and thoracic muscles. Gl-E75 had highest sequence identity with MeE75 from a shrimp (*Metapenaeus ensis*). Based on its expression pattern and structural similarity, Gl-E75 may be a homolog of DmE75A from *Drosophila melanogaster*. The F domain in Gl-E75 was longer than that in MeE75 and had some consensus sequences with DmE75, while MeE75 did not. These data suggest that the F domain may have a similar function with that of insects. At 1 day after eyestalk ablation, Gl-EcR and Gl-CalpT mRNA increased significantly in claw muscle not in thoracic muscle. This suggests that sensitivity for ecdysone may be different between the two skeletal muscles. At 3 days

after eyestalk ablation, Gl-EcR and Gl-CalpT mRNA decreased in claw muscle suggesting some other mechanism can inhibit ecdysteroidogenesis without MIH from eyestalk ganglia. Gl-CalpB mRNA level increased in limb regenerates while Gl-EcR expression was not significantly upregulated. In both eyestalk ablation and limb regenerate experiments, Gl-CalpT and Gl-EcR mRNA level were significantly correlated, suggesting that Gl-CalpT is directly regulated by ecdysteroids.

Introduction

Molt-induced muscle atrophy is specific to the claw muscle (Schmiege et al., 1992). Muscle in the walking legs, which lack an enlarged distal segment, does not atrophy (Mykles and Skinner, 1982). Atrophy is coincident with increasing concentrations of ecdysteroids in the hemolymph. In the large claw muscles of fiddler crab (*Uca pugilator*), ecdysone receptor (UpEcR) mRNA level is increased significantly in $D_{1\text{-}4}$ stage (Chung et al., 1998). These results suggest that 20-hydroxyecdysone (20E) is involved in myofibrillar degradation through an ecdysteroid signaling pathway.

Ecdysteroid signaling has been well studied in insects. Ecdysteroids are steroid hormones that regulate growth, development, reproduction, and molting in arthropods (Chang, 1993; Thummel, 1996). Ecdysteroids, like vertebrate steroid hormones, regulate ecdysteroid response gene transcription after binding to a nuclear ecdysteroid receptor (EcR). Usually, EcR itself cannot bind to hormone; it heterodimerizes with a vertebrate RXR homolog, ultraspiracle (USP) in insects (Yao et al., 1993). EcR/USP heterodimers bind to a specific response motif to induce expression of early response genes, such as E74 and E75. These early response gene products are usually transcription factors and induce an ecdysteroid cascade reaction (Huet et al., 1995).

EcR, USP and E75 share the typical nuclear receptor domain structure (Renaud and Moras, 2000). The N-terminal A/B domain is the least conserved in amino acid sequence and varies in length. In fact, many insects have different A/B domains that are generated by different transcription start sites or alternative splicing (Talbot et al., 1993; Segraves and Woldin, 1993). The A/B domain contains a transcriptional activation function (AF-1) sequence (Onate et al., 1998; Hadzic et al., 1995). The C domain is highly conserved

among different steroid nuclear receptors and primarily serves as the DNA-binding domain (DBD) containing two zinc-finger motifs. The E domain is the ligand-binding domain (LBD) of about 250 amino acids and is moderately conserved. In addition to various ligands for the specific nuclear receptors, multiple proteins, such as homo-/heterodimermic partners, co-repressors, and co-activators, can interact with domain E. The D domain is the hinge region connecting the DBD and LBD. The F domain, which varies in sequence and length (and sometimes is entirely absent), has no known function.

In crustaceans, the EcR (UpEcR) and retinoid-X receptor (UpRXR) homologs were first cloned from fiddler crab (*U. pugilator*) (Chung et al., 1998). Both the DBD and LBD of UpEcR are closely related to those domains from insects. UpRXR shares the greatest similarity to insect USPs in the DBD, while its LBD shares greater amino acid similarity to vertebrate RXR (Chung et al., 1998). Insect EcRs usually occur in two or three A/B domain isoforms. However, only one A/B domain isoform has been identified in crustacean EcR, while several alternative spliced isoforms around the LBD have been identified (Durica et al., 2002). For the insect, some of the RXR homologs (USPs) have isoforms with different N-terminal A/B regions (Jindra et al., 1997; Kapitskaya et al., 1996). Each isoform is expressed independently, showing expression patterns varying with physiological or developmental states. In crustaceans, the UpRXR has two splicing variants in the hinge region, while no A/B domain variant has been identified (Durica et al., 2002). One isoform has a five-amino acid insertion within the "T" box, which is a highly conserved domain in the DBD boundary. The T-box is essential for DNA binding (Wilson et al., 1992). The other isoform has a 33-amino acid deletion in the flexible loop region connecting the LBD and DBD. This isoform may be important for interacting with

EcR or other transactivators. A cDNA encoding another crustacean nuclear receptor family protein, MeE75, has been isolated from a shrimp (*Metapenaeus ensis*) (Chan, 1998). E75s in insects have two or three isoforms in the A/B domain and the expression pattern of each isoform varies with the molting cycle and ecdysteroid level (Segraves and Hogness, 1990; Jindra et al., 1994). MeE75 has three isoforms in the A/B domain (75A, 75C and 75D). MeE75A is constitutively expressed, while MeE75C is expressed highly during the transition from early premolt to late premolt.

The hormonal regulation of ecdysteroid synthesis differs between crustaceans and insects. In insects, the prothoracic gland is stimulated to synthesize ecdysteroid by prothoracicotropic hormone (PTTH), a neuropeptide produced in the brain (Gilbert et al., 2002). However, in crustaceans no stimulatory mechanism for ecdysteroid synthesis has been identified. Instead, the Y-organ is negatively regulated by molt-inhibiting hormone (MIH), a neuropeptide produced in the neural ganglia in the eyestalks. In insects, a critical titer of 20E inhibits ecdysteroid production as a feedback regulation (Beydon, 1983; Sakurai and Williams, 1989). This down-regulation is mediated by the EcR/USP complex. After treatment with 20E, small forms of USP are expressed predominantly when ecdysteroid production is decreased (Beydon, 1983). Phosphorylation of USP affects ligand and DNA binding of the EcR/USP heterodimer. These results suggest USP plays an important role in negative feedback regulation of ecdysteroidogenesis in insect. In crustaceans ecdysteroid inhibits ecdysteroidogensis in Y-organ, but the mechanism is not known.

In order to understand the role of 20E in regulating molt-induced muscle atrophy, we cloned the ecdysteroid receptor (Gl-EcR), retinoid-X receptor (Gl-RXR), and E75

homolog (Gl-E75) from the land crab, *Gecarcinus lateralis*. The tissue distribution of each nuclear receptor was determined by RT-PCR. The relationship between the expression of Gl-EcR and three different calpains (Gl-CalpM, Gl-CalpB, and Gl-CalpT was quantified using real-time PCR. These results suggest that Gl-EcR may directly induce Gl-CalpT expression.

Materials and Methods

Animals

Adult land crabs (*Gecarcinus lateralis*) were collected from San Miguel Reserve near Fajardo, Puerto Rico. They were kept in covered plastic cages with aspen beddings moistened with tap water at 27 °C and 50% humidity and were fed cat chow, carrots, and lettuce twice a week. A 12 h dark/12 light cycle was used. Walking legs were autotomized to induce limb regeneration. Eyestalks were ablated to derepress the Y-organs to increase ecdysteroid levels in the hemolymph. Wounds were cauterized with a heated spatula to minimize loss of hemolymph.

Cloning of ecdysteroid-related genes

Partial sequences encoding ecdysone receptor (EcR), retinoic-X receptor (RXR), and E75 were initially obtained by nested RT-PCR using degenerate primers directed to highly conserved sequences in a wide variety of proteins for each nuclear receptor in the GenBank database (http://www.ncbi.nlm.nih.gov). Those protein sequences were aligned using the web-based ClustalW program (http://www.ebi.ac.uk/clustalw/index.html). Two forward primers (EcR F1 and EcR F2) were designed within domain C (DNA binding

domain) and two reverse primers (EcR R1 and EcR R2) were designed within domain E (ligand binding domain) of EcR (Table 3-1). In RXR, domain E was not well aligned due to multiple alternative splicing isoforms within domain E. Consequently, two sets of degenerate primer pairs (RXR F1, F2 and RXR R1, R2) were made within domain C of RXR (Table 3-1). E75s were the least conserved and only one forward primer (E75 F1) on the margin of domain C could be made and two reverse primers were designed by only one amino acid shift (E75 R1,R2) (Table 3-1). All the primers were synthesized and purified by Integrated DNA Technologies, Inc. (Des Moines, Iowa).

Total RNA was isolated from the claw muscle using the RNeasy mini kit (Qiagen Inc.USA). cDNA was synthesized according to the manufacturer's protocol using Superscript II RNase H- reverse transcriptase first strand synthesis system (Invitrogen, Inc.). Briefly, 12 µl of a mixture containing 1 µl oligo (dT)12-18 (500 µg/ml), 1 µg total RNA, and 1 µl 10 mM dNTPs was heated to 65 °C for 5 min and chilled on ice for 1 min. 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT, and 1 µl RNaseOUT recombinant ribonuclease inhibitor (40 units/µl) were added. The mixture was incubated at 42 °C for 2 min. The reaction was initiated by the addition of 1 µl (200 units) of SuperScript II at 42 °C for 50 min. The reaction was inactivated by heating at 70 °C for 15 min. PCR reactions were performed using an ABI 9600 thermal cycler (Perkin-Elmer, Inc.). The first PCR reaction contained 3 µl cDNA mixture, 3 µl 10 X Takara EX Taq buffer, 2 µl 250 µmol dNTPs, 1 µl forward degenerate primer, 1 µl reverse degenerate primer, 0.2 µl Takara EX Taq DNA polymerase (5 units/ µl), and 18.8 µl PCR grade deionized water. Initial denaturation at 95 °C for 5 min was followed by 35 amplifying cycles (95 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 1 min) and final extension at 72 °C for 7 min.

For the second PCR reaction, 0.2 μl of the first PCR reaction was used for each nuclear receptor. Nested PCR primers for each gene were used, except for E75, for which we could not make a nested forward primer and therefore used the same forward primer for the PCR (Table 3-1). Other reaction components and PCR conditions were same as those in first reaction.

The PCR products were separated with 1.2% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were purified from gel slices using Qiaquick gel extraction kit (Qiagen, Inc.), ligated into TA plasmid vector with the TOPO TA cloning kit (Invitrogen, Inc.), and transformed into One Shot TOP 10 *E. coli* strain (Invitrogen). The ligation and transformation methods followed manufacturer's protocol. Transformants were first selected by blue-white colony screening on LB agar medium containing 50 μg/ml ampicillin (Sigma-Aldrich, USA), followed by PCR with T7 and M13- Reverse vector primers to estimate insert size. Plasmids were purified using a Qiagen spin mini prep kit (Qiagen, Inc.) and sequenced using T7 and M13 reverse vector primers (Davis Sequencing, CA, USA). If needed, gene-specific primers were used for further sequence information.

RACE (Rapid Amplification of cDNA Ends) of mRNA was used to obtain full-length sequences. Poly (A^+) RNA was isolated from total claw muscle RNA using Oligotex mRNA kit (Qiagen, USA). For the 3' sequence, the RACE System (Invitrogen Inc.,USA) was used. Briefly, first-strand cDNA synthesis reactions contained 200 ng poly(A^+) RNA and adaptor primer (5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3'). First-round PCR on the cDNA (20 ng) included a universal amplification primer (5'-

CUACUACUACUAGGCCACGCGTCGACTAGTAC-3') and gene-specific forward primers (Table 3-2) under following conditions: denaturation at 96 °C for 5 min, 35 cycles (96 °C for 30 sec, 60 °C for 30 sec, and postextention at 72 °C for 2 min) and 72 °C for 10 min. Nested PCR on 30 μl of each reaction was conducted with gene-specific primers (Table 3-2) and abridged universal amplification primer (5'-GGCCACGCGTCGACTAGTAC-3') under the same conditions as the first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

SMART™ RACE cDNA amplification Kit (BD Biosciences, Inc., USA) was used to get the 5' sequences of the each gene. The first-strand cDNA synthesis reaction contained 3 μl poly(A+) RNA (100 ng), 1 μl 5' CDS primer [10 mM, 5'–(T)25N-1N–3'], and 1 μl SMART II A oligo (10 mM, 5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'), was incubated at 68 °C for 2 min. After chilling the reaction on ice for 2 min, 2 μl 5X First-Strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 30 mM $MgCl_2$], 1 μl DTT (20 mM), 1 μl dNTPs (10 mM), and 1 μl PowerScript reverse transcriptase were added. The reaction was covered with 20 μl paraffin oil and incubated at 42 °C for 1.5 hr in a ABI 9600 DNA thermal cycler (Perkin-Elmer Inc., USA). The reaction mixture was diluted 10-fold with autoclaved distilled water and was used for first-round PCR with 10X universal primer A Mix (0.4mM 5'-CTAATACGACTCACTATAG GGCAAGCAGTGGTATCAACGCAGAGT-3' and 2 mM 5'-CTAATACGACTCACTATAGGGC-3') and gene-specific reverse primers (Table 3-2) under the following conditions: denaturation at 96 °C for 5 min, 35 cycles (96 °C for 30

sec, 66 °C for 15 sec and 72 °C for 3 min) and postexteinsion at 72 °C for 10 min. Second-round PCR was conducted using nested gene-specific primers (Table 3- 2) and nested universal primer A (10 mM, 5'-AAGCAGTGGTATCAACGCAGAGT-3'). The PCR conditions were the same was the same as those used for first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Purified products were sequenced to confirm identities.

Expression of ecdysteroid-related genes

Total RNA was isolated from tissues using RNeasy mini kit (Qiagen, Inc.). Isolated RNA was treated with DNaseI (Invitrogen, Inc.). cDNA was synthesized using Superscript II RNase H- reverse transcriptase first strand synthesis system (Invitrogen, Inc.) and quantified by absorbance at 260 nm. PCR on 5 µl cDNA was performed with gene-specific primers (Table 3-3) under the following conditions: denaturation at 96 °C for 5 min, 40 amplification cycles (96 °C for 30 sec, 62 °C for 15 sec and 72 °C for 40 sec) and postextension at 72 °C for 10 min. A pair of Elongation Factor 2 (EF2) primers were used as an internal control.

Transcript levels were quantified by real-time PCR. Primers for three calpains (Gl-CalpB, Gl-CalpM, and Gl-CalpT), ecdysone receptor (Gl-EcR), retinoid-X receptor (Gl-RXR), ecdysone response gene (Gl-E75), and elongation factor 2 (EF2)(Table 3-4) were designed using the IDT BioTools program (http://biotools.idtdna.com/gateway). EF2, which is constitutively expressed, served as an internal control. Identities of each PCR product was confirmed with regular PCR. PCR products were ligated into pCR2.1 TOPO vector using TOPO TA Cloning Kit (Invitrogen, Inc.). Standards curves were generated

using serial dilutions from 10 ng to 10 fg of plasmids containing each PCR product (Yu and Mykles, 2003; Medler and Mykles, 2003). A Cepheid Smart Cycler instrument was used. The reaction mixture contained 2 µl LightCycler FastStart Reaction Mix (Roche; 10X buffer, Fast Taq DNA polymerase, and dNTPs), 2 µl $MgCl_2$ (25 mM), 2 µl cDNA template, 1 µl forward primer (10 pmol/µl), 1 µl reverse primerv(10 pmol/µl) and 12 µl PCR grade water. The PCR conditions were denaturation at 96 °C for 5 min, 40 amplification cycles (96 °C for 20 sec, 65 °C for 15 sec and 72 °C for 30 sec) and melting curve analysis (60 °C +0.2/sec).

DNA molecular weights were calculated by the Schepartz lab biopolymer calculator (http://paris.chem.yale.edu/extinct.html) and copy numbers were calculated with the equation:

Copy number = (weight of the plasmid (g)/molecular weight of the plasmid (g/mol)) x 6.25 x 10^{23} (number/mol)

From the equation above 1 ng plasmid is equal to 4.6 x 10^8 in copy number when $\log^{-2}$ from the standard curve. In the standard curve the -Log value was converted into actual copy number using following equation:

Log copy number = $\text{Log}(10^{(\text{-log value at a critical cycle number})} \text{ x } (4.6 \text{ x } 10^8))/10^{-2})$

The Statview Program (SAS Institute, Inc. USA) was used for statistical analysis and SigmaPlot program (SPSS Inc. USA) was used for each graph.

Results

Cloning of ecdysteroid genes

An initial 700-bp product from nested PCR using degenerate EcR primer sets (Table 3-1) was 95% identical in deduced amino acid sequence with EcR from fiddler crab (accession number: AAC33432). 3' RACE using sequence-specific primers failed. Since the fiddler crab EcR (UpEcR) has a very long 3' UTR region (~about 4 kb) the land crab EcR (Gl-EcR) may also have a long 3'-UTR and thus be difficult to amplify. 5' RACE yielded a 300-bp product, which extended the 5' sequence another 100 bp. Additional 3' sequence was obtained by PCR using gene-specific forward primers (cEcRF1 and cEcRF2; Table 3-2) and degenerate primer sets (EcR F3 and EcR F4; Table 3-2). The consensus partial sequence of Gl-EcR was 1005 bp and encoded a deduced 335-amino acid sequence encompassing part of the C domain and all of the D and E domains (Fig. 3-1). The partial amino acid sequence of Gl-EcR was aligned with full-length sequences of EcR genes from fiddler crab (UpEcR), and locust (LmEcR) (Fig. 3-2). The Gl-EcR sequence was 93% identical with homologous regions of UpEcR and 66% identical with LmEcR.

A single 200-bp product was obtained with nested PCR using degenerate RXR primer sets (Table 3-1); its deduced amino acid sequence was 100% identical to the DNA binding domain of the fiddler crab RXR (accession number AAC32789) and 88% identical to the DNA binding domain of rat RXR-alpha (accession number NP036937). The complete ORF was obtained with 3' and 5' RACE. 3' RACE produced three Gl-RXR isoforms within the "T" box, which is a conserved domain at the DBD boundary (Fig.3-4). One isoform had a 5-amino acid insertion (VQEVGAVEERQR) while the other did not (VQEERQR). In a third isoform a 7-amino acid sequence (VQEERQR) was replaced by a 8-amino acid sequence (AGHRRGQW). A second alternative splicing site was

located in the flexible loop region connecting LBD and DBD. In one isoform, a 33-amino acid sequence was deleted while the other was not. The final splicing site was located in the LBD. One isoform had a 35-amino acid deletion in the LBD while the other did not. In addition to the three internal alternative splicing sites, two truncated isoforms lacking LBD were obtained from 3' RACE. 5' RACE yielded a single sequence and contained the rest of the ORF. The deduced Gl-RXR and UpRXR amino acid sequences were very similar, except that the Gl-RXR sequence was 32 amino acids shorter at the N-terminus and lacked a 11-amino acid sequence in the A/B domain. The longest Gl-RXR cDNA was 1515 bp with an ORF containing a 436-amino acid sequence with all the insertion sequences (Fig. 3-3).

The full-length amino acid sequence of Gl-RXR was aligned with RXR sequences from fidller crab (UpRXR), locust (LmRXR), snail (BgRXR), and human (hRXR) (Fig. 3-5). Gl-RXR had the highest amino acid sequence identity (95%) to UpRXR in the C, D, and E domains. The DBD (domain C) and LBD (domain E) were particularly well conserved. The Gl-RXR C domain was 100% identical to the UpRXR sequence, 92% identical to LmRXR, 87% identical to BgRXR, and 86% identical to human RXR. In addition, the Gl-RXR C domain had the 8 cysteine residues of two zinc finger motifs essential for DNA binding. The Gl-RXR E domain had 99% sequence identity with UpRXR, 78% identity with LmRXR, 78% identity with BgRXR, and 74% identity with human RXR.

A single product of about 700 bp was obtained with nested PCR using degenerate E75 primer sets (Table 3-1). The deduced amino acid sequence was 94% identical to the E75 nuclear hormone receptor from the shrimp accession number O77245). 5' RACE

yielded a single product of about 800 bp; it contained the 5' UTR and the remainder of the coding sequence. 3' RACE yielded a 2.3-kb product, which contained the remainder of the C-terminal coding sequence and 3' UTR. The full length cDNA of Gl-E75 was 3258 bp and encoded a protein 828 amino acids (Fig 3-6).

The Gl-E75 deduced full-length amino acid sequence was aligned with that from a shrimp, *Metapenaeus ensis* (MeE75) and with three insect alternatively spliced isoforms from *Drosophila melanogaster* (DmE75A, B, and C) and *Bombyx mori* (BmE75A, B, and C) (Fig. 3-7). As in other nuclear receptors, there was no significant homology in the A/B domain. Crustacean E75s and BmE75A and C had a short A/B domain, while DmE75A and C had a long A/B domain. In domain C, Gl-E75 was 100% identical to all the other sequences, except DmE75B and BmE75B, which lacked one or two zinc finger motifs. The domain E of Gl-E75 had 90% amino acid sequence identity with MeE75, 58% with BmE75s, and 56% with DmE75. Domain E of Gl-E75 had lower amino acid identity with other species than domain E of EcR and RXR. Gl-E75 had a longer F domain (455 amino acids) than shrimp (233 aimino acids). DmE75B has C terminal splicing variant different from A and C. In addition, there were consensus sequences in domain F between Gl-E75 and DmE75 and BmE75, but not in MeE75.

Ecdysteroid genes expression in muscle tissues

Both Gl-EcR and Gl-E75 were expressed in intermolt muscle tissues (Fig. 3-9). EF2 was used as internal control to confirm the quality of the RT-PCR. Gl-RXR expression was more complex. In thoracic muscle, isoforms containing only the 5 amino acid insertion (GAVEG) in the DBD domain (DBD2 +5 aa) were expressed, while isoforms

containing both the 5 amino acid insertion and deletion in the DBD were expressed in claw muscle. In addition, two alternatively spliced sites and two truncation sites generated all possible splicing combinations. However, neither isoform containing the 8 amino acid replacement in the DBD domain (DBD1) or the 35 amino acid deletion in the LBD domain was amplified in thoracic muscle. Isoforms containing the 35 amino acid deletion and a truncated isoform containing a 8 amino acid replacement (DBD1-33 LDB) was weakly expressed in claw muscle.

Calpain and ecdysteroid receptor gene expression in skeletal muscles and limb regenerates

The effect of eyestalk (ES) ablation on mRNA levels of EcR and three calpains in thoracic and claw muscles was quantified by real-time PCR. The EF2 transcript was used as an internal control. In thoracic muscle, ES ablation had no significant effect on expression of the Gl-EcR and three calpains (Fig 3-10). In contrast, Gl-EcR expression in claw muscle increased about 15-fold ($p < 0.0001$) at one day after ES ablation but then decreased to about 2.8-fold ($p = 0.002$) above the intermolt level at three days after ES ablation (Fig. 3-12). Gl-CalpM and B expression was not altered by ES ablation, while Gl-CalpT mRNA was increased about 19.3-fold above the intermolt level one day after ES ablation and about 4.3-fold higher at three days after ES ablation in the claw muscle. The correlation between Gl-EcR and CalpT was highly significant both in claw muscle ($p < 0.0001$) (Fig. 3-13) and thoracic muscle ($p = 0.0105$) (Fig. 3-11). There was no correlation between EcR expression and either Gl-CalpM or Gl-CalpB.

Gl-EcR, Gl-CalpM and Gl-CalpT expression was not significantly different between

limb regenerates and intact legs, while Gl-CalpB was expressed 15-fold ($p = 0.0068$) higher in secondary limb regenerates than in the intact legs. There is no significant difference in the EcR and three calpain mRNA levels between primary and secondary limb regenerates. As in the claw and thoracic muscle, the correlation between Gl-EcR and Gl-CalpT was significant ($p = 0.0066$).

Discussion

A partial clone of Gl-EcR has the highest amino acid identity with UpEcR (95%). Alternative A/B domain isoforms have not been found in UpEcR and UpRXR (Durica et al., 2002). Some insects have A/B domain isoforms and that differ in physiological function (Minakuchi et al., 2002; Wang et al., 2002; Wang et al., 2000). These N-terminal isoforms are generated from the use of different promoters and/or alternative splicing. The different RXR isoforms may play key roles in regulating the ecdysteroid signaling pathway. The UpRXR has two alternative splicing sites in the C and D domains; these isoforms have different DNA binding elements and dimerization patterns (David Durica, personal communication).

The Gl-RXR has the same isoforms as the UpRXR, including a 5-amino acid insertion in the C domain and 33-amino acid deletion in the D domain (Fig. 3-4). RT-PCR shows that the 5-amino acid insertion isoform is highly expressed in claw muscle, but not in thoracic muscle (Fig. 3-9). In addition, we found two additional isoforms that were not described for UpRXR. Two truncated isoforms that lack the E domain were also expressed (Fig. 3-9). The E domain is necessary for ligand binding, as well as binding partners such as co-repressors and co-activators. There is no previous report of truncated

EcRs or RXRs (USP) in arthropods. Its role in the ecdysteroid signaling pathway is unclear. It may act independently without interacting with retinoid. Another alternative isoform has a 35 amino acid deletion in the E domain and is expressed at low levels only in claw muscle and not in thoracic muscle. Although there are no Gl-EcR alternative isoforms, the different RXR isoforms would create different combinations of EcR/RXR heterodimers differing in functional properties.

The A/B domain in Gl-E75 and MeE75 is shorter than those of insects. The long N-terminus of *Drosophila* E75 serves as a built-in mechanism for regulating the timing of the onset and duration of the early ecdysone response (Thummel, 1992). This suggests that the regulation of E75 in crustaceans may be different from that in *Drosophila*. MeE75 has three N-terminal isoforms (Chan, 1998), but only one isoform was found in land crab. Based on the N-terminal sequence and RT-PCR, Gl-E75 appears to be the homolog of MeE75A. As the E75B and C isoforms are induced by ecdysteroids and then decline quickly, it is likely the message level was too low to clone them. Since the DNA binding motif is identical with that of insect E75, Gl-E75 may have the same DNA binding properties. However, since the E domain has low amino acid identity with insects, the Gl-E75 may have different properties in binding ligands and/or protein partners. The ligand-binding properties of crustacean E75 is unknown. Moreover, Gl-E75 has a longer F domain compared with MeE75 and some specific regions within the F domain of Gl-E75 have high identities with those in *Drosophila* E75. This suggests that MeE75 is a truncated form of crustacean E75 generated by alternative splicing. Similarly, the C-terminus of DmE75B is different from A and C because of alternative splicing. However, detailed studies of the functions of the F domain have not been done, although

the similarities in the F domain between Gl-E75 and Dm-E75 suggest that this domain functions in regulating E75 activity.

Real time PCR shows that Gl-EcR and Gl-CalpT expression are induced by eyestalk ablation in claw muscle, but not in thoracic muscle. Premolt atrophy occurs specifically in the claw muscle (Mykles, 1998), suggesting that sensitivity to ecdysteroid may be important in initiating molt-induced muscle protein degradation. The expression of Gl-EcR and Gl-CalpT was highly correlated in all tissues examined, including claw and thoracic muscle and 1° and 2° limb regenerates. These results suggest that expression of Gl-EcR and Gl-CalpT is linked. One possibility is that the EcR/RXR complex binds directly to the promoter of the Gl-CalpT gene, which induces its expression. Another possibility is that ecdysone early response genes, such as the E75 or E74 are induced by EcR/RXR complex and they, in turn, induce the expression of Gl-CalpT. In *Manduca sexta,* the EcR/RXR complex induces E75 within 30 min (Zhou et al., 1998). Although Gl-CalpT expression is correlated with EcR expression, there is no direct evidence that Gl-CalpT has a major role in muscle atrophy. There is a preferential degradation of thin filaments during atrophy (Mykles and Skinner, 1981). CDP I and CDP IIb (possibly CalpB) more efficiently degrade thin filaments than CDP IIa and CDP III (CalpM) (Mattson and Mykles, 1993). CDP III is encoded by CalpM, but the identities of CDPs I, IIa, and IIb have not yet been established. If CDP IIa is encoded by Gl-CalpT, increased Gl-CalpT may cause an increase in the degradation of thick filaments. If other calpains, such as CalpB or CalpM are involved, then their activities are not controlled at the transcriptional level. Instead, activating Gl-CalpB or inhibiting Gl-CalpM by intracellular factors, such as Ca^{2+}, phosphorylation/ dephosphorylation, or endogenous activators or

inhibitors, may cause preferential thin filament loss. Further work is needed to determine the role of each calpain in claw muscle atrophy.

Table 3-1. Forward (F) and reverse (R) degenerate primers used for RT-PCR to obtain initial partial cDNAs encoding land crab EcR, E75, and RXR.

Name	Conserve sequence	Primer sequences
EcR F1	MRRKCQ	5'-ATG MGN MGN AAR TGY CAR-3'
EcR F2	CRL(K/R)KC	5'-TGY MGN YTN VSN AAR TG-3'
EcR F3	AEIWDV	5'-AC RTC CCA DAT YTC NGC-3'
EcR F4	PFLAEI	5'-DAT YTC NGC NAR RAA NGG-3'
ECR R1	EYALL(T/A)A	5'-CNG YNA RNA RNG CRT AYT C-3'
ECR R2	DQI(A/T)LLK	5'-TTN ARN ARN GYD ATY TGY TC-3'
RXR F1	(S/A)(I/V)CGDR	5'-DSN RTN TGY GGN GAY MG-3'
RXR F2	SGKHYGV	5'-SN GGN AAR CAY TAY GGN G-3'
RXR R1	QEERQR	5'-NCK YTG NCK YTC YTC YTG -3'
RXR R2	GMKREAV	5'-CNG CYT CNC KYT TCA TNG G-3'
E75 F1	A(V/I)RFGR	5'-DSN RTN TGY GGN GAY MG-3'
E75 R1	DRPGL(R/L)N	5'-TNM DNA RNC CNG GNC KRT C-3'
E75 R2	(G/P)DRPGL	5'-NAR NCC NGG NCK RTC NSS -3'

Table 3-2. Sequence-specific forward(F) and reverse (R) primers used for 3'and 5' RACE of land crab EcR, E75, and RXR.

NAME	SEUQNECE	TM (C°)	DESCRIPTION
cEcR F1	5'-GTTTGAGCAGCCAACTGAAGCAGATG-3'	62.3	EcR 3'RACE first round forward Primer
cEcR F2	5'-ATTCAGGCACATAACCGAGATGACGATC-3'	62.2	EcR 3'RACE nested forward Primer
cEcR R1	5'-CATCTGCTTCAGTTGGCTGCTCAAAC-3'	62.3	EcR 5'RACE first round reverse Primer
cEcR R2	5'-CACTGGACTAATAGGAGCAGCCTTGTC-3'	62.2	EcR 5'RACE nested reverse Primer
cE75 F1	5'-TGCGTCGTGAAGCTCTGCACACGTCAGTC-3'	69.1	E75 3'RACE first round forward Primer
cE75 F2	5'-CGCACGCTTCTTAGTGGACTCCATGTTC-3'	64.7	E75 3'RACE nested forward Primer
cE75 R1	5'-GCGAATGATGGCGGCGGTGACTC-3	66.1	E75 5'RACE first round reverse Primer
cE75 R2	5'-CCAACACTGCCCTCTCCTGTGACCTG-3'	66.3	E75 5'RACE nested reverse Primer
cRXR F1	5'-GCAAGGGGTTCTTCAAACGGAC-3'	60	RXR 3'RACE first round forward Primer
cRXR F2	5'-TACTGCCGCTACCAGAAGTGCTTG-3'	62.6	RXR 3'RACE nested forward Primer
cRXR R1	5'-TCAAGCACTTCTGGTAGCGGCAG-3'	62.9	RXR 5'RACE first round reverse Primer
cRXR R2	5'-TCACGGCAGGCATATGTCAGGTCTTTG-3'	64.5	RXR 5'RACE nested reverse Primer

Table 3-3. Sequence-specific forward (F) and reverse (R) primer pairs used for RT-PCR of EF2, EcR, E75, and RXR mRNAs in land crab tissues.

NAME	SEUQNECE	TM (C°)	DESCRIPTION
RcEF2 F1	5'-TTC TAT GCC TTT GGC CGT GTC TTC TC-3'	62.6	elongation factor 2 forward primer
RcEF2 R1	5'-TGA TGG TGC CCG TCT TAA CCA GAT AC-3'	62.1	elongation factor 2 reverse primer
cEcR F11	5'-AAGAATGCCGTGTACCAGTGTAAATATG-3'	59.2	Gl-EcR forward primer
cEcR R11	5'-GCTGCTCGAAGCATCATGACCTC -3'	61.5	Gl-EcR reverse primer
cRXR F11	5'-GAGAAGCCGCAGGTCACAGGAG-3'	63	Gl-RXR forward primer (DBD1)
cRXR F12	5'-CGAGAAGCCGTCCAGGAGGAG-3'	62.2	Gl-RXR forward primer (DBD2)
cRXR F13	5'-CGTCCAGGTAGGGGCTGTAGAG-3'	61.6	Gl-RXR forward primer (DBD2+5a.a.)
cRXR R11	5'-ACTCACCACGTCCTGCCCTTG-3'	62.9	Gl-RXR reverse primer (-33a.a.)
cRXR R12	5'-ACGTCCTGCAGAGGGTTAGCAC-3'	62.5	Gl-RXR reverse primer (+34a.a.)
cRXR R13	5'-CAGTACAAGTCAGTTGGTTGTCTTACCAGA-3'	62.8	Gl-RXR reverse primer (ΔEF)
cRXR R14	5'-CACCACCACCACCACGTCCTG-3'	63.5	Gl-RXR reverse primer (-33a.a.ΔEF)
cRXR R15	5'-CTCTCGCAGAATCTCCACCAGCTC-3'	62.4	Gl-RXR reverse primer (-35a.a.)
cRXR R16	5'-CTCTCGCAGAATCTCCACATCATTGCA-3'	62.6	Gl-RXR reverse primer (+35a.a.)
cE75 F11	5'-CAGGTCACAGGAGAGGGCAGTGTTGG-3'	66.3	Gl-E75 forward primer
cE75 R11	5'-GAACATGGAGTCCACTAAGAAGCGTG -3'	61	Gl-E75 reverse primer

Table 3-4. Forward (F) and reverse (R) primers used for real-time PCR quantification of land crab EF2, calpains, and EcR mRNAs.

Name	Sequence	TM(°C)
RcEF2 F1	5'-TTC TAT GCC TTT GGC CGT GTC TTC TC-3'	62.6
RcEF2 R1	5'-TGA TGG TGC CCG TCT TAA CCA GAT AC-3'	62.1
RcCalpM F1	5'-GGC GGC TGC AGG AAT TAC ATT AAC AC-3'	62.2
RcCalpM R1	5-'TGT ACC TGA AGA AAT CGA CGT CCA GC-3'	62.2
RcCalpB F1	5'-GTT CAA CTT TGA GGG CTT CAG CAA GG-3'	62.2
RcCalpB R1	5'-GAT AAC CAG CAG AGT TGA GGG CTT GA-3'	62.3
RcCalpT F1	5'-TCT CTG ATG TGC TGT GCC ATA ACT CC-3'	62.3
RcCalpT R1	5'-TGA TGC AGA GAC CTG TGA CCA TTC TG-3'	62.2
RcEcR F1	5'-CAC GAA GAA TGC CGT GTA CCA GTG TA-3'	62.3
RcEcR R1	3'-CAT CTG CTT CAG TTG GCT GCT CAA AC-3'	62.4

Figure 3-1. Nucleotide sequence and deduced amino acid sequence of partial cDNA encoding ecdysone receptor (Gl-EcR) from land crab. The cDNA sequence (1005 bp) encoded a partial protein sequence containing 335 amino acids. Locations of degenerate primers used for nested RT-PCR to obtain the initial cDNA are underlined and bolded. Lines with arrows indicate locations of sequence-specific primers used for 5' and 3' RACE to obtain additional sequence in the ORF.

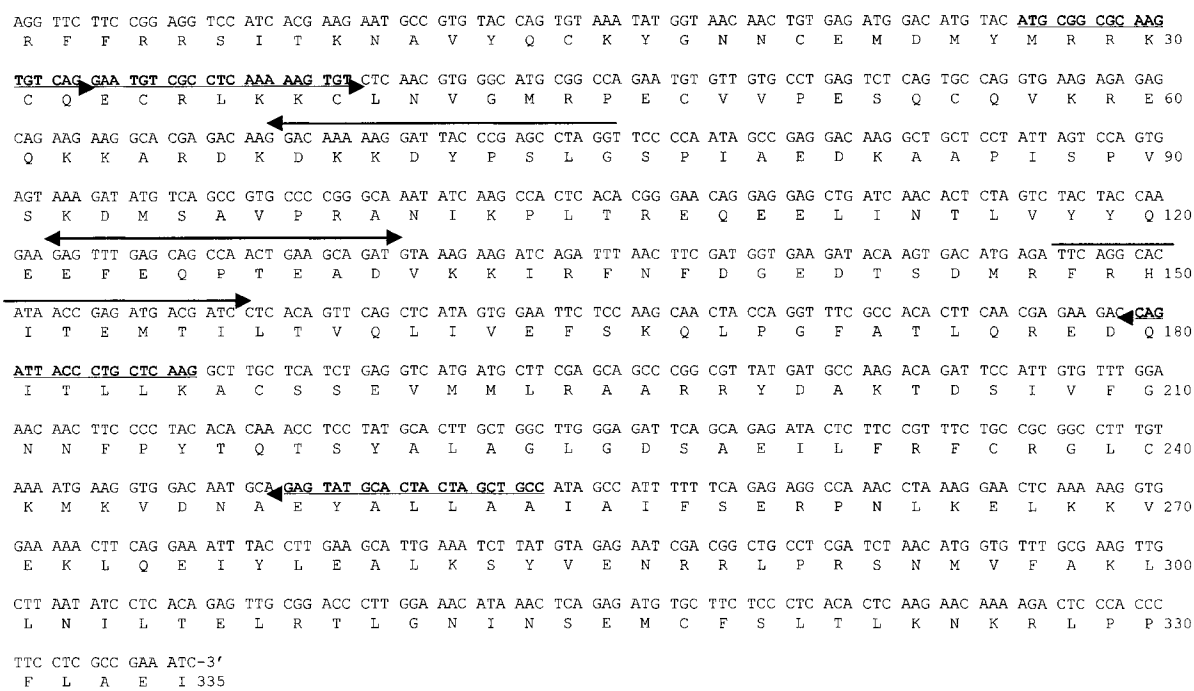


Figure 3-2. Comparison of deduced amino acid sequences of ecdysone receptor (EcR) from land crab, fiddler crab, and locust. The partial Gl-EcR sequence is aligned with EcR sequences from fiddler crab, *Uca pugilator* (UpEcR, AAC33432) and locust, *Locusta migratoria* (LmEcR, AAD19828). Gl-EcR had the highest sequence identity with other EcR sequences in the DNA-binding (domain C) and ligand-binding (domain E) domains. Overall identities were 93% between Gl-EcR and UpEcR and 66% between Gl-EcR and LmEcR. Amino acid identities in all three sequences are highlighted in black. Boundaries between domains are indicated by capital letters.

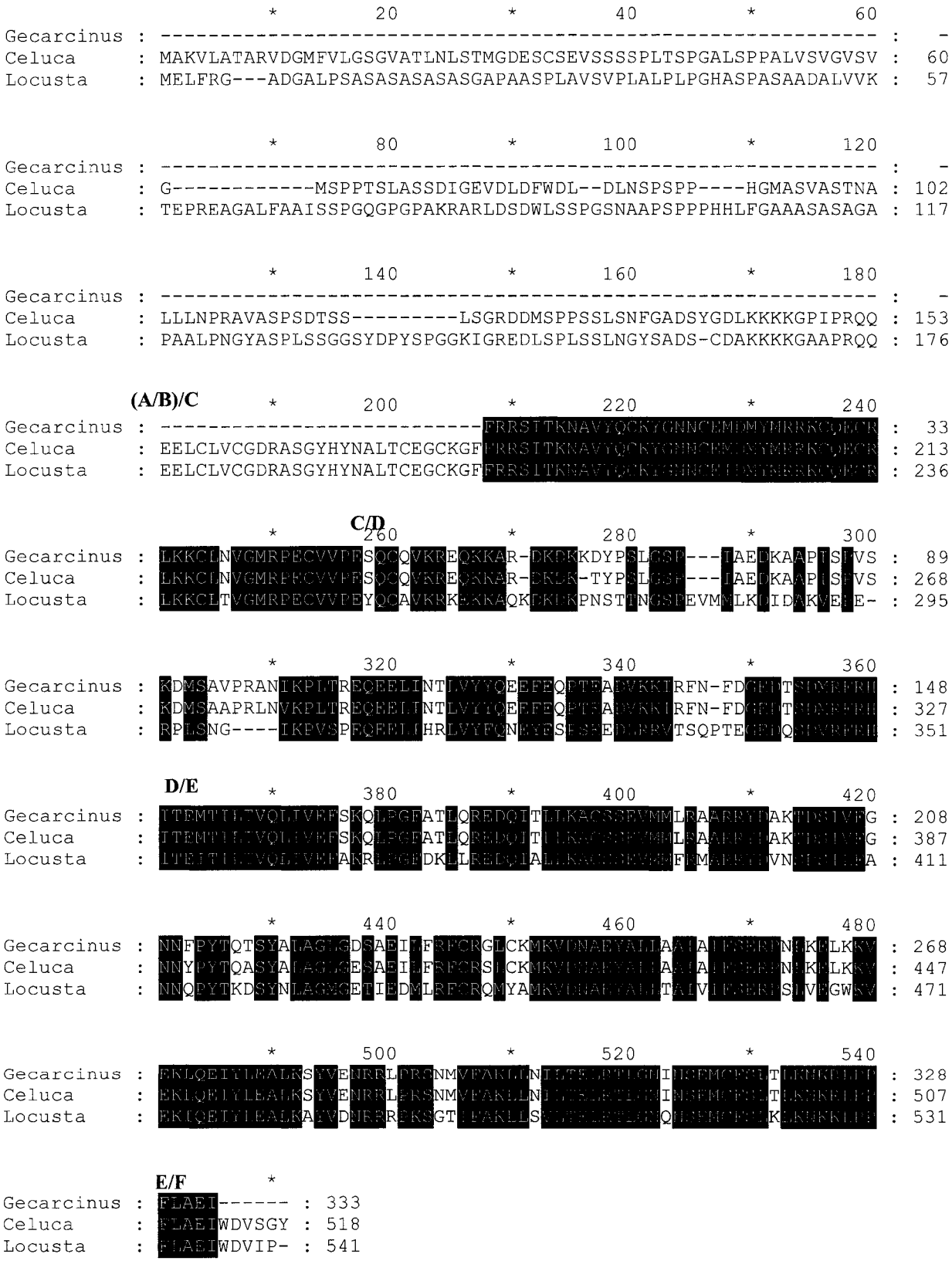


Figure 3-3. Nucleotide sequences and deduced amino acid sequences of land crab retinoid-X receptor (Gl-RXR) isoforms. cDNAs encoding seven alternatively-spliced sequences were identified. Two of the sequences introduce a stop codon to produce truncated proteins lacking domains E and F. Locations and directions of degenerate primers used for nested PCR to obtain the initial cDNAs are shown by bold letters and arrows with dashed lines. Arrows with solid lines indicate locations and directions of sequence-specific primers for 5' and 3' RACE. Sequences of the alternatively-spliced isoforms are boxed.

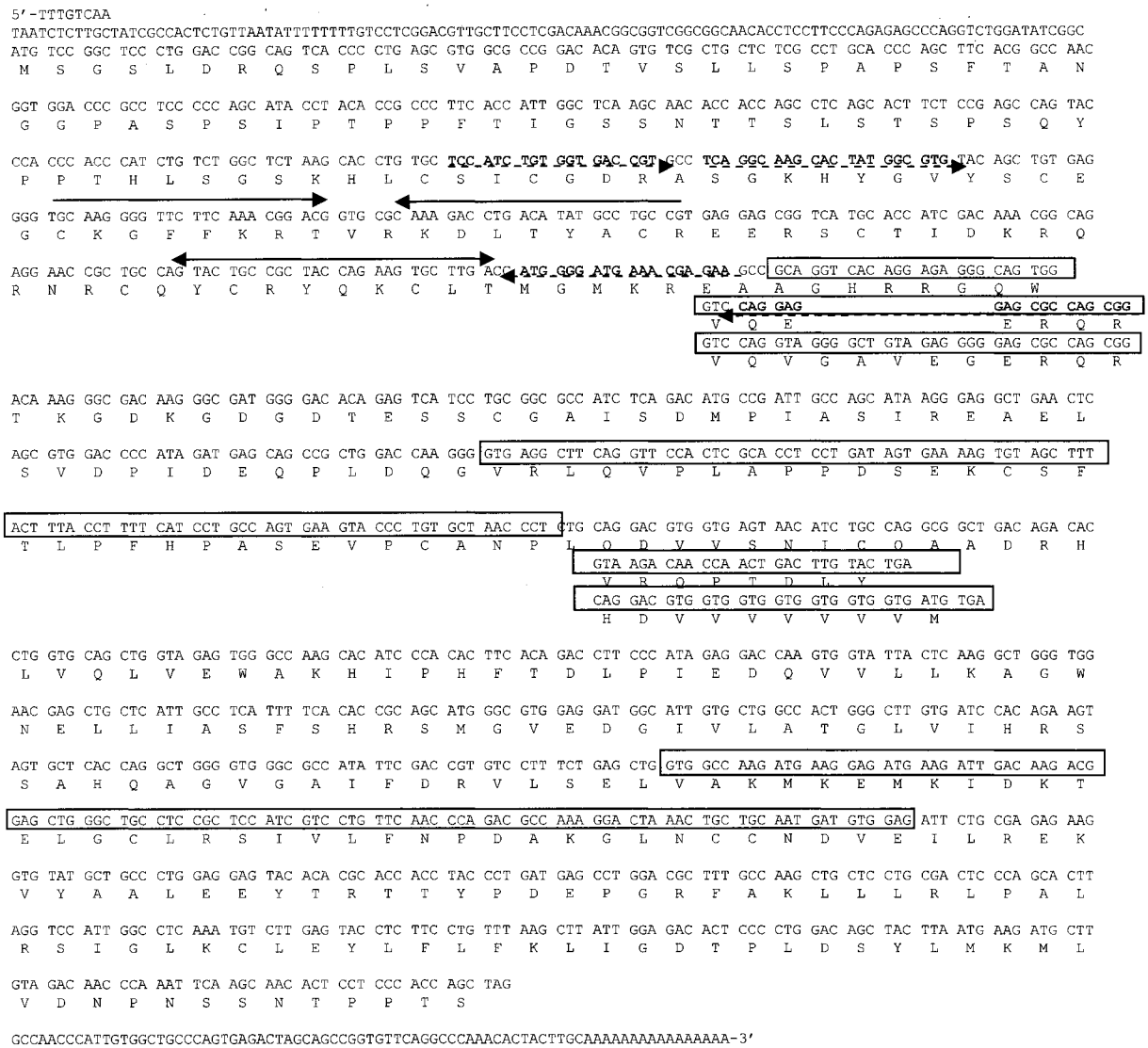
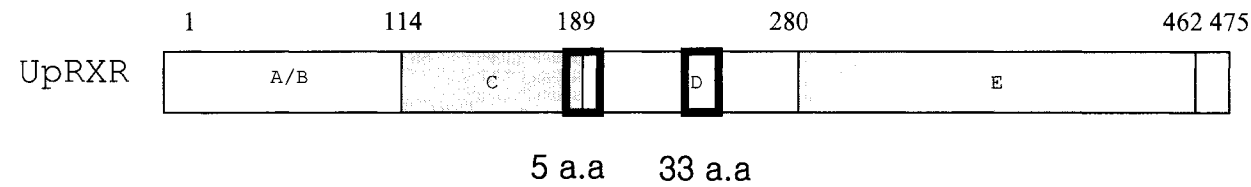
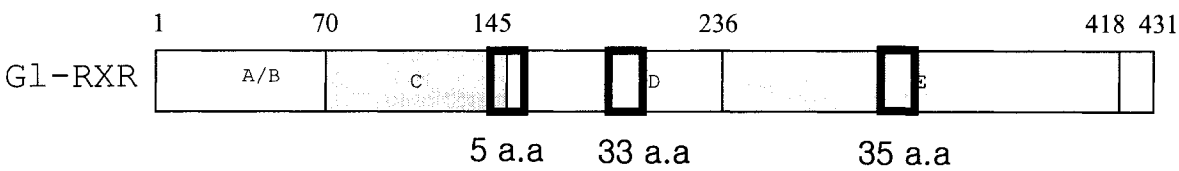


Figure 3-4. Domain organization and alternative splicing sites in crustacean retinoid-X receptor (RXR). RXR cDNAs from fiddler crab (UpRXR) and land crab (Gl-RXR) are depicted. Domains are indicated by capital letters. Boxes indicate locations of alternative splicing sites. In addition to the two alternative splicing sites in domains C and D of UpRXR, Gl-RXR has additional splicing site in the E domain. Alternatively splicing at these sites generates seven different mRNA sequences. Two of the alternatively spliced variants at the D domain introduce stop codons, producing truncated proteins of 233 and 235 amino acids that lack domains E and F (Gl-RXR Δ E).





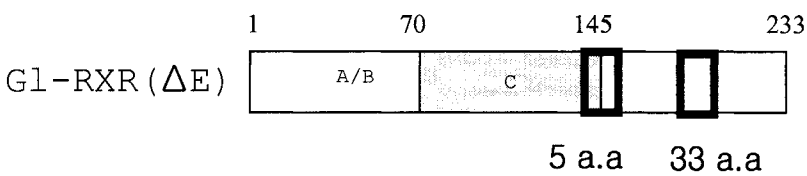


Figure 3-5. Comparison of deduced amino acid sequences of RXR cDNAs from crustaceans, insect, mollusk, and human. Amino acid sequences were aligned using ClustalW (see Materials and Methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical between all the sequences are hignlighted in black. Solid triangles indicate conserved cysteine residues in the zinc-finger DNA-binding motif in domain C. There is a high degree of sequence identity in the DNA-binding (domain C) and ligand-binding (domain E) domains. Arrows indicate alternative splicing regions. Capital letters indicate boundary of each functional domain. Accession numbers: *Celuca (Uca) pugilator,* AAC32789; *Locusta migratoria,* AAQ55293; *Biomphalaria glabrata,* AAL86461; *Homo sapiens,* NP002948.

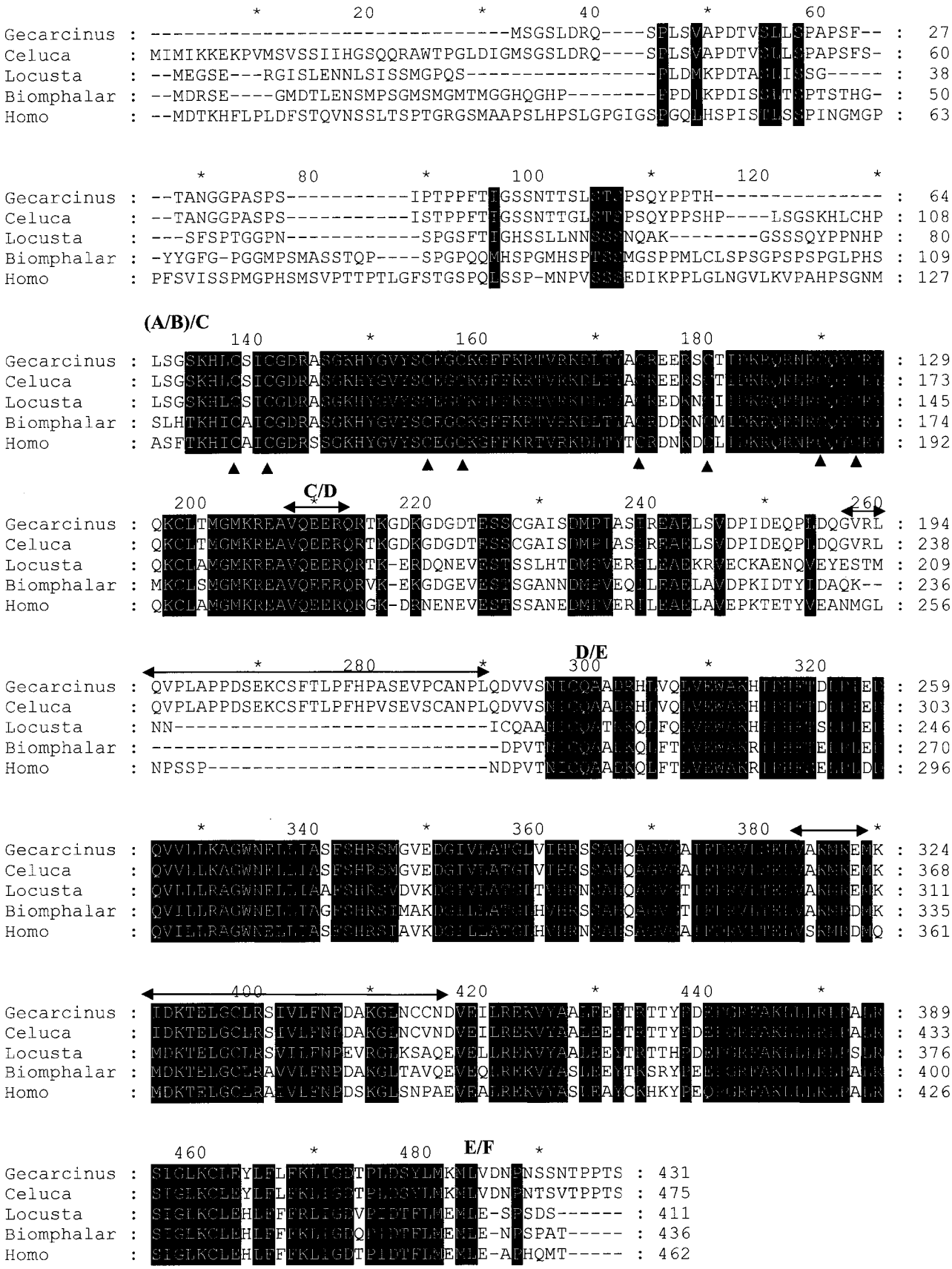
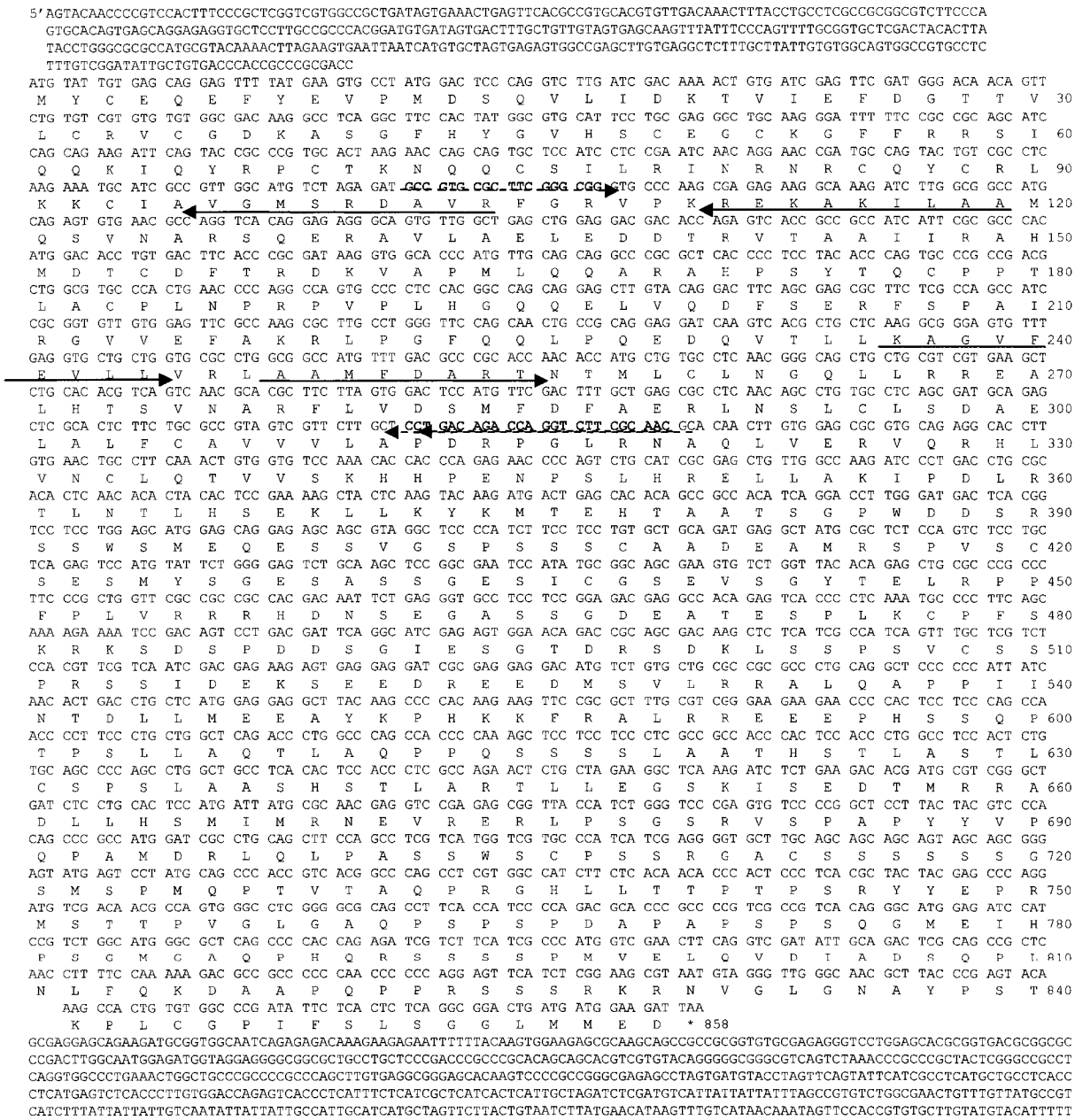


Figure 3-6. The nucleotide and deduced amino acid sequences of land crab E75 ecdysone-responsive gene (Gl-E75). The cDNA sequence (3258 bp) contained the complete ORF encoding a 858-amino acid protein. Locations and directions of degenerate primers used for semi-nested PCR to obtain the initial partial cDNA are indicated by bold letters and arrows with dashed lines. Arrows with solid lines indicate locations and directions of sequence-specific primers used for 5' and 3' RACE. The poly(A) signal is bolded and underlined.



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TTTGTCGGATATTGCTGTGACCCACCGCCCGCGACC

ATG TAT TGT GAG CAG GAG TTT TAT GAA GTG CCT ATG GAC TCC CAG GTC TTG ATC GAC AAA ACT GTG ATC GAG TTC GAT GGG ACA ACA GTT
M Y C E Q E F Y E V P M D S Q V L I D K T V I E F D G T T V 30
CTG TGT CGT GTG TGT GGC GAC AAG GCC TCA GGC TTC CAC TAT GGC GTG CAT TCC TGC GAG GGC TGC AAG GGA TTT TTC CGC CGC AGC ATC
L C R V C G D K A S G F H Y G V H S C E G C K G F F R R S I 60
CAG CAG AAG ATT CAG TAC CGC CCG TGC ACT AAG AAC CAG CAG TGC TCC ATC CTC CGA ATC AAC AGG AAC CGA TGC CAG TAC TGT CGC CTC
Q Q K I Q Y R P C T K N Q Q C S I L R I N R N R C Q Y C R L 90
AAG AAA TGC ATC GCC GTT GGC ATG TCT AGA GAT GCC GTG CGC TTC GGG CGC GTG CCC AAG CGA GAG AAG GCA AAG ATC TTG GCG GCC ATG
K K C I A V G M S R D A V R F G R V P K R E K A K I L A A M 120
CAG AGT GTG AAC GCC AGG TCA CAG GAG AGG GCA GTG TTG GCT GAG CTG GAG GAC GAC ACC AGA GTC ACC GCC GCC ATC ATT CGC GCC CAC
Q S V N A R S Q E R A V L A E L E D D T R V T A A I I R A H 150
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L A C P L N P R P V P L H G Q Q E L V Q D F S E R F S P A I 210
CGC GGT GTT GTG GAG TTC GCC AAG CGC TTG CCT GGG TTC CAG CAA CTG CCG CAG GAG GAT CAA GTC ACG CTG CTC AAG GCG GGA GTG TTT
R G V V E F A K R L P G F Q Q L P Q E D Q V T L L K A G V F 240
GAG GTG CTG CTG GTG CGC CTG GCG GCC ATG TTT GAC GCC CGC ACC AAC ACC ATG CTG TGC CTC AAC GGG CAG CTG CTG CGT CGT GAA GCT
E V L L V R L A A M F D A R T N T M L C L N G Q L L R R E A 270
CTG CAC ACG TCA GTC AAC GCA CGC TTC TTA GTG GAC TCC ATG TTC GAC TTT GCT GAG CGC CTC AAC AGC CTG TGC CTC AGC GAT GCA GAG
L H T S V N A R F L V D S M F D F A E R L N S L C L S D A E 300
CTC GCA CTC TTC TGC GCC GTA GTC GTT CTT GCT CCT GAC AGA CCA GGT CTT CGC AAC GCA CAA CTT GTG GAG CGC GTG CAG AGG CAC CTT
L A L F C A V V V L A P D R P G L R N A Q L V E R V Q R H L 330
GTG AAC TGC CTT CAA ACT GTG GTG TCC AAA CAC CAC CCA GAG AAC CCC AGT CTG CAT CGC GAG CTG TTG GCC AAG ATC CCT GAC CTG CGC
V N C L Q T V V S K H H P E N P S L H R E L L A K I P D L R 360
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T L N T L H S E K L L K Y K M T E H T A A T S G P W D D S R 390
TCC TCC TGG AGC ATG GAG CAG GAG AGC AGC GTA GGC TCC CCA TCT TCC TCC TGT GCT GCA GAT GAG GCT ATG CGC TCT CCA GTC TCC TGC
S S W S M E Q E S S V G S P S S S C A A D E A M R S P V S C 420
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S E S M Y S G E S A S S G E S I C G S E V S G Y T E L R P P 450
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F P L V R R R H D N S E G A S S G D E A T E S P L K C P F S 480
AAA AGA AAA TCC GAC AGT CCT GAC GAT TCA GGC ATC GAG AGT GGA ACA GAC CGC AGC GAC AAG CTC TCA TCG CCA TCA GTT TGC TCG TCT
K R K S D S P D D S G I E S G T D R S D K L S S P S V C S S 510
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N T D L L M E E A Y K P H K K F R A L R R E E E P H S S Q P 600
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D L L H S M I M R N E V R E R L P S G S R V S P A P Y Y V P 690
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GTAATATTTGGGAGTTCAAATTTTGAGTCTTCA**AATAAA**TGTGCAAAGAAAAAAAAAAAAAAAAA-3'

Fig. 3-7. Comparison of deduced amino acid sequences of E75 cDNAs from crustaceans and insects. Amino acid sequences were aligned using ClustalW (see Materials and Methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical between all the sequences are hignlighted in black. Solid triangles indicate conserved cysteine residues in the zinc-finger DNA-binding motif in the C domain. Capital letters indicate boundary of each functional domain. Accession numbers: *Metapenaeus ensis* (MeE75, O77245); *Drosophila melanogaster* (DmE75A, NP730321; DmE75B, NP730323; DmE75C, P13055); and *Bombyx mori* (BmE75A, AF332550; BmE75B, BAA89263; BmE75C, AF332551).

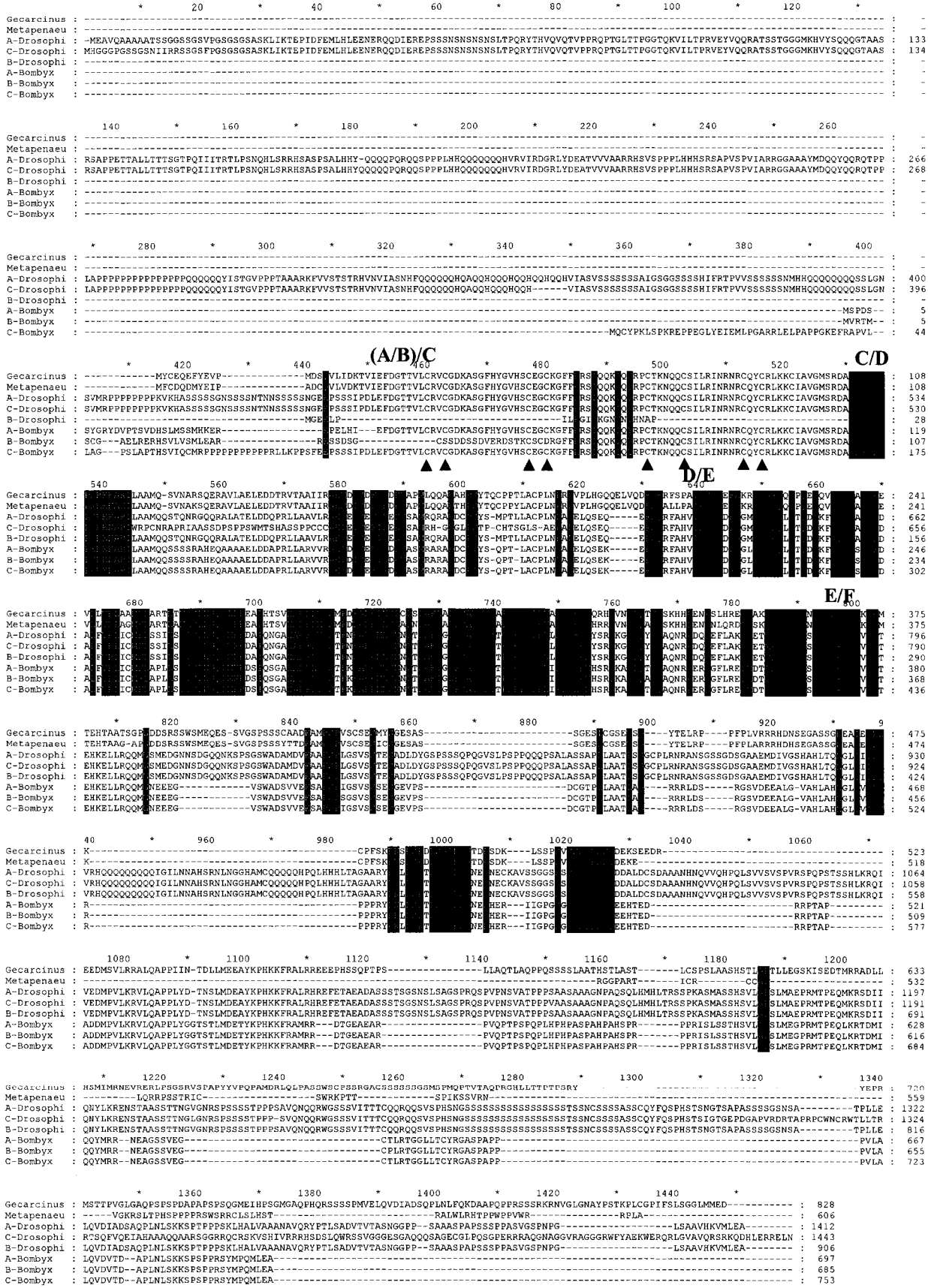


Figure 3-8. Domain organization and alternatively-spliced isoforms of arthropod E75 cDNAs. E75s from land crab (Gl-E75), shrimp (MeE75), fruit fly(DmE75A, B, and C), and silk moth (BmE75A, B, and C) are depicted. Domains are indicated by capital letters. Isoforms that differ in domain A/B are generated by alternative splicing. DmE75B, which lacks a DNA binding domain C, may act as negative regulator.

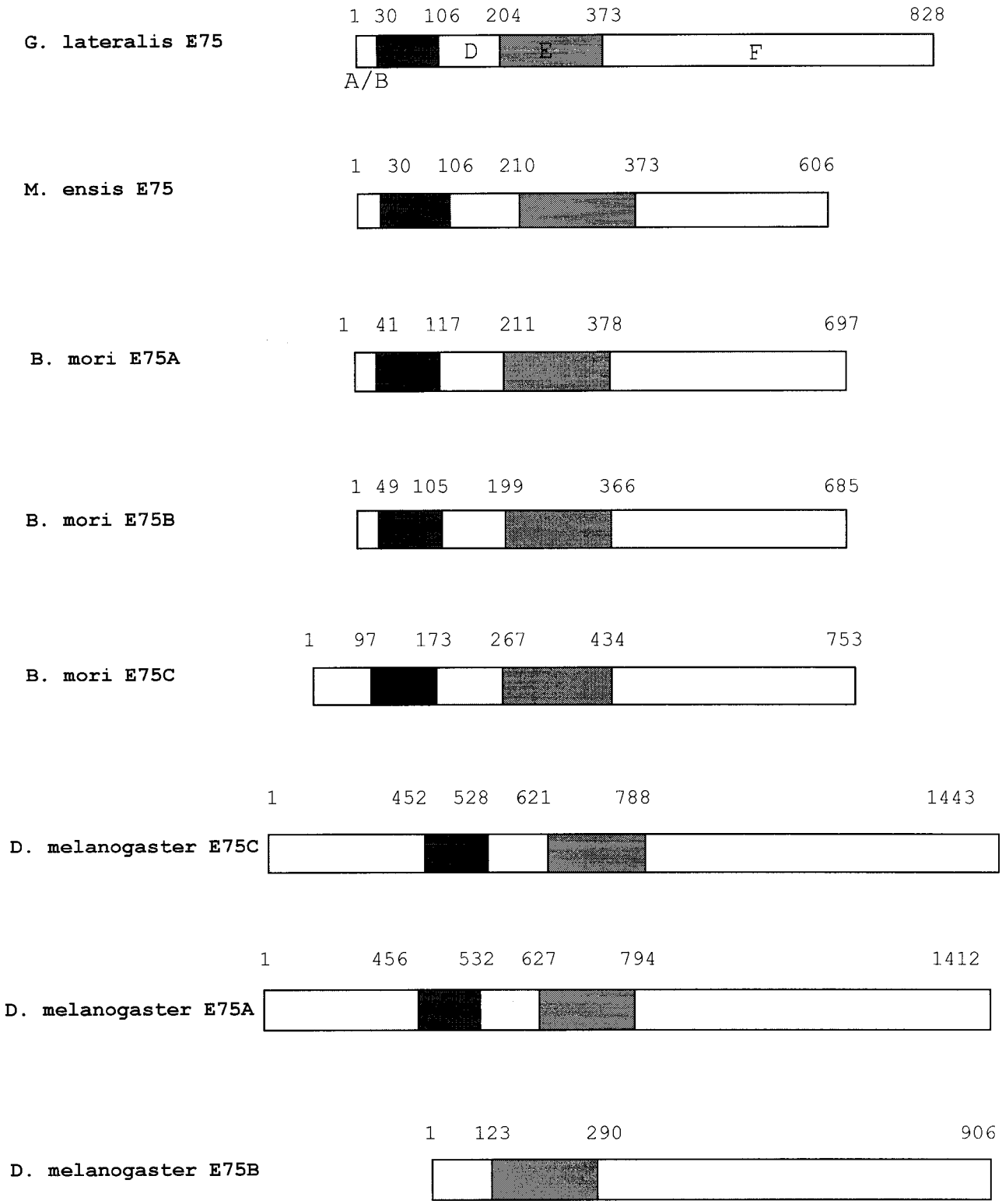


Figure 3-9. Expression of EcR, RXR, and E75 in land crab skeletal muscles using RT-PCR. Total RNA from thoracic muscle (top) and claw muscle (bottom) were DNase-treated, reverse-transcribed, and PCR-amplified using sequence-specific primers (see Materials and Methods). Shown is a reverse image of ethidium bromide-stained agarose gels of the PCR products. All three genes were expressed in both tissues, although RXR mRNA was higher in claw muscle than in thoracic muscle. However, only an isoform without the GAVEG sequence in the DNA-binding domain (DBD2) was expressed in thoracic muscle. Neither an 8-amino acid (AGHRRGQW) replacement variant in the DBD domain nor a 35-amino acid deletion variant in the LBD domain was amplified in either tissue.

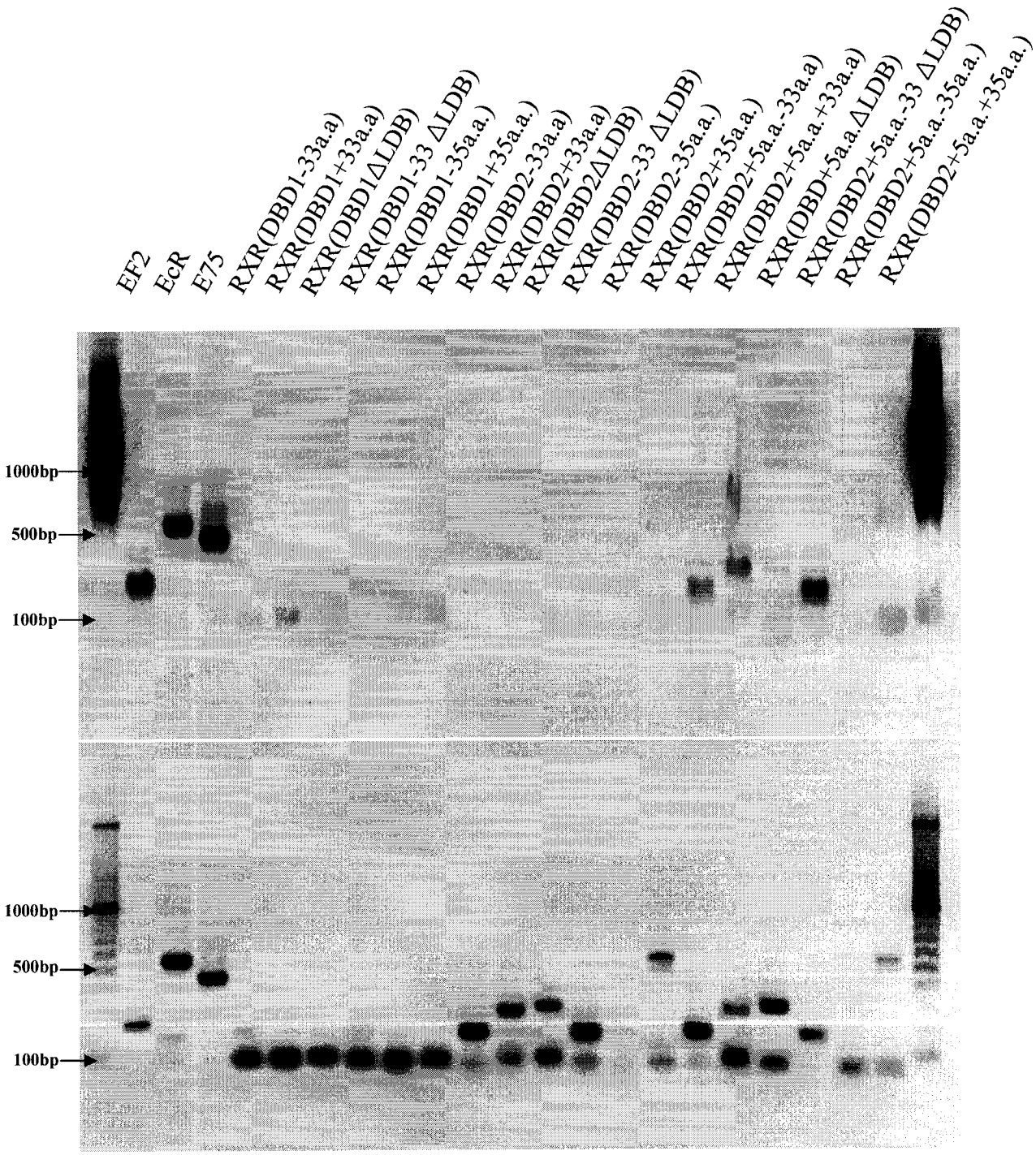


Figure 3-10. Effect of eyestalk ablation on expression of land crab EcR and calpains in thoracic muscle. Transcript levels, expressed as Log copy number, were quantified using real-time PCR. Elongation factor 2 (EF2) mRNA served as an internal standard to optimize the PCR reactions. P-values between means for each gene are given at the top of each graph (see Materials and Methods for statistical analysis). There was no significant effect of eyestalk ablation (1 or 3 days post-ablation), which increases hemolymph ecdysteroid levels, on EcR and calpain expression.

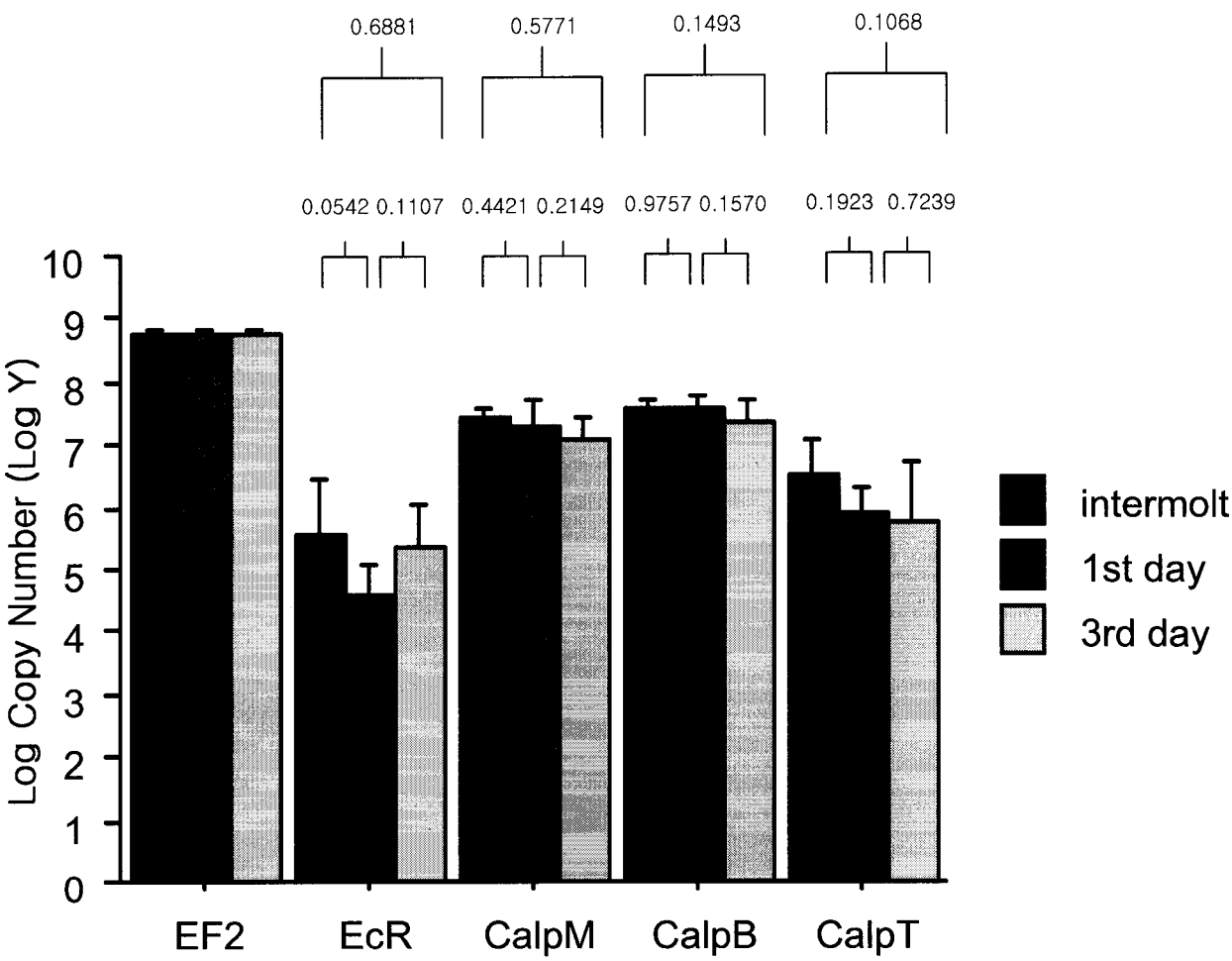
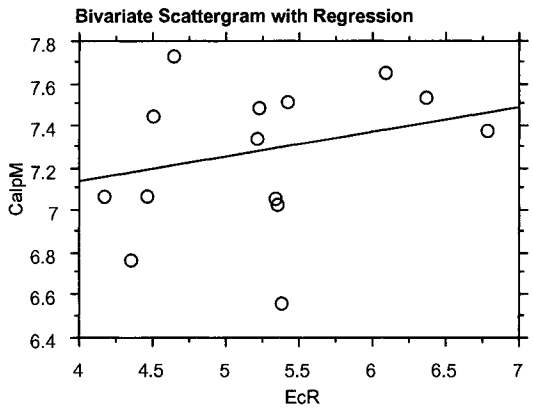
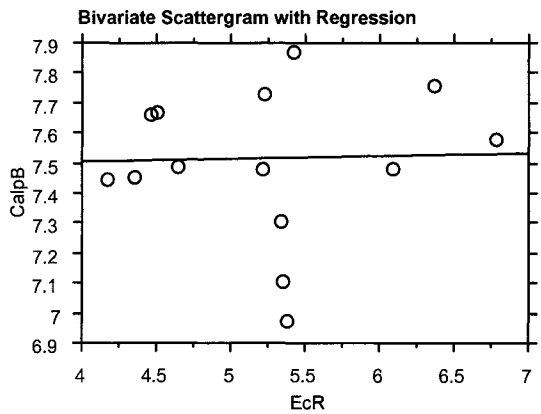


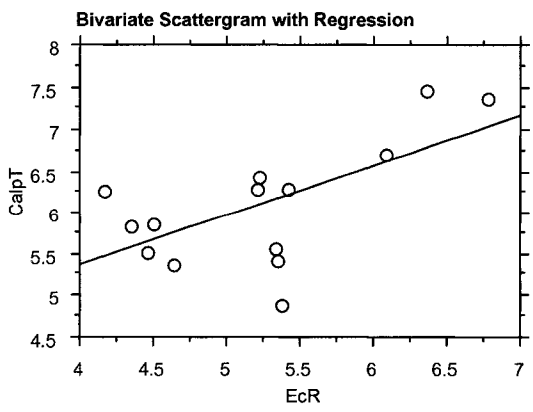
Figure 3-11. The relationship between EcR and calpain expression in thoracic muscle from intact and 1- or 3-day eyestalk-ablated land crabs. Correlations between each calpain mRNA and EcR mRNA were determined with Statview (see Materials and Methods). Only EcR and CalpT expression was significantly correlated (P = 0.0105).



CalpM = 6.676 + .116 * EcR; R^2 = .072 (P=0.3325)



CalpB = 7.467 + .009 * EcR; R^2 = .001 (P=0.9190)



CalpT = 2.988 + .596 * EcR; R^2 = .407 (P=0.0105)

Figure 3-12. Effect of eyestalk ablation on expression of land crab EcR and calpains in claw muscle. Transcript levels, expressed as Log copy number, were quantified using real-time PCR. Elongation factor 2 (EF2) mRNA served as an internal standard to optimize the PCR reactions. P-values between means for each gene are given at the top of each graph (see Materials and Methods for statistical analysis). Eyestalk ablation (1 and 3 days post-ablation) had a significant effect on EcR and CalpT mRNA levels, but no effect on CalpM and CalpB mRNA levels.

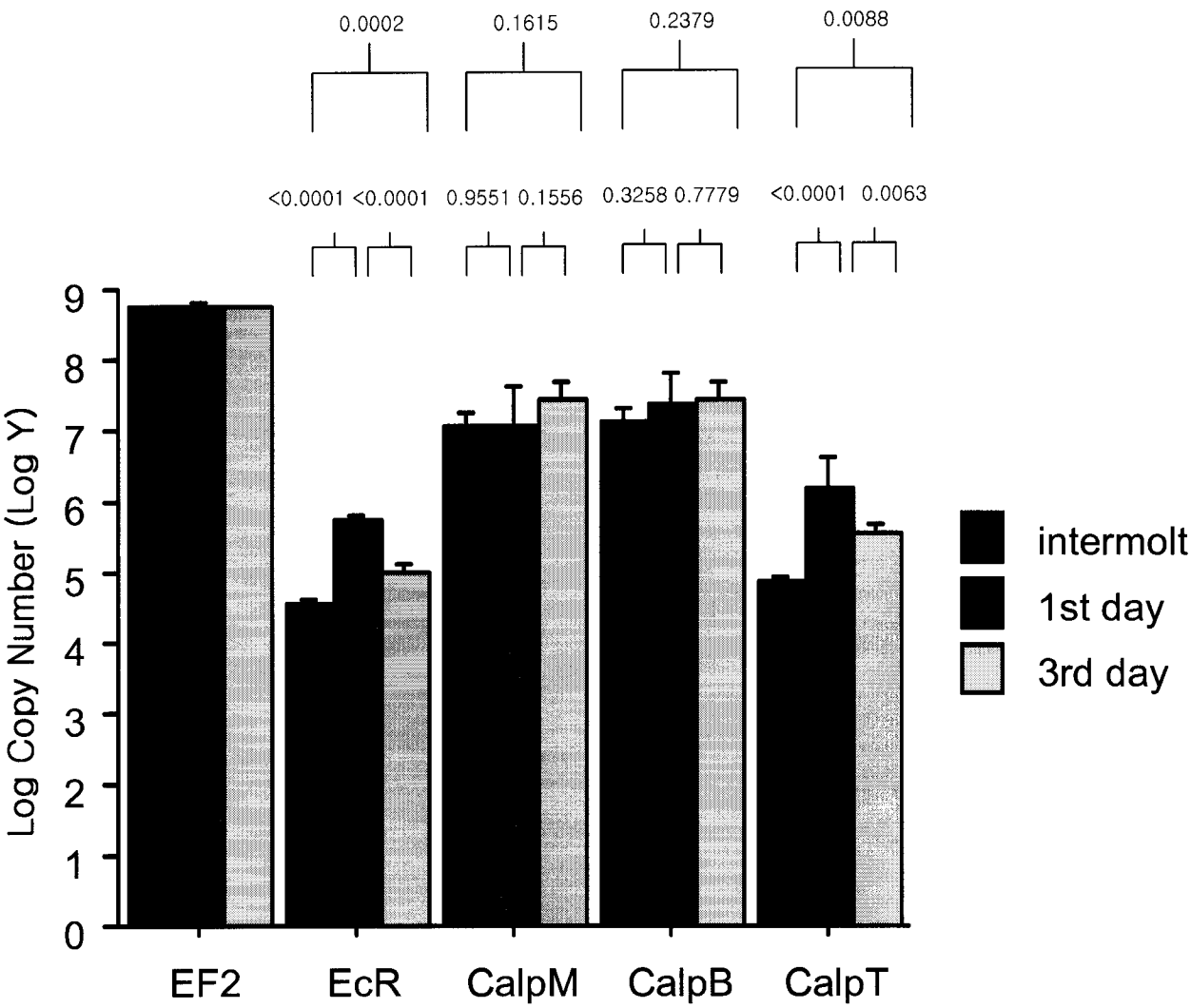
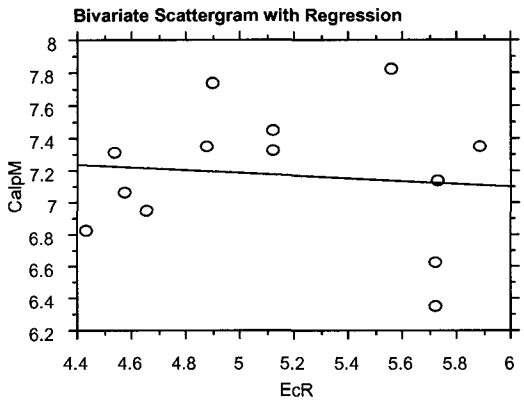
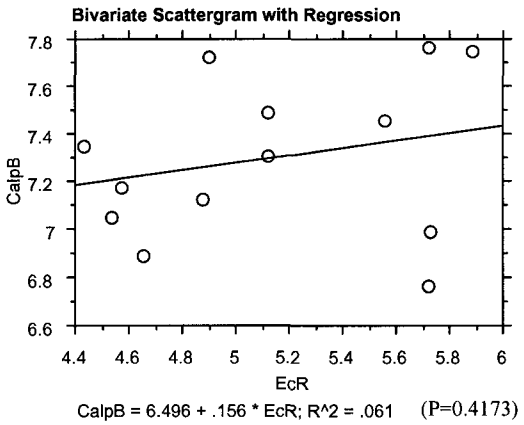


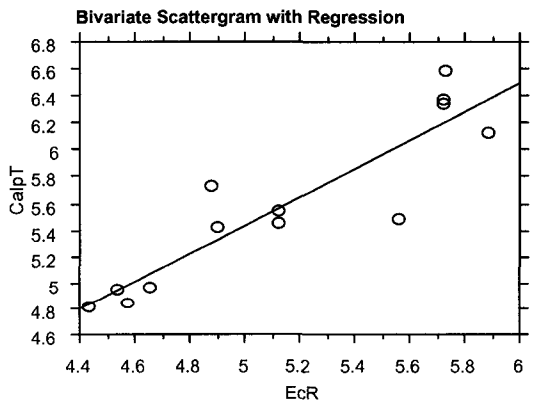
Figure 3-13. The relationship between EcR and calpain expression in claw muscle from intact and 1- or 3-day eyestalk-ablated land crabs. Correlations between each calpain mRNA and EcR mRNA were determined with Statview (see Materials and Methods). Only EcR and CalpT expression was significantly correlated ($P < 0.001$).



CalpM = 7.619 - .086 * EcR; R^2 = .012 (P=0.7259)



CalpB = 6.496 + .156 * EcR; R^2 = .061 (P=0.4173)



CalpT = .142 + 1.06 * EcR; R^2 = .827 (P<0.001)

Figure 3-14. Expression of EcR and calpains in leg muscle and limb regenerates. Transcript levels were quantified by real-time PCR (see Materials and Methods) and expressed as Log copy number (mean ± 1 S.D.). P-values between means for each gene are given at the top of the graph (see Materials and Methods for statistical analysis). Differences between means were not statistically significant, except for the difference in CalpB means between leg muscle and 2° limb regenerates (P = 0.0068).

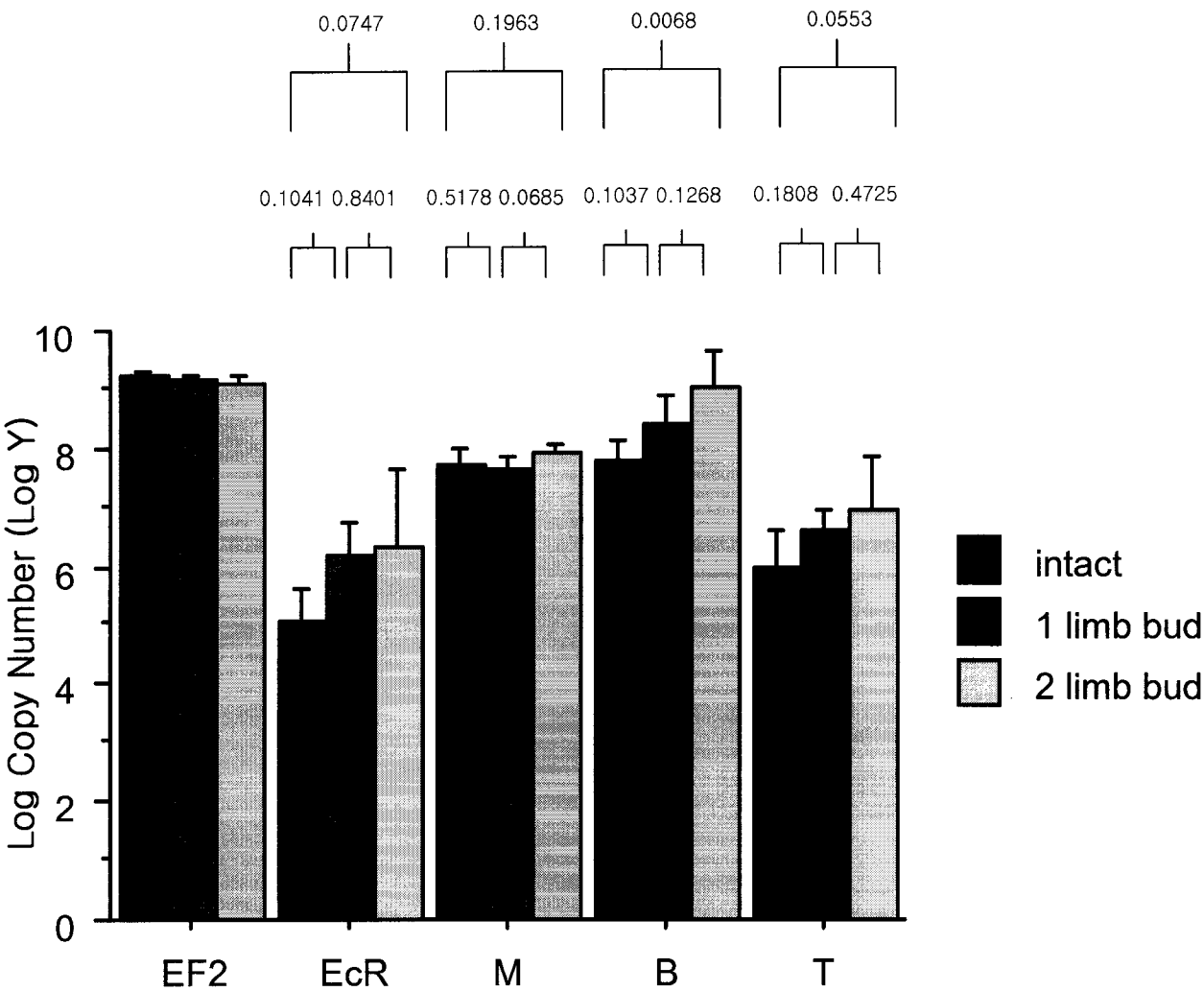
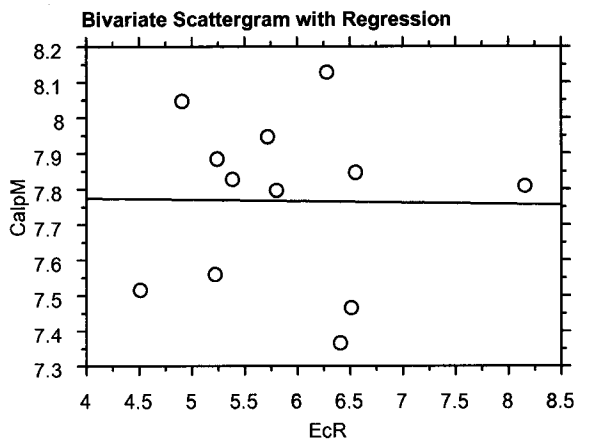
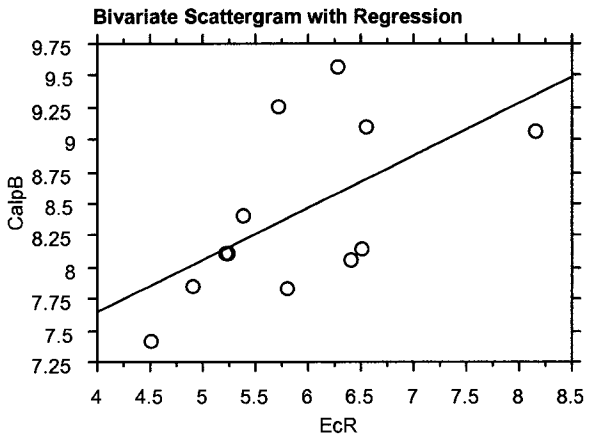


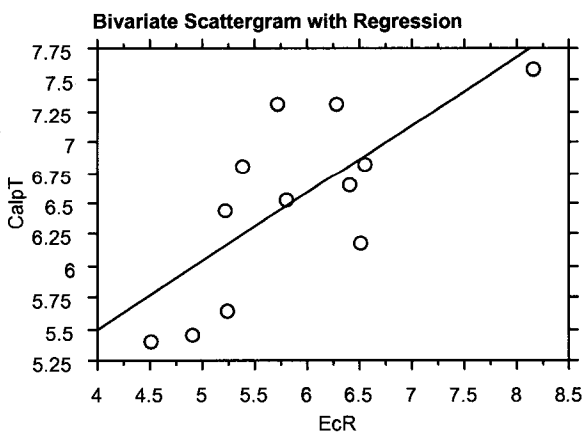
Figure 3-15. The relationship between EcR and calpain expression in leg muscle and limb regenerates. Correlations between each calpain mRNA and EcR mRNA were determined with Statview (see Materials and Methods). The mRNA levels of Calp B and CalpT were significantly correlated with EcR mRNA levels ($P = 0.0425$ and $P = 0.0066$, respectively).



CalpM = 7.793 - .004 * EcR; R^2 = 2.72E-4 (P=0.9594)



CalpB = 6.021 + .407 * EcR; R^2 = .351 (P=0.0425)



CalpT = 3.3 + .547 * EcR; R^2 = .538 (P=0.0066)

CHAPTER FOUR

MOLECULAR CLONING OF THE LAND CRAB NITRIC OXIDE SYNTHASE FROM NON-NEURONAL TISSUES

Abstract

NO signaling is involved in many physiological processes in invertebrates. To study the possible roles of nitric oxide synthase (NOS) in molting, the first NOS cDNA from a crustacean was cloned. RT-PCR with degenerated primers and 5' and 3' RACE generated the full-length sequence (3982 bp) of land crab NOS cDNA (Gl-NOS). The ORF encoded a protein of 1199 amino acids with an estimated mass is about 136 kDa. Gl-NOS had the highest sequence homology with *Drosophila* NOS. The motifs for binding tetrahydrobiopterin in the oxygenase domain, binding calmodulin, and binding FMN, FAD and NADPH in the reductase domain were well conserved. Interestingly, Gl-NOS had amino acid isoforms in all three motifs for binding FAD, which distinguished it from other NOS cDNAs. RT-PCR showed that the Gl-NOS was expressed in testis, gill, ovary, eyestalk neural ganglia, and Y-organ. Although Gl-NOS had high homology with mammalian neuronal NOS, its expression in thoracic ganglion was often below detectable levels. Interestingly, NOS expression varied between preparations of Y-organ and gill, while expression was at consistently high levels in the ovary, testis, and eyestalk ganglia. This is the first report of NOS expression in crustaceans non-neuronal tissues, which suggests that NOS has functions in addition to neuromodulation. NO may be involved in

regulating ecdysteroid synthesis in the Y-organ.

Introduction

Nitric oxide (NO) appears to have evolved as a signaling molecule before the radiation of the metazoans (Feelish, 1995). NO is generated by nitric oxide synthase (NOS) from L-arginine, O_2, and NADPH and diffuses freely across the cell membrane to induce responses in neighboring cells. The best known NO signaling pathway is one in which NO activates a soluble guanylyl cyclase (sGC). Activated NO-sensitive sGC produces cyclic 3',5'-guanosine monophosphate (cGMP) which, in turn, activates cGMP-dependent protein kinase (PKG). In mammals, NO/cGMP signaling is involved in vasodialtion, neurotransmission, and the immune response (Bredt and Snyder, 1994). In insects, NO signaling is involved in many physiological processes (Davies, 2000). NO regulates nervous system development and maintenance in insects (Truman et al., 1996). The hematophagous insect, *Rhodnius prolixus,* produces NO, which dilates blood vessels and inhibits platelet aggregation in the host (Ribeiro and Nussenzveig, 1993). Recent studies show that NO/cGMP pathway is involved in the insect immune response (Luckhart et al., 1998; Imamura et al., 2002; Weiske and Wiesner, 1999). The NO/cGMP signaling inhibits steroid synthesis in the ovary in blow fly (Maniere et al., 2003).

In mammals there are three NOS genes: nNOS, neuronal NOS, eNOS, endothelial NOS, and iNOS, inducible NOS (Nathan and Xie, 1994). Although their expression and biological roles vary, they share a common structural organization (Kone et al., 2003). The N-terminal oxygenase domain contains the binding motif for a P450-like cysteine

thiolate-ligate heme, and tetrahydrobiopterin (H4B). The C-terminal reductase domain contains the binding motif for FAD, FMN, and NADPH. Those two domains are linked by a calmodulin binding motif. The nNOS and eNOS are constitutively expressed and their enzymatic acitivities are regulated by the intracellular Ca^{2+} concentration through binding of Ca^{2+} to calmodulin (CaM)(Roman et al., 2002). They contain 40-50 amino acid inserts linked to the FMN binding motif that act as autoinhibitory loops blocking electron transfer from FMN to the heme in the absence of Ca^{2+}/CaM (Salerno et al., 1997; Nishida and Montellano, 2001). In contrast, iNOS lacks the autoinhibitory loop and binds CaM with high affinity at low Ca^{2+} levels; its activity is regulated predominantly at the transcriptional level (Nathan and Xie, 1994).

Insect NOSs have the highest sequence identity with nNOS and share the same organization in the oxygenase, CaM-binding, and reductase domains (Davies, 2000). Insect NOSs require NADPH, Ca^{2+} and CaM for enzymatic activity. They are expressed in a variety of tissues, including abdominal nerve cord, antenna, midgut, embryo, and Malpighian tubule (Nighorn et al., 1998; Luckhart et al., 1998; Broderick et al., 2003). Isoforms of NOS are generated by alternative splicing. The *Drosophila* NOS gene contains at least four alternative promoters (Stasiv et al., 2001). Some truncated alternative splicing variants of the *Drosophila* NOS lacking the reductase domain may act as dominant negative regulators, as heterodimers would lack enzyme activity (Stasiv et al., 2001). In mosquito, *Anopheles stephensi*, alternative transcripts are more complex (Luckhart and Li, 2001). A total of 18-22 NOS transcripts are expressed, and three transcripts differing in size are significantly induced in *Plasmodium*-infected mosquitoes. Although a single NOS gene is present, it has a variety of roles in many physiological

processes and transcriptional regulation is key to regulating invertebrate NOS activity.

In crustaceans, NO signaling plays a role in olfaction and neuronal development and regulation (Scholz et al., 1998; Scholz et al., 2001; Johansson and Mellon, 1998). NOS is expressed in the somatogastric and cardiac ganglia. The NO/cGMP signaling pathway is required for the dynamic assembly of the neuronal circuit that drives rhythmic movement in crabs (Scholz et al., 2001; Scholz et al., 2002). NOS activity was also identified in eyestalk ganglion, which suggests that NO may be involved in vision and in regulating the X-organ/sinus gland neurosecretory complex (Lee et al., 2000). The biochemical properties of crustacean NOS are similar to mammalian nNOS, as it also requires NADPH, Ca^{2+} and CaM for activity (Scholz et al., 2002; Lee et al., 2000).

NOS/cGMP signaling pathway may be involved in regulating molting in crustaceans. Molting inhibiting hormone (MIH), a neuropeptide synthesized in a neurosecretory center (X-organ/sinus gland complex) in the eyestalk acts as a negative regulator of ecdysteroidogenesis in the molting gland or Y-organ (Lachaise et al., 1993). The signal transduction pathway is poorly understood. cDNA encoding high affinity MIH receptors in the Y-organ membrane have yet to be characterized. Both cAMP and cGMP have been implicated as second messengers, although cGMP appears to be the main messenger regulating Y-organ ecdysteroidogesis. MIH induces 2-fold increase in cAMP and 60-fold increase in cGMP in Y-organ (Saïdi et al., 1994). PKG is involved in MIH inhibition of the Y-organ *in vitro* (Baghdassarian et al., 1996).

In this study we cloned a cDNA encoding NOS (Gl-NOS) from the land crab (*Gecarcinus lateralis*) and determined the tissue expression of NOS mRNA and protein. NOS is expressed in non-neuronal tissues, including the Y-organ. These data suggest that

NOS may be involved in regulating ecdysteroid synthesis by MIH.

Materials and Methods

Animals

Adult land crabs (*Gecarcinus lateralis*) were collected from San Miguel Reserve near Fajardo, Puerto Rico. They were kept in covered plastic cages with aspen beddings moistened with tap water at 27 °C and 50% humidity and were fed cat chow, carrots, and lettuce twice times a week. A 12 h dark/12 light cycle was used.

Cloning of Gl-NOS cDNA

Partial NOS cDNAs were initially obtained by the nested RT-PCR using degenerate primers directed to highly conserved sequences in a wide variety of NOS genes in the GenBank database (http://www.ncbi.nlm.nih.gov), including those from six insects and three human (nNOS, eNOS, iNOS) and aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html). Two sets of degenerate primes were designed to anneal to DNA sequences encoding F(S/N)GWYM, VF(H/F)QEM or TFGNG(E/D)PP: NOS F1, 5'-TT(C/T) (A/T)(G/C/A)(A/G/T/C) GG(A/G/T/C) TGG TA(C/T) ATG-3'; NOS F2, 5'-GT(A/G/T/C) TT(C/T) (C/T)(A/T)(C/T) CA(G/A) GA(G/A) ATG-3'; NOS R1, 5'-GG(A/G/T/C) GG(A/G/T/C) TC(A/G/T/C) CC(G/A) TT(A/G/T/C) CC-3'; R2, 5'-G(A/G/T/C) TC(A/G/T/C) CC(G/A) TT(A/G/T/C) CC(G/A) AA(A/G/T/C) G-3'). All the primers were synthesized and purified by Integrated DNA Technologies, Inc. (Des Moines, Iowa ,USA).

Total RNA was isolated from thoracic ganglia and Y-organ using RNeasy Protect

mini kit (Quiagen). About 20 mg of tissue and 600 μl of RTL reagent were used for each spin column unit. Total RNA (100 μg) was used for isolating mRNA with a Oligotex mRNA isolation kit (Qiagen). cDNA was synthesized according to the manufacturer's protocol using the Superscript II RNase H- reverse transcriptase first-strand synthesis system (Invitrogen, Inc.). Briefly, 12 μl of a mixture containing 1 μl oligo (dT)12-18 (500 μg/ml), 100 ng RNA, and 1 μl 10 mM dNTPs was heated to 65 °C for 5 min and chilled on ice for 1 min. 4 μl 5X First-Strand Buffer, 2 μl 0.1 M DTT, and 1 μl RNaseOUT, recombinant ribonuclease inhibitor (40 units/μl) were added. The mixture was incubated at 42 °C for 2 min. The reaction was initiated by the addition of 1 μl (200 units) SuperScript II at 42 °C for 50 min. The reaction was inactivated by heating at 70 °C for 15 min. PCR reactions were performed using an ABI 9600 thermal cycler (Perkin-Elmer, Inc.). The first PCR reaction contained 3 μl cDNA, 3 μl 10 X Takara EX Taq buffer, 2 μl 250 μM dNTPs, 1 μl forward degenerate primer (NOS F1), 1 μl reverse degenerate primer (NOS R1), 0.2 μl Takara EX Taq DNA polymerase (5 units/μl), and 18.8 μl PCR grade deionized water. Initial denaturation (95 °C for 5 min) was followed by 35 amplifying cycles (95 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 1 min) and final extension at 72 °C for 7 min. For the second PCR reaction, 0.1 μl of the first PCR reaction and the nested degenerate NOS F2 and NOS R2 primers were used. Other reaction components and PCR conditions were same as those in the first reaction.

PCR products were separated with 2% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were purified from gel slices using QIAquick Gel Extraction mini kit (Qiagen), ligated into PCR2.1 vector with the TOPO TA Cloning kit (Invitrogen), and transformed into One Shot TOP 10 *E. coli* strain (Invitrogen), followed

by PCR with T7 and M13 reverse vector primers. Plasmids were purified using Qiagen spin mini prep kit and sequenced using T7 and M13 reverse vector primers (Davis Sequencing, Davis, CA,USA).

RACE (Rapid Amplification of cDNA Ends) of mRNA was used to obtain full-length sequences. Poly (A^+) RNA was isolated from total RNA using Oligotex mRNA kit (Qiagen). For the 3' sequence, the RACE System (Invitrogen) was used. Briefly, first-strand cDNA synthesis reactions contained 200 ng claw poly(A^+) RNA and adaptor primer (5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3'). First round PCR on the cDNA (20 ng) included a universal amplification primer (5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3') and gene-specific forward primer, cNOS F1 (5'-CACTATGGCTGAGTGTGTCT ACCAGAAG -3') under the following conditions: denaturation at 96 °C for 5 min, 35 amplification cycles (96 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 2 min), and final extension at 72 °C for 10 min. Nested PCR on 30 µl of each reaction was conducted with a gene-specific primer, cNOS F2 (5'-AGCTGAGGTCCATTGTGCAGGAGCATG-3') and an abridged universal amplification primer (5'-GGCCACGCGTCGACTAGTAC-3') under the same conditions as the first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

The SMART™ RACE cDNA amplification kit (BD Biosciences, Inc.) was used to obtain the 5' sequence. The first-strand cDNA synthesis reaction contained 3 µl poly(A+) RNA (100 ng), 1 µl 5' CDS primer [10 mM, 5'-(T)25N-1N-3'], and 1 µl SMART II A oligo (10 mM, 5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') and was incubated at 68 °C for 2 min. After chilling the reaction on ice for 2 min, 2 µl 5X First-

Strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 30 mM $MgCl_2$], 1 µl DTT (20 mM), 1 µl dNTPs (10 mM), and 1 µl PowerScript Reverse Transcriptase were added. The reaction was covered with 20 µl paraffin oil and incubated at 42 °C for 1.5 h in a ABI 9600 DNA thermal cycler (Perkin-Elmer). The reaction mixture was diluted 10-fold with autoclaved distilled water and was used for first-round PCR with 10X universal primer A Mix (0.4mM 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAA CGCAGAGT-3' and 2 mM 5'-CTAATACGACTCAC TATAGGGC-3') and gene-specific reverse primer, cNOS R1 (5'-CGAAGT CCTCCCCATTCTCAGGAG -3' under the following conditions: denaturation at 96 °C for 5 min, 35 amplification cycles (96 °C for 30 sec, 66 °C for 15 sec and 72 °C for 3 min), and final extension at 72 °C for 10 min. Second-round PCR was conducted using nested gene-specific primer cNOS R2 (5'-AGCTTACTTGTGAACTTGACGGCTCTG-3') and nested universal primer A (10 mM, 5'-AAGCAGTGGTATCAACGCAGAGT-3'). The PCR conditions were the same as those used for first-round PCR. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Purified products were sequenced to confirm identities.

Gl-NOS expression by RT-PCR

Integument, thoracic ganglia, testis, ovary, heart, digestive gland, gill, claw muscle, eyestalk neural ganglia and Y-organ were dissected from 3-5 crabs and immediately placed in RNAlater RNA stabilization eagent (Qiagen). Tissues were stored at -20 °C until RNA extractions could be performed. Total RNA was isolated from pooled tissues using either the RNeasy mini or midi kit according to the manufacturer's instructions (Qiagen).

RNA concentration was determined by UV absorbance at 260 nm and stored at –80 °C. About 1 μg of total RNA was used for the reverse transcription reaction. RNA was first treated with DNase I to degrade any contaminating genomic DNA. First-strand cDNA was synthesized in a 20 μl reaction volume containing 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 40 units of RNaseOUT ribonuclease inhibitor, 1 ng oligo dT primer and 200 units of moloney murine leukemia virus reverse transcriptase (Invitrogen). The reaction was incubated for 50 min at 37 °C, heat-inactivated, and stored at -20 °C.

The quality of the cDNA was first verified by performing PCR with land crab elongation factor 2 primers (cEF F1, 5'-TTCTATGCCTTTGGCCGTGTCTTCTC-3'; cEF R1, 5'-TGATGGTGCCCGTCTTAACCAGATAC-3'). The PCR conditions were an initial denaturation at 95 °C for 2 min, then 35 amplification cycles (denaturation at 94 °C for 30 sec, annealing at 61 °C for 30 sec, and extension at 72 °C for 30 sec), followed by 2 min at 72 °C as a final extension.

NOS PCR was then performed in a 20-μl reaction mixture as described above using 2 μl of the first strand cDNA and a NOS gene-specific primer pair (cNOS EXF, 5'-CAACTTGAGAAGGAATAAAAGGGGAGGATG-3'; cNOS R31, 5'-CTGCTGAAGCTGCTGCCTCTGTCTTGAG -3'), each at a final concentration of 0.2 μM. The PCR conditions were an initial denaturation at 95 °C for 2 min, then 35 amplification cycles (denaturation at 96 °C for 20 sec, annealing at 62 °C for 20 sec, and extension at 72 °C for 90 sec), followed by 4 min. at 72 °C as a final extension. This primary PCR reaction was then used as template with a nested NOS primer pair (cNOS F1, 5'-GTACAAGCAGGAGGACGGGAG-3'; cNOS R5, 5'-

AGCTTACTTGTGAACTTGACGGCTCTG-3'), each at a final concentration of 0.2 μM as described above. The primary PCR reaction was diluted 1:10,000 in water and 2 μl used in the reaction. The PCR conditions were an initial denaturation at 95 °C for 2 min, then 35 amplification cycles (denaturation at 96 °C for 20 sec, annealing at 62 °C for 20 sec, and extension at 72 °C for 50 sec), followed by 4 min at 72 °C as a final extension. All PCR reactions were analyzed by separating some or all of the 20 μl reaction volume on 1% to 2% agarose gels with a 100 bp PCR Molecular Ruler DNA size ladder (BioRad).

Results

Cloning of the Gl-NOS

Nested PCR using NOS F2 and R2, amplified a product of about 400 bp. The deduced amino acid sequence had 72% identity with the calmodulin-binding and flavodoxin-like domain of *Drosophila* NOS (accession number AAC46882). 3'-RACE PCR did not generate any products using specific forward primers (cNOS F5: 5'-TCTTCGGTCACACCTTCAAT GCTC-3' or F6: 5'-CAAGTCAGAGATGTACGCCAAGAAG-3'). Instead, another reverse degenerate primer (NOS R3: 5'-RAADTARTCYTCRTGRTANC-3') was used for extending NOS cDNA fragment. A cDNA (1913bp) was amplified by PCR with the cNOS F5 and NOS R3 primer sets. The deduced amino acid sequence was 50% identical to NOS from the insect *Rhodnius prolixus* (accession number Q26240). A 1.5-kb PCR product was identified after 5' RACE PCR and a 500 bp product was amplified in 3' RACE PCR. The PCR products contained a full-length 3982-bp sequence of land crab NOS cDNA (Gl-

NOS).

The Gl-NOS cDNA encoded a protein containing 1199 amino acids with an estimated mass of about 136 kDa (Fig. 4-1). The Gl-NOS amino sequence was aligned with NOS sequences from five insects, one mollusk and three human types (Fig. 4-2). The N-terminal region was varied among different NOS genes, but the oxygenase domain in Gl-NOS was 70% identical with the *Drosophila* NOS (accession number Q27571), 68% identical with *Aplysia californica* NOS (accession number AAK83069), and 66% identical with human nNOS. In the oxygenase domain, the heme-binding motif was well conserved, including the cysteine residue that acts as an axial ligand. The motif for binding tetrahydrobiopterin cofactor (H4B) and CaM were also well-conserved. The reductase domain contained all conserved binding motifs for FMN, FAD and NADPH. Interestingly, the Gl-NOS has amino acid variants in all three motifs for binding FAD while no such variation is reported in other NOS cDNAs (Fig.4-2)

The phylogenetic relationships of various NOS sequences was determined using sequence alignments of the oxygenase and reductase domains (Fig.4-3). Insect NOS sequences grouped according to major taxonomic groups: Lepidoptera (*M. sexta* and *B. mori*) Diptera (*A. stephensi* and *D. melanogaster*), and Hemiptera (*R. prolixus*). Molluscan NOS (*A. california*) and vertebrate NOS formed distinct groups. Within the vertebrates, the inducible NOS (iNOS) and noninducible NOS (nNOS,eNOS) were divided. Since few NOS genes have been obtained from lower invertebrates (e.g., nematode), Gl-NOS could not be grouped with any other NOS, although overall homology comparison showed that Gl-NOS most closely related to the *Drosophila* NOS.

Gl-NOS expression by RT-PCR

The cNOS EXF and R31 PCR product (2110 bp) was amplified in testis, gill, ovary, eyestalk neural ganglia, and Y-organ after first-round PCR (Fig. 4-4). cDNA quality was confirmed by PCR with an elongation factor 2 primer pair. Nested PCR on first-round PCR product using cNOS F1 and R5 primers confirmed the identities of the 795-bp product as the NOS sequence (Fig. 4-4). NOS expression varied in Y-organ, thoracic ganglion (data not shown), and gill. In contrast, NOS was expressed at consistently high levels in eyetalk ganglia, ovary, and testis.

Discussion

To study the possible roles of NOS in molt regulation, a cDNA encoding a crustacean NOS was cloned and sequenced. The Gl-NOS has the highest homology to insect NOS, which is the homolog of nNOS in vertebrate species. It is divided into two major domains with a CaM-binding domain in between (Fig. 4-2). The oxygenase domain has a well-conserved binding motif for heme and tetrahydrobiopterin and the reductase domain has FAD, FMN and the NADPH binding sites. Interestingly, the Gl-NOS has a single amino acid change in all three FAD-binding motifs, but its effect on enzyme activity is unknown. Since the FAD-binding motif participates in electron transfer within the reductase domain, it may affect affinity of FAD binding, and therefore affect the rate of NO production.

NOS is expressed in the nervous system of crustaceans. The estimated mass of the Gl-NOS protein is 136 kDa, which is similar to the mass of a protein reacting to a universal anti-NOS antibody in immunoblots of extracts of crayfish eyestalk ganglia (Lee

et al., 2000). The NOS mRNA is found at high levels in land crab eyestalk ganglia (Fig. 4-4). Immunocytochemistry indicated two major locations of NOS in the crayfish eyestalk ganglia. NOS immunolocalization in the first chiasma suggests that NO may be play a role in the processing of visual information. Immunolocalization in the sinus gland suggests that NO may regulate the release of neuropeptides.

A 110-kDa protein from the cardiac ganglion of the rock crab (*Cancer productus*) reacts strongly the universal anti-NOS (Scholz et al., 2002), suggests that there may be a different type of NOS in crustaceans. Insect NOS isoforms differing in sizes are produced by alternative splicing (Stasiv, 2001). A truncated isoform may regulate NOS activity as a dominant repressor. A truncated form of Gl-NOS in the Y-organ was obtained by 3'-RACE, but its expression has not been studied in any detail (data not shown).

This is the first report of the presence of NOS in non-neuronal tissues of a crustacean. Its expression in gonad, Y-organ, and gill suggests that the NO/cGMP signaling pathway regulates a greater variety of physiological processes in crustaceans that was previously supposed. Since cGMP appears to be the main messenger regulating Y-organ ecdysteroidogesis, there are two possible signaling pathways. The increase in intracellular cGMP may result from the activation of a soluble NO-sensitive guanylyl cyclase (GC-I) and/or a membrane receptor guanylyl cyclase (GC-II). We have cloned partial cDNAs encoding the membrane and two soluble GCs (GC-I and GC-III) that are expressed in Y-organs and other tissues (see Appendix; data not shown). However, the effects of MIH on NOS and GC activities have not been performed. The NOS/cGMP signaling pathway is involved in regulating steroidogenesis in blowfly ovary; activation of a NO-sensitive GC inhibits ecdysteroid synthesis (Maniere et al., 2003).

In summary, the cloning and tissue expression of the first NOS from a crustacean is reported. The NO/cGMP signal pathway may be involved in regulating both ecdysteroidogenesis in the Y-organ and neuropeptide secretion in the sinus gland, thus playing a important role in coordinating organismal growth and reproduction.

Figure 4-1. The nucleotide and deduced amino acid sequences of land crab nitric oxide synthase (Gl-NOS) cDNA. The cDNA (3982 bp) containd a complete ORF encoding a protein of 1199 amino acids. Locations and directions of degenerate primers used for nested PCR to obtain the initial cDNA are indicated by bold letters and arrows with dashed lines. Arrows with solid lines indicate locations and directions of sequence-specific primers used for 5' and 3' RACE. The poly(A) signal is boxed.

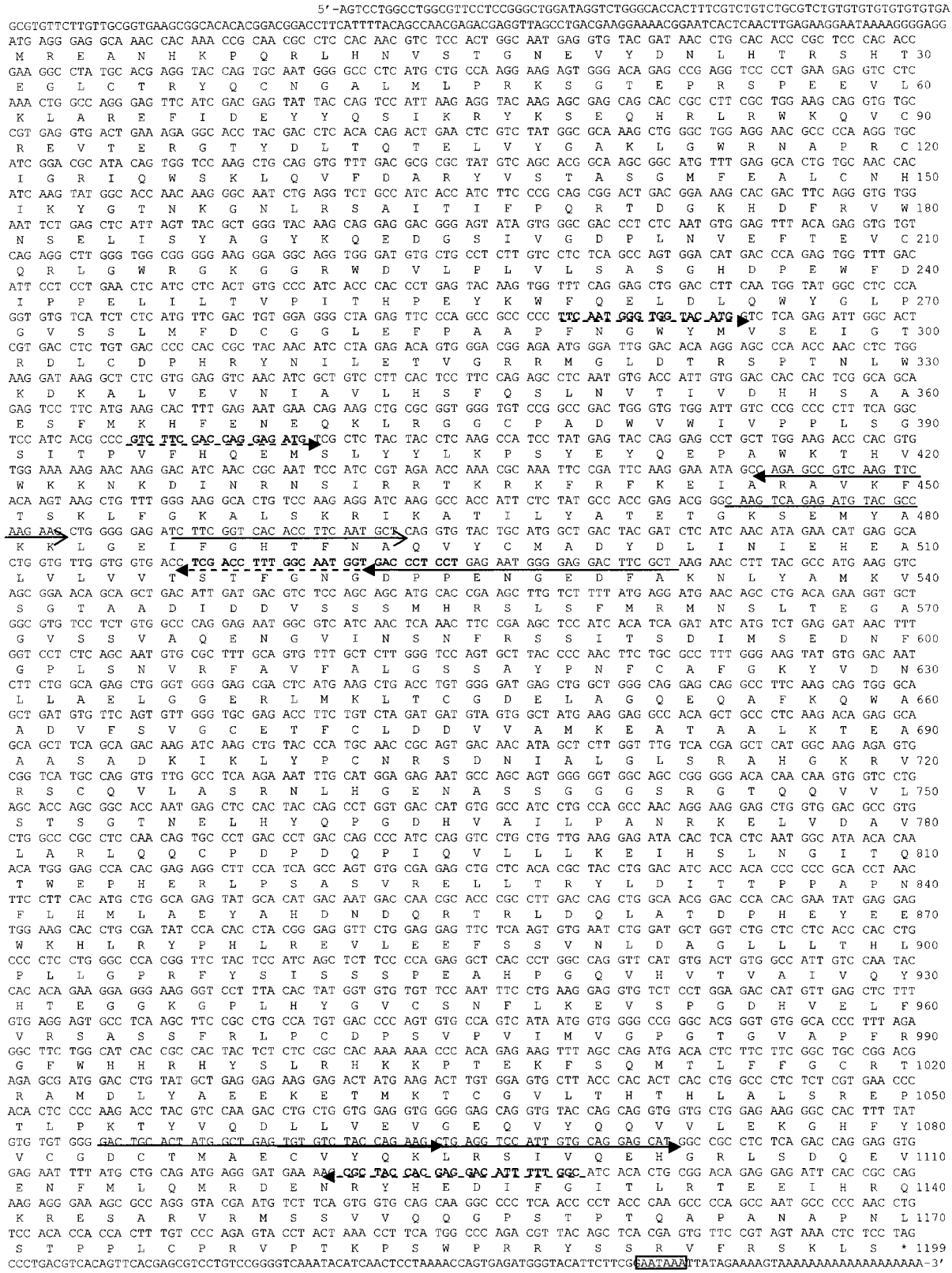


Figure 4-2. Comparison of deduced amino acid sequences of NO synthase from land crab, insects, mollusk, and human. Land crab (*Gecarcinus lateralis*) NOS was aligned with NOS sequences from insects (*Manduca sexta, Bombyx mori*, *Rodnius prolixus, Anopheles stephensi* and *Drosophila melanogaster*), mollusk (*Aplysia californica*) and human (nNOS, eNOS). Identities in all 8 sequences are highlighted in black. Boxes identify highly-conserved binding domains for heme, tetrahydrobiopterin (H4B), calmodulin, FMN, FAD(ADP) flavin, FAD(ribose), NADPH(ribose), NADPH(ADP), and NADPH. Inverted triangles indicate amino acid sequence deviation in the FAD-binding motif of Gl-NOS. Regular triangles indicate the two conserved cysteine residues in the zinc tetrathiolate cluster.

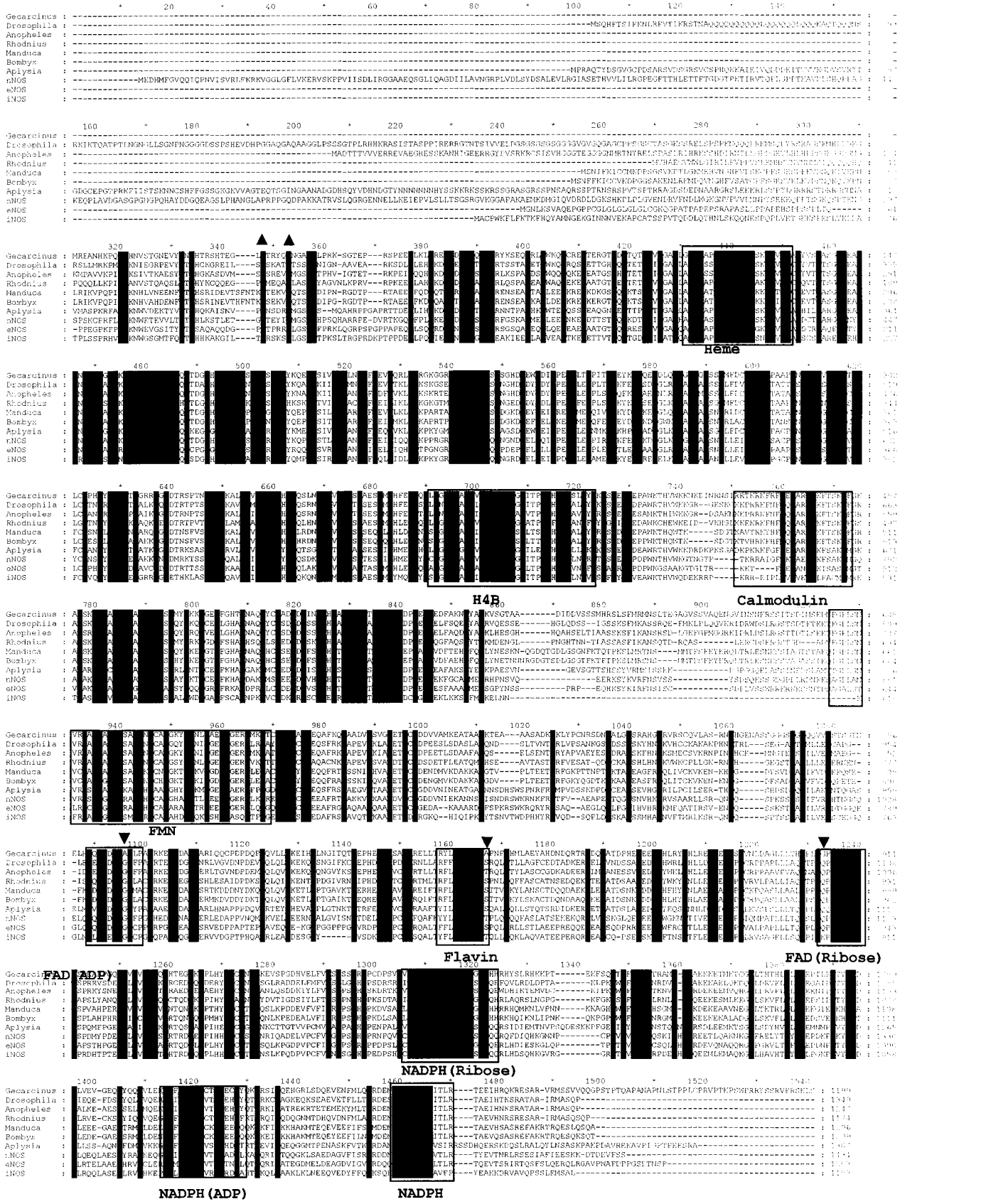


Figure 4-3. Phylogenetic relationships of NO synthases from arthropods, mollusk, and mammals. The deduced amino acid sequences of the oxygenase domain were analyzed using ClustalW and Treeview software (see Materials and Methods). Arthropod NOS sequences form a group diverged from molluscan and mammalian NOS sequences. Within the arthropods, the land crab NOS has diverged from those from insects. Within Insecta, NOS sequences group according to major taxonomic lineages: Lepidoptera (*Manduca* and *Bombyx*), Diptera (*Drosophila* and *Anopheles*), and Hemiptera (*Rhodnius*). Accession numbers: *Rhodnius prolixus*, Q26240; *Anopheles stephensi*, O61608; *Bombyx mori*, BAB85836; *Drosophila melanogaster*, Q27571; *Manduca sexta*, T30555; *Aplysia californica*, AF288780; and mammal iNOS (AAB49041), eNOS (NP000594) and nNOS (NP000611).

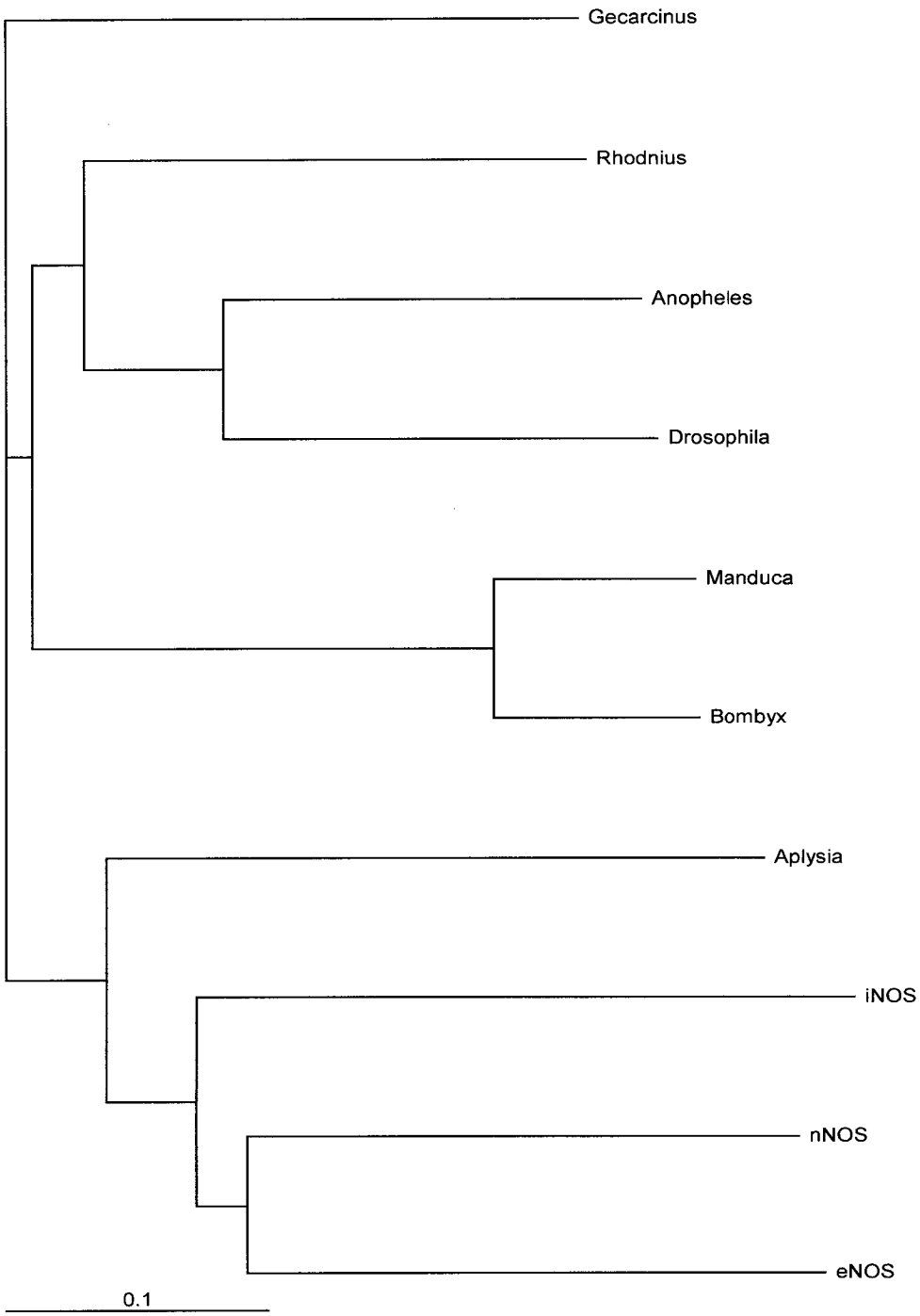
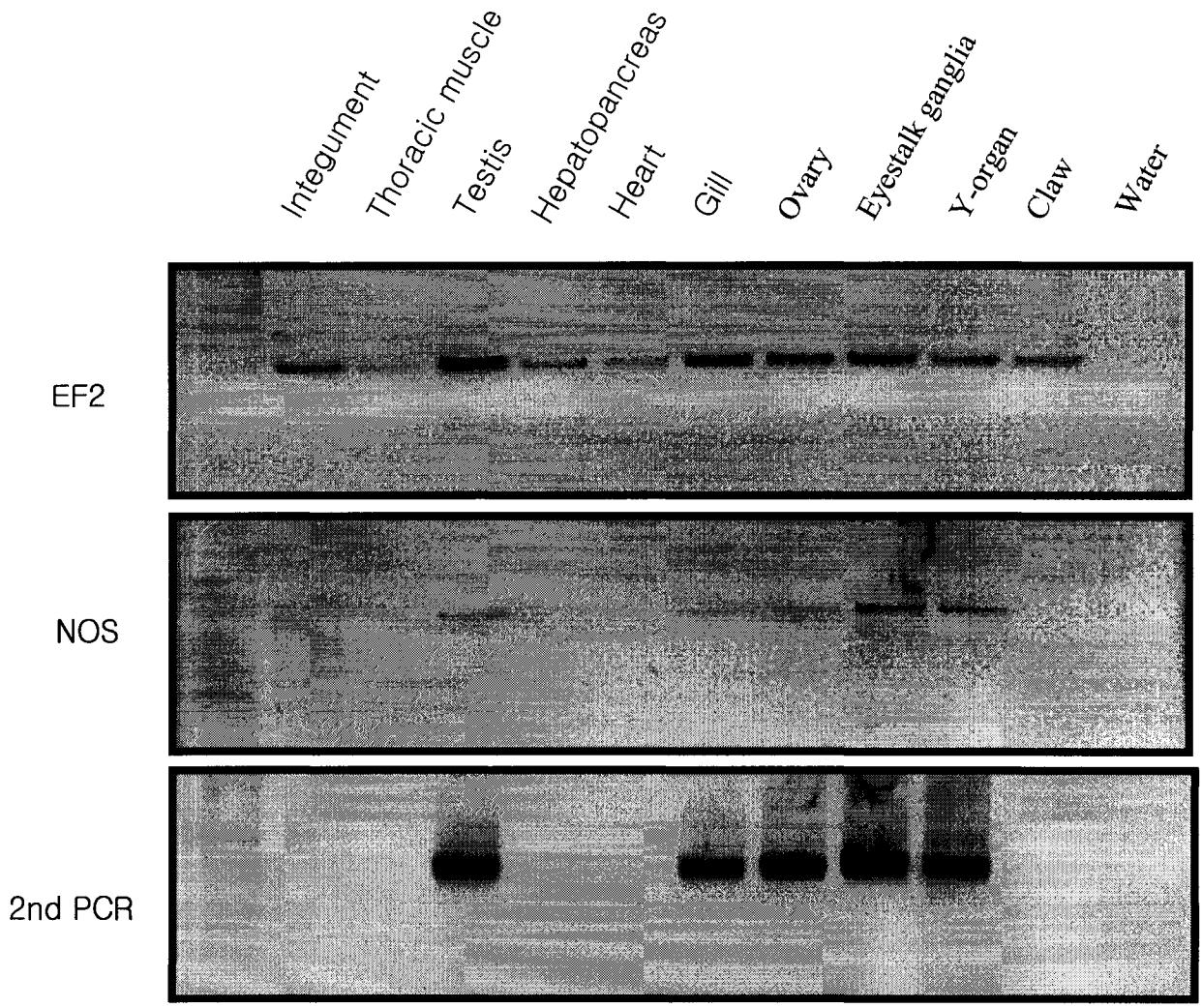


Figure 4-4. Expression of NO synthase (Gl-NOS) in land crab tissues. Total RNA was DNase-treated, reverse-transcribed, and PCR-amplified using sequence-specific primers (see Materials and Methods). Shown are reverse images of ethidium bromide-stained agarose gels of the PCR products. Elongation factor 2 (EF2) served as internal positive control (top). Gl-NOS was expressed in testis, gill, ovary, eyestalk neural ganglion, and Y-organ (middle). Nested PCR of the initial PCR confirmed the identity of the product as Gl-NOS (bottom). In other experiments, a Gl-NOS product was obtained from thoracic ganglion. Reactions without template (water) served as negative control.



CHAPTER FIVE

SUMMARY AND FUTURE RESEARCH DIRECTIONS

Molting is essential for organismal growth in arthropods. Claw muscle atrophy enables successful withdrawal of the claws from the old exoskeleton at ecdysis (Mykles and Skinner, 1990). Calpains play a major role in this claw muscle atrophy. Four activities (CDP I, IIa, IIb, and III) that degrade myofibrillar proteins to acid-soluble products have been characterized biochemically. In this study, three calpain genes were isolated (Gl-CalpM, Gl-CalpB, and Gl-CalpT) from land crab limb regenerates. Gl-CalpM and Ha-CalpM encode the CDP III activity. CalpM may be involved in normal myofibrillar protein turnover, as it is highly expressed in skeletal muscles and its expression is not altered by molt stage or by elevated ecdysteroid induced by eyestalk ablation (Yu and Mykles, 2003). The Ha-CalpM mRNA level is 4-fold higher in fast fibers than in slow fibers, which suggests that it is involved in maintaining the fast-fiber phenotype or that protein turnover differs between the two fiber types. In mammalian muscle, increased μ-calpain activity is involved in controlled proteolysis during fast-to-slow fiber-type transformation (Sultan et al., 2001). Gl-CalpM is also highly expressed in ovary and integument, suggesting it may have different roles in these tissues.

Gl-CalpB appears to encode CDP IIb, which is a homodimer of a 95-kDa subunit and the homolog of Dm-CalpA and Dm-CalpB (Beyette et al., 1993). It may have a housekeeping function, as it is expressed in all tissues examined. It may maintain the 9:1 thin:thick filament ratio in intermolt muscle, as CDP IIb, as well as CDP I, preferentially

hydrolyzes thin filament proteins and are the major calpain activities in claw muscle. The higher Gl-CalpB mRNA level in secondary limb regenerates suggest that Gl-CalpB may also be involved in muscle regeneration and growth. In mammals, activated m-calpain is translocated to the cell membrane in regenerating muscle (Sultan et al., 2000) and calpain 3 is increased in developing muscle (Tang et al., 2000).

Gl-CalpT, human Capn5, and nematode TRA-3 are members of the calpain T family. TRA-3 is a component of the pathway regulating sex determination in *C. elegans* (Barnes and Hodgkin, 1996), but the functions of the mammalian and crustacean orthologs are unknown. The expression patterns of crustacean and mammalian T calpains differ significantly (Tissue expression in nematode has not been determined). Calp5 is highly expressed in the colon, small intestine, and testis, whereas Gl-CalpT is highly expressed in hindgut, heart, digestive gland, ovary, and integument. Although Gl-CalpT is expressed at lower levels in intermolt skeletal muscle, it is the only calpain that is up-regulated in claw muscle in response to eyestalk ablation. This suggests that it plays a role in the initiation of muscle atrophy. Future work will determined its expression during the molting cycle.

Atrophy is coincident with increasing concentrations of ecdysteroids in the hemolymph, suggesting that protein degradation is under the regulation of 20E. In order to better understand the relationship between ecdysteroids and molting processes, ecdysone receptor genes (EcR and RXR) and an ecdysone early-response gene (E75) were cloned from the land crab. The ecdysone receptor is a heterodimer of EcR and RXR. Unlike insects, crustacean EcR and RXR lack alternative A/B domain isoforms (Durica et al., 2002), which have distinct physiological functions in some insect species (Minakuchi

et al., 2002; Wang, et al., 2002; Wang et al., 2000). However, other alternatively-spliced RXR isoforms may produce different functional receptors when complexed with the EcR. The Gl-RXR has same isoforms as UpRXR, including a 5-amino acid insertion in the C domain and a 33-amino acid deletion in the D domain. In addition, the Gl-RXR has two truncated isoforms that lack an E domain; another alternative isoform has a 35-amino acid deletion in the E domain.

The shrimp E75 has three N-terminal isoforms (Chan, 1998). The Gl-E75 is homologous to MeE75, but only one isoform was identified. Since the DNA binding motif is identical with that of insects, Gl-E75 may have the same DNA-binding properties as insect E75s. On the other hand, since the E domain has low amino acid homology with insects, its ligand may differ from that of insects. As the E domain is functionally the most important domain, not only binding for ligands but also for binding with multiple protein partners, the low identity in the E domain indicates that the crustacean and insect E75s differ in regulatory and transcriptional activation properties. Further study of the ligand-binding domain in crustacean E75 is needed. The Gl-E75 has a longer F domain compared with MeE75, and some specific regions within the F domain of Gl-E75 have high homology with those of *Drosophila* E75. These results suggest that MeE75 may be a truncated form of crustacean E75 generated by alternative splicing form. Similarly, the C-terminus of DmE75B differs from that of isoforms A and C because of alternative splicing.

Real time PCR shows that Gl-EcR and Gl-CalpT expression are induced by eyestalk ablation in the claw muscle, but not in the thoracic muscle. Atrophy is specific to the claw muscle (Mykles and Skinner, 1982) and these results indicate that claw and thoracic

muscle differ in sensitivity to 20E, even though they consist of the same slow-fiber type. The expression of Gl-EcR and Gl-CalpT was highly correlated in all the tissues examined, including claw muscle, thoracic muscle, and 1° and 2° limb regenerates. These results suggest that Gl-EcR controls Gl-CalpT expression. One possibility is that the EcR/RXR complex binds directly to the promoter of the Gl-CalpT gene and induces its expression. A second possibility is that ecdysone early response genes, such as E75 and E74, are induced by the EcR/RXR complex and they, in turn, bind to the Gl-CalpT promoter. Since the EcR/RXR complex induces E75 within 30 min, these early-response genes may induce Gl-CalpT. Additional study is needed to determine the time-course of EcR, RXR, E75, and CalpT gene activation by ecdysteroids.

Although Gl-CalpT expression appeared to relate with EcR expression, there is no direct evidence that Gl-CalpT have major role in muscle atrophy. In addition muscle atrophy is preferentially occurred in thin filaments (Mykles and Skinner, 1981) and CDPI and CDP IIb more efficiently degrade thin filaments than CDP IIa and CDP III (Mattson and Mykles, 1993). If CDP IIa is Gl-CalpT increased Gl-CalpT will cause to increase degrading thick filament. If not, there will be another calpain gene in claw muscle. One more possible explanation is that calpain activity is not mainly controlled in transcription level. In stead, activating Gl-CalpB or inhibiting Gl-CalpM by intracellular factors such as Ca2+, phosphorylation/ dephosphorylation, or other proteins cause preferential thin filament muscle atrophy. Once specific antibodies for each calpain are raised more information on calpain action for molt-induced muscle atrophy.

To study the possible roles of NOS in molt cycle we cloned NOS cDNA for the first time in crustacean. The isolated Gl-NOS (136 kDa) has the highest homology with insect

NOS which is homologue of nNOS in vertebrates. Interestingly, Gl-NOS has single amino acid replacement in all three FAD binding motif but its effect in NO production can not be expected. Since FAD motif participates in electron transfer within the reductase domain it may affect binding affinity of FAD and NO production consequently.

The possible roles of NOS in molting cycle in crab may be harmonized events in three tissues. First, NOS in the eye stalk ganglia, a neurohemal organ, suggests that NO may act on regulating endocrine system by NOS/cGMP pathway. Although we have no data to show whether NOS is expressed in the X-organ or in the sinus gland in land crab, NOS expression was detected in the sinus gland using α-universal NOS in crayfish suggesting NOS is involved in secretion of neuropeptides (Lee et al., 2000). If NOS/cGMP pathway regulates MIH or other neuropeptide secretion, it will affect on regulating ecdysteroidogenesis on Y-oragan. It is generally known that MIH level decreases in premolt (Lee et al., 1998; Nakatsuji and Sonobe, 2003) MIH level change and it effects during molt are still controversial (Chung and Wester, 2003; Ohira et al., 1997).

Second, presence of NOS in Y-organ suggests that NOS may be involved in regulating molting cycle by controlling ecdysteroid synthesis. Since cGMP appears to be the main messenger regulating Y-organ ecdysteroidogesis there are two possible signaling pathways. One is that NOS activates soluble GC and the other is that receptor GC regulated cGMP level. The other is receptor GC regulate cGMP level to regulate ecdysteroid synthesis. We also cloned three partial GCs from Y-organ (see Appendix). Since there is no data to show expression level change of NOS and three GCs in molting cycle, it is still unknown what roles of NOS and GCc in Y-organ and more study should

be made to understand link between cGMP level and ecdysteroidogesis.

Third, in addition to its expression in Y-organ, Gl-NOS was highly expressed in the ovary and testis. It is reported that cGMP (probably by NOS) inhibits steroidogenesis in the insect ovary (Maniere et al., 2003). Our immunocytochemistry data shows that NOS is concentrated around nucleus in ovarian cell while NOS is spread overall Y-organ (data not shown). This result is similar to the observation in insect (Maniere et al., 2003). They observed NOS in ovary was located in a few chambers which prematurately aborted vitellogenesis. Both Y-organ and ovary can synthesize ecdysteroid and this may be regulated by NOS/cGMP pathway in crustacean like in insects. However, whether or not MIH affects on ovary is still unknown in crustaceans. In insect, brain factors did not affect on ecdysteroidogenesis suggesting its regulation may be autocrine or paracrine (Maniere et al., 2003). Although it may not be regulated by MIH, ecdysteroids synthesized in the ovary or testis affect on overall titer in blood stream in crustacean. This may explain individual variation in ecdysteroid titer in molt cycle.

NOS study in crustacean will help to understand the physiological regulation in various aspects. Growth, molting and reproduction should be linked and controlled by each other and NOS/cGMP signaling pathways in three different tissues may play important roles in communicating different tissues. Since the land crab is a good model system in crustacean biology as explained introduction, Gl-NOS study will lead us to understand not only molt cycle but also other various physiological questions like reproduction or growth.

APPENDIX

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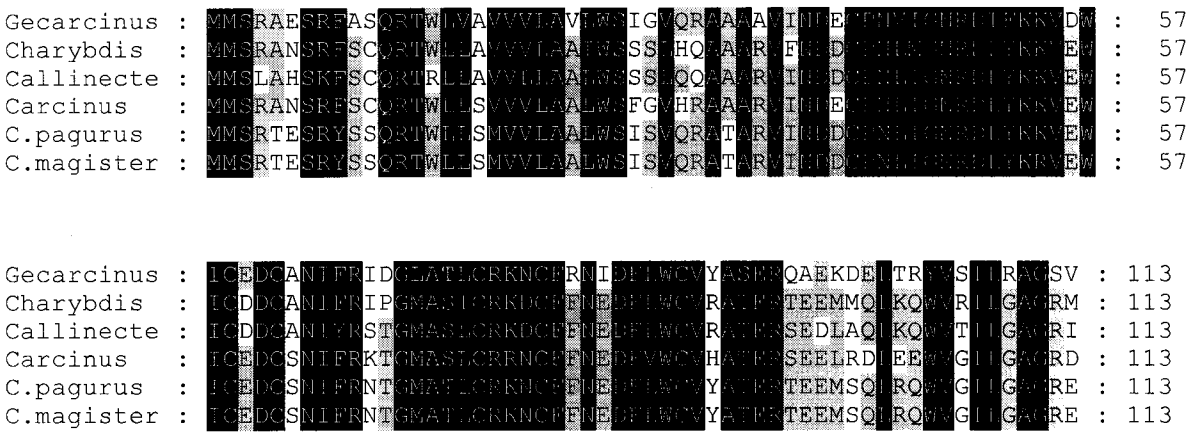
5'-GCTGAACACCAGACAGACGCCTCCTCCTGCTCCTGCTTCCACCTACTCCTTCCAC
GGCGCCCCTCAGCCTCATTATTCCCATTTCTCCCTCACTCCTTCAGCGCCTCCGTCCA
CGCCTCCGTCACCTCGAGCACCTCAAAGCCAAC

ATG ATG TCA CGC GCT GAA TCC AGA TTT GCC TCC CAG AGG ACG TGG
 M   M   S   R   A   E   S   R   F   A   S   Q   R   T   W
CTG GTG GCG GTG GTG GTT CTG GCT GTC CTG TGG AGC ATC GGT GTC
 L   V   A   V   V   V   L   A   V   L   W   S   I   G   V
CAG CGG GCC GCG GCA GCC GTC ATT AAC GAT GAA TGT CCA AAC GTG
 Q   R   A   A   A   A   V   I   N   D   E   C   P   N   V
ATT GGA AAC CGC GAC ATT TTC AAG AAG GTG GAC TGG ATC TGC GAG
 I   G   N   R   D   I   F   K   K   V   D   W   I   C   E
GAC TGT GCC AAT ATC TTC CGC ATC GAC GGC CTG GCC ACC CTC TGC
 D   C   A   N   I   F   R   I   D   G   L   A   T   L   C
AGG AAG AAC TGC TTC AGA AAC ATT GAC TTC CTG TGG TGT GTA TAT
 R   K   N   C   F   R   N   I   D   F   L   W   C   V   Y
GCC TCG GAG CGC CAG GCA GAG AAG GAC GAG CTC ACA CGC TAC GTC
 A   S   E   R   Q   A   E   K   D   E   L   T   R   Y   V
AGC ATC CTC AGG GCC GGC AGC GTG TAA
 S   I   L   R   A   G   S   V   *

GCGTACCACCCCCTCCTCCTCTCCCTCCCTCCTCAGGGGATCCGCCGCTACTGGAGCT
GCCCCGAGGGTGATGCCCGTCTTATTGAAGCGCCTTGAAGTGAAGAGTGAAGTGATTC
TCCCCAGCAACCGTCCCTCTTTAGCTGCTCTTCGTTCACGGTGTAGGAACTCAGCGGC
TAAAGCCTTGGTTGTGTATCTCTCGAGGAGTTAGTTCAGTCGATTTGTCTCCGTTCAC
CTCCGTTCAGCCGTTTGCCTCTTCTCAGATATTCAGCCTCTTTTCAGCCGTTCAGCCG
TTTGCCTCCGTTCACCAGTTCGTCTTCGTTCACTCGTTCGTCTCTTGTTTAGCTGTTC
ACTTCCGTTCACCTGTTCCCTCGTTTTACACACAAAGATCCACGTGTTGCTACTCTCA
TTGCTCCTTCAGTAGTGTGTCTTGTATATCAGGAATGACTGCAGTCCGTATGTCAGGA
GCAACAGTACGTCACCACCTTAATTGGAGTTTACACAATATGGGGGCTGACAATTTTT
TTTTTCTTAGACGTGTGCAAGTTTGCATGTAGGAGGCTGTTGTAGTATTTGTAGTAGC
TGAAGCAGAAGGAGAAGATGGACGAGGAGAAGAGGAGGAAAAGAAGGATGAGAAGAGG
AAGAAGCTGGAGGAGTAGAGATAAAACTGCAAGCTGAAAGGCACCTCATTGCAACAGT
ATTCTCAAAACTCTAGTCATCTCTAACGCTGTTTGAGCTTTTTTTTGCGTATGTGCGT
CAACTTTTTTTGTATCCTAGTTTAGTATTTTATAATTCTTCTCGGCGTTAACTTGTTT
CGTATTTCGTGTTTTTGTGTTGCTCCTAGTGCTGTCTTTATTTATTTAAGTTGTTGTA
CTGATTAAGACCTGAGCTGCTGTGTTCTTTCACGTTTTAGAATTAATTATGACAATGA
TAAAAATAGAAACGAAAGAGCCCTAAAAAAAAAAAAAAAAAAAAAAAAA-3'
```

The full-length sequence of molt-inhibiting hormone cDNA from land crab (Gl-MIH). cDNA (1465 bp) encoded a protein of 113 amino acids. Locations of degenerate primers used for initial RT-PCR are bolded and underlined.

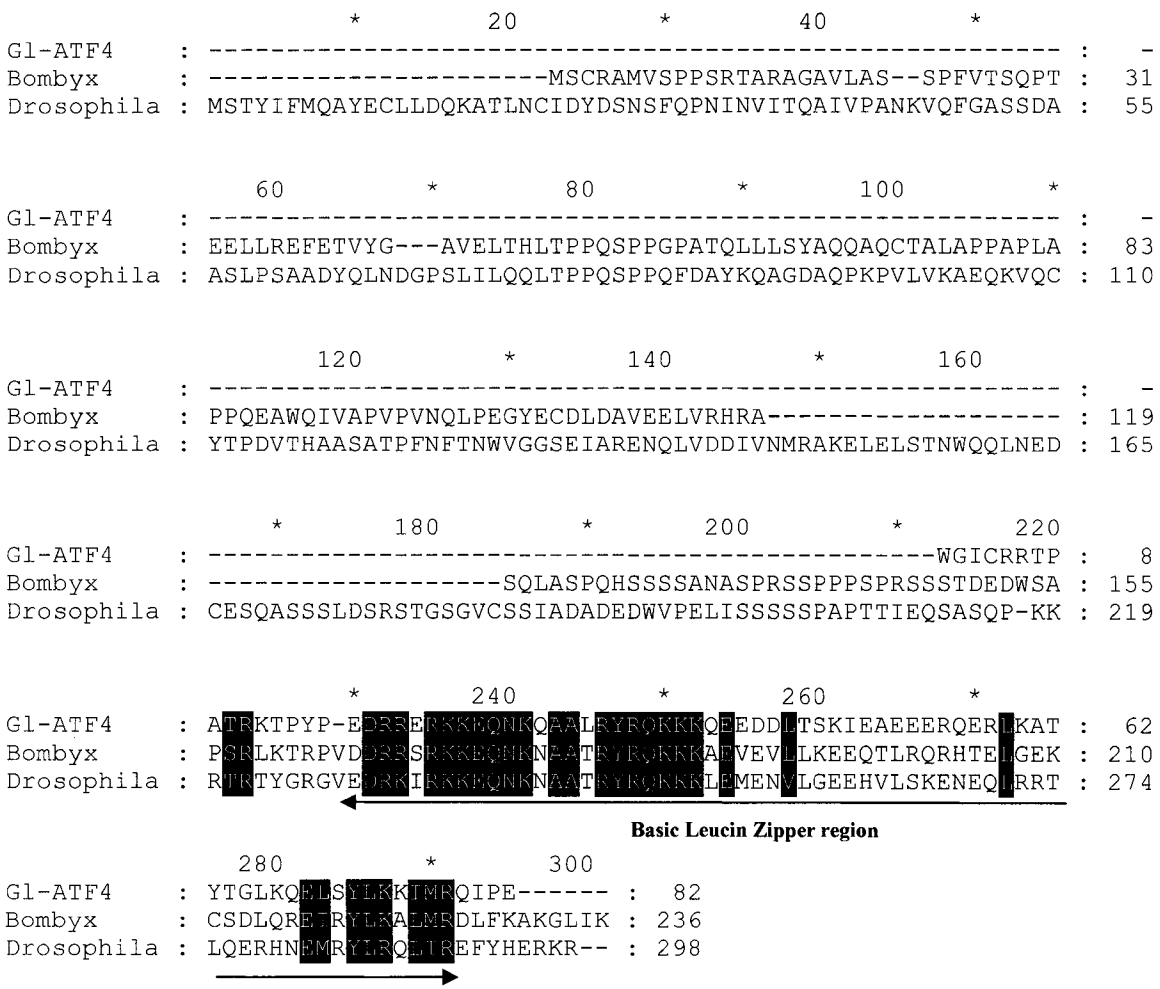


Deduced amino acid sequence comparison of crustacean molt-inhibiting hormone (MIH) cDNAs. MIH sequences from *Gecarcinus lateralis, Cancer pagurus (CAC05346), Cancer magister* (O61389), *Carcinus maenas* (Q27225), *Charybdis feriatus* (O96605), and *Callinectes sapidus* (P55321) are included. Gl-MIH has 71% identity with *Cancer pagurus* and 70% identity with *Cancer magister.* Gl-MIH has the lowest identity with *Callinetes sapidus* (61%).

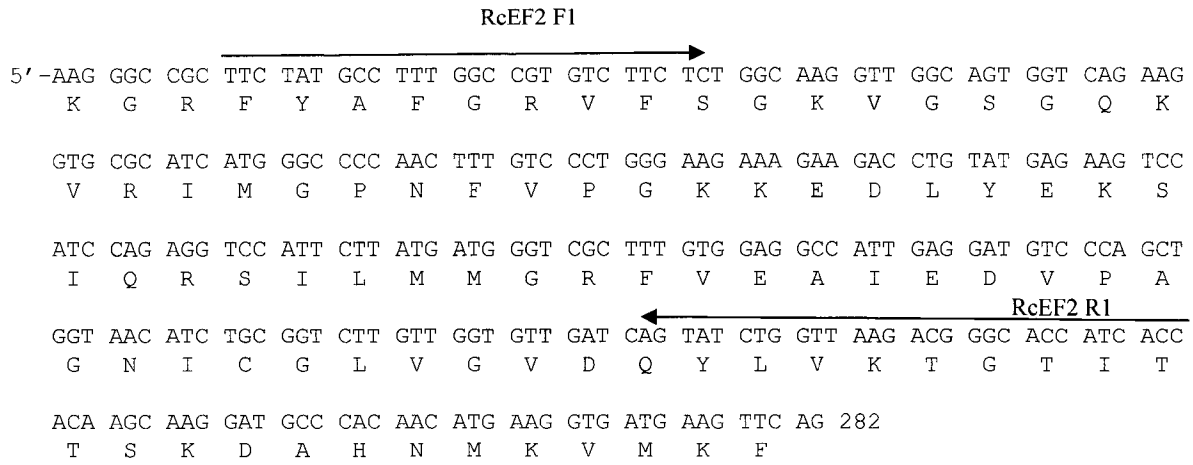
```
5′-TGG GGT ATC TGC CGC AGG ACC CCA GCC ACC AGG AAA ACA CCA TAC CCA GAG GAC CGC AGG
   W   G   I   C   R   R   T   P   A   T   R   K   T   P   Y   P   E   D   R   R
  GAA CGC AAG AAG GAG CAG AAC AAG CAG GCA GCG CTA CGA TAC AGA CAG AAG AAG AAG CAG
   E   R   K   K   E   Q   N   K   Q   A   A   L   R   Y   R   Q   K   K   K   Q
  GAG GAA GAC GAC CTG ACG AGC AAG ATT GAG GCA GAG GAG GAG CGC CAA GAG AGG CTT AAG
   E   E   D   D   L   T   S   K   I   E   A   E   E   E   R   Q   E   R   L   K
  GCC ACC TAC ACT GGC CTG AAG CAG GAG CTT AGC TAC CTG AAG AAG ATC ATG CGG CAA ATA
   A   T   Y   T   G   L   K   Q   E   L   S   Y   L   K   K   I   M   R   Q   I
  CCC GAG GGA CGA TGA
   P   E   G   R   *

CTAAACTGCTGAAAATAGAGAAATACTGACTATGACCAATAGCAAGTTGCAGGCTGATGTGTTGTGACCAGCACTCCCCTTA
GTACAAAATTTTCTTGGTGGCAGGATGGTCTCAGCAAGTCCATTTCTCATGCTACTTCTCTCTTTTTCGGTGGGGGGCATTT
TCATCTGTTAGTGGCCAGATTAAGCCTCTCATCATGTAACACAGTATTATTAATTCTAGAGAGCTACATATGGCAATTTAAA
TTTTACATATTTGAGGATGTGAACTGTGCTGTAGGGTGGTCTGTGTCCTTTCTGGCTTTTCCATTCTATCAGTACAACACAG
ATCTCAGGCCTGTAAAGTGTTCATGGTGTTTCTAGGAAGTCCAGCATGCAGTGGAAATGCCCATTTCAGAGATGAGTGTCAT
GAAGCAGAGGCTTAGCTATCTGCACTCTTTACTTTTGCCTTTTTATCTTTTCTTCCTCATTGATCCTGAGTTCTAGGAAGGA
GTAGTTTTGGCCCCAGTCTGCACTCCTAGCTCATAAGGAGGGCCAGGGAAGGGGGCTGTGGCACAGAGCCAGTGTCCTGAAC
TTGGTGAAGGCTCTTGGGGCTGGAAGATGCAGGACATACTGTTCTGCCCCAGCCCCCTGCCTGAGCCATAGTAACATTTCAT
CATAGTAGTTTCCATATCTTATTAGTATGAGTATGGTAATAAGGTGTGGAATATGTTTTATATTATATCCATCATAATATAT
ATATTTGGGTTTCCACTTCTGGTTTTCTCTCGACTTTATTCCAGAATAAGTGATAGTGTCATGTTTCGATTTTGTCTTTTCA
TAGTAAACGTCTAACCAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
```

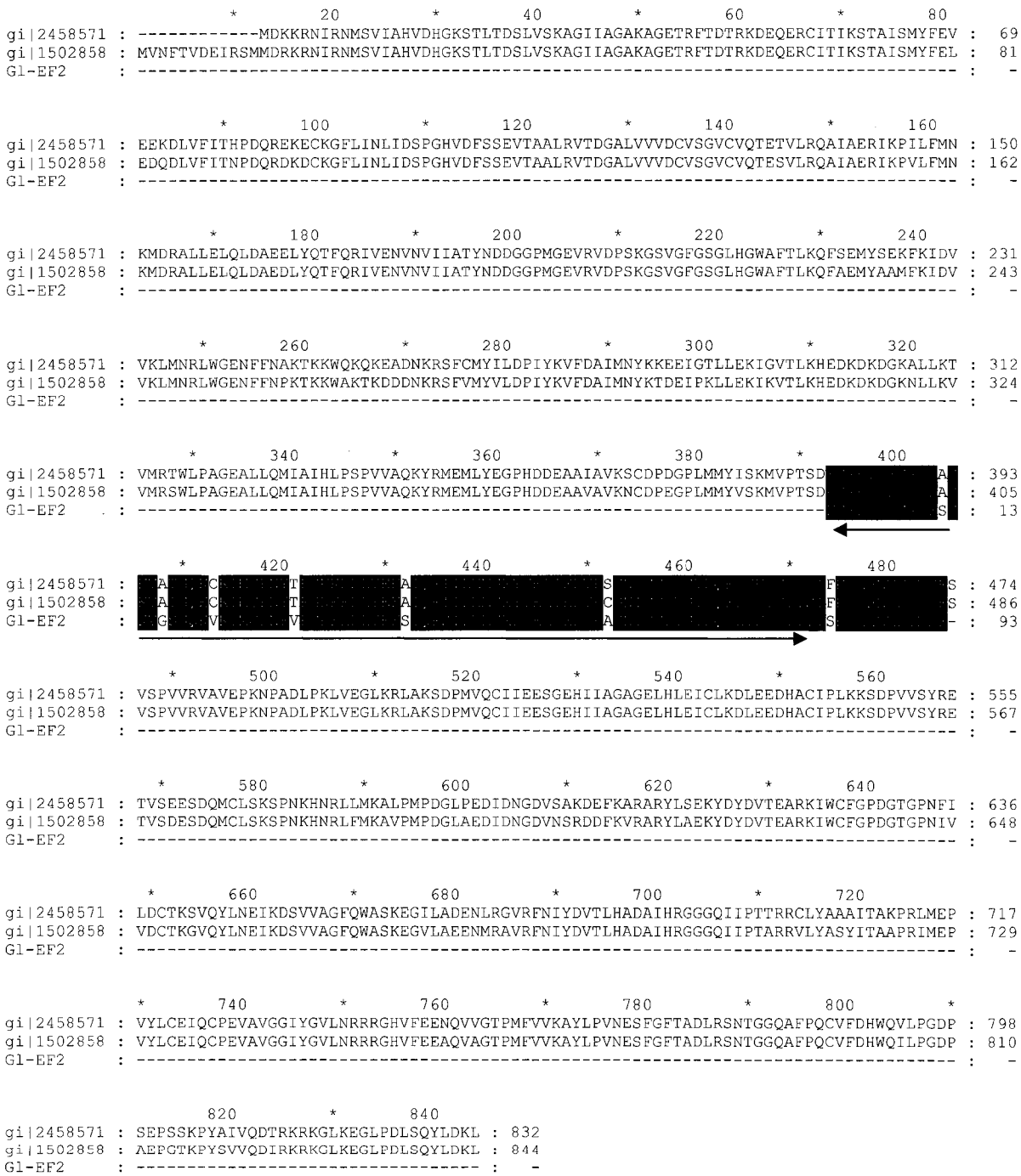
The partial cDNA sequence of ATF-4 transcription factor from land crab (Gl-ATF-4). The cDNA (1119 bp) encoded a protein sequence of 84 amino acids (bottom). The poly(A) signal in the 3-UTR is bolded and underlined.



Amino acid alignment of arthropod ATF transcription factors (ATFs). ATF cDNAs from *Gecarcinus lateralis*, *Bombyx mori* (accession number AAG45935), *Drosophila melanogaster* (accession number AAL39237) were aligned. Identical amino acids in all three sequences are highlighted in black. The basic leucine zipper region is highly conserved (arrow).



The partial cDNA sequence of elongation factor 2 from land crab (Gl-EF2). The cDNA (281 bp) encoded a protein of 93 amino acids. Arrows indicate locations and directions of primers used for RT-PCR.



Amino acid alignment of arthropod elongation factor 2 (EF2) cDNAs. EF2s from *Gecarcinus lateralis*, *Aedes aegypti* (accession number AAK77225) *Drosophila melanogaster* (accession number NP724357) are aligned. Identical amino acids in all three sequences are highlighted in black. Arrow indicates the highly-conserved Tu domain 2, which is one of two domains involved in binding charged tRNA.

```
5'-TAT AAG GTG GAG ACG ATG GGG GAC AAA TAT ATG GCT GTG AGT GGT CTG CCA GAG GCT TGT
   Y   K   V   E   T   M   G   D   K   Y   M   A   V   S   G   L   P   E   A   C
  GAT CAC CAC GCC CGC TGC ATC GGC AAC CTC GCT CTC GAC ATG ATG GAC AAG GCT GCG GGA
   D   H   H   A   R   C   I   G   N   L   A   L   D   M   M   D   K   A   A   G
  GTG ATC GTG GAC GGC CAG CGA GTG CAA ATC ACC ATC GGT ATC CAT ACG GGC GAA GTG GTG
   V   I   V   D   G   Q   R   V   Q   I   T   I   G   I   H   T   G   E   V   V
  ACG GGA GTG ATC GGA CAG AGG ATG CCA CGC TAC TGT CTG TTT GGC AAC ACT GTC AAC ATC
   T   G   V   I   G   Q   R   M   P   R   Y   C   L   F   G   N   T   V   N   I
  ACT TCG AGG ACG GAA ACG ACG GGG GAG AAA GGA CGC ATC AAC GTG TCC GAG GTA TCC TAC
   T   S   R   T   E   T   T   G   E   K   G   R   I   N   V   S   E   V   S   Y
  AGG TAC TTG CAG CAG CAA GAG AAC CAA GAC TCC GGG TTC GCC TTC ACC TAC CGC GGC CCC
   R   Y   L   Q   Q   Q   E   N   Q   D   S   G   F   A   F   T   Y   R   G   P
  GTG CCC ATG AAG GGG AGG AAG GAG CCC ATG CAG GTG TGG TTC CTC AGC AGG CGA AGG GCA
   V   P   M   K   G   R   K   E   P   M   Q   V   W   F   L   S   R   R   R   A
  GCG TAG
   A   *
GGCGGACGAGCAGGACGAAGGCTGTGAACGGCTGGGAGGAGGACGGGCGGGGAAGGAAATGAATGTTTCGCCTCGCTT
CGTACAGTAATCATTGCCAAAGAGTTTGATGCTTTAAGGAAAATATTTCTTTTTAGTGTATGCATTTATTTTTTTTTC
CTGCCCTTTGTATATGCAGCGTCACAGTTCAGTATGTGTTTATGAATTTTAATCAACATTGTTCTGTACATTGACGAA
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GAAATATATATGCTGCATGGGACTGTAAAAAAAAAAAAAAAAAAAAA-3'
```

The partial cDNA sequence of a soluble NO-sensitive guanylyl cyclase beta subunit from land crab (Gl-GCIß-1) The cDNA (863 bp) encoded a protein of 141 amino acids, which contains the GTP-binding domain and 3' UTR.

```
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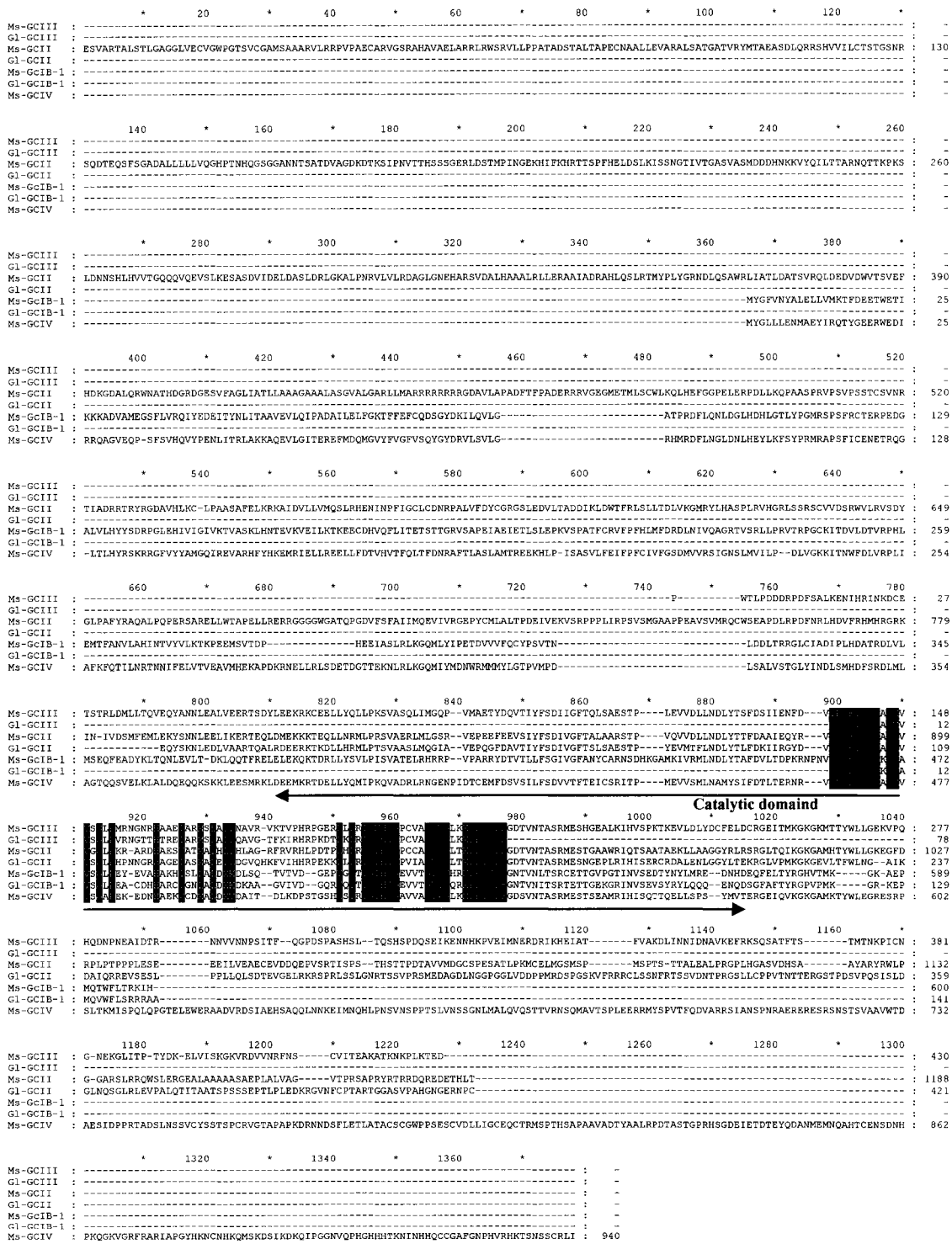
   E   Q   Y   S   K   N   L   E   D   L   V   A   A   R   T   Q   A   L   R   D
  GAG GAG AGA AAG ACC AAG GAC CTA CTA CAC CGC ATG CTG CCG ACG TCT GTG GCA GCC AGC
   E   E   R   K   T   K   D   L   L   H   R   M   L   P   T   S   V   A   A   S
  CTC ATG CAA GGG ATA GCT GTG GAG CCC CAA GGG TTT GAT GCC GTC ACC ATC TAC TTC AGT
   L   M   Q   G   I   A   V   E   P   Q   G   F   D   A   V   T   I   Y   F   S
  GAT ATT GTT GGG TTC ACT TCA CTG TCT GCC GAG AGC ACA CCA TAC GAG GTG ATG ACT TTC
   D   I   V   G   F   T   S   L   S   A   E   S   T   P   Y   E   V   M   T   F
  CTG AAT GAC CTG TAC ACA CTC TTC GAC AAG ATC ATC CGT GGC TAT GAT GTG TAC AAA GTG
   L   N   D   L   Y   T   L   F   D   K   I   I   R   G   Y   D   V   Y   K   V
  GAG ACC ATA GGC GAT GCT TAT ATG GTT GTG TCT GGC CTG CCA CAC CCC AAC AAC GGT AGA
   E   T   I   G   D   A   Y   M   V   V   S   G   L   P   H   P   N   N   G   R
  CAC GCT GGG GAG ATT GCA TCC ATG GCC TTG GAA CTG CTG GAT GGG GTA CAA CAC AAG TTC
   H   A   G   E   I   A   S   M   A   L   E   L   L   D   G   V   Q   H   K   F
  GTT ATC CAC CAC CGT CCA GAG AAG AAG CTG CTG CTG AGA ATA GGC CTC CAC ACT GGG CCT
   V   I   H   H   R   P   E   K   K   L   L   L   R   I   G   L   H   T   G   P
  GTC ATT GCT GGA GTG GTT GGC CTC ACC ATG CCT AGA TAT TGC CTG TTT GGC GAC ACT GTG
   V   I   A   G   V   V   G   L   T   M   P   R   Y   C   L   F   G   D   T   V
  AAC ACT GCC TCA AGA ATG GAA AGC AAT GGG GAG CCG CTG CGC ATT CAC ATC TCC GAG CGG
   N   T   A   S   R   M   E   S   N   G   E   P   L   R   I   H   I   S   E   R
  TGT AGG GAT GCA TTG GAG AAC CTT GGG GGG TAC CTG ACA GAG AAG CGT GGC CTT GTT CCC
   C   R   D   A   L   E   N   L   G   G   Y   L   T   E   K   R   G   L   V   P
  ATG AAG GGC AAG GGT GAG GTG CTG ACA TTT TGG CTC AAT GGG GCA ATC AAG GAT GCC ATA
   M   K   G   K   G   E   V   L   T   F   W   L   N   G   A   I   K   D   A   I
  CAG AGG AGG GAG GTG AGT GAG TCC CTG CCG CCA CTG CTG CAG CTA AGT GAC ACA GAA GTG
   Q   R   R   E   V   S   E   S   L   P   P   L   L   Q   L   S   D   T   E   V
  GGT GAA CTT AGA AAG AGG AGC CCA CGA CTC AGC TCA CTT GGA AAC CGC ACC TCC AGT GTG
   G   E   L   R   K   R   S   P   R   L   S   S   L   G   N   R   T   S   S   V
  CCC AGG AGT ATG GAA GAT GCT GGG GAC CTA AAC GGG GGA CCT GGG GGC CTT GTG GAT GAC
   P   R   S   M   E   D   A   G   D   L   N   G   G   P   G   G   L   V   D   D
  CCC CCC ATG AGA GAC TCC CCT GGG TCA AAG GTC TTC CGC CGC CGC TGC CTC AGT AGT AAC
   P   P   M   R   D   S   P   G   S   K   V   F   R   R   R   C   L   S   S   N
  TTC CGC ACA TCC AGC GTT GAC AAC ACA CCC AGA GGT AGT TTA CTC TGT CCA CCA GTC ACC
   F   R   T   S   S   V   D   N   T   P   R   G   S   L   L   C   P   P   V   T
  AAC ACG ACC GAG AGA GGA TCA ACG CCT GAT TCT GTC CCC CAG AGC ATC AGT TTA GAT GGC
   N   T   T   E   R   G   S   T   P   D   S   V   P   Q   S   I   S   L   D   G
  CTT AAC CAG TCT GGG CTG AGG CTA GAG GTC CCC GCA CTT CAG ACC ATC ACT GCC GCC ACC
   L   N   Q   S   G   L   R   L   E   V   P   A   L   Q   T   I   T   A   A   T
  TCC CCA TCC TCC TCT GAG CCT ACG CTT CCA CTG GAA GAT AAA AGA GGT GTT AAT TTC TGC
   S   P   S   S   S   E   P   T   L   P   L   E   D   K   R   G   V   N   F   C
  CCC ACT GCA CGC ACG GGT GGT GCC TCA GTT CCA GCA CAT GGG AAT GGT GAG CGG AAC CCA
   P   T   A   R   T   G   G   A   S   V   P   A   H   G   N   G   E   R   N   P
  TGC TGA
   C   *

GTGGGGAGGAGGGGGAAGAAGCCTGGGTGGAGGAGGTGGGGACGAGAGGGGAAGGCAGCCCTATCCCGGGCAGTCCTGTAG
TTAAATCAAATTCCCTAAAAGCTTGGTTTTCAGGCTTTTTCCGCCACCCTAAACCACCCCCAAGGATCACATCCAAGTTTA
CGCTGAATGGACTACGGCGGGAGAATGCGGTGTGAGTGTTTGTATGTGTGTGTGTGTGTGTGTGTTTCTTGGGACTCAA
AACGATACCCAGGGATGCTGTAGTAATGTCATCCAGGGTTTTTCCTTCCTTCATTCACTCTAATGTGGTTCTTTTCTTGCT
GCTGCTGCTAAAATTTTGTATGTATGTGTGTGTATCAATGGAACATTCTGTGGCACATCTCAGAAACATTGTGATACAGAC
TTGGATGTTCTGGTGTGTTGGTGACTGGTGG-3'
```

The partial cDNA sequence of a membrane receptor guanylyl cyclase from land crab (Gl-GCII). The cDNA (1702 bp) encoded a protein of 421 amino acids, which contained the GTP-binding site and 3' UTR.

```
5′-TAT AAG GTG GAG ACC GTC GGG GAC GCC TAC ATG GTG GTG TCG GGG CTG CCT GTG AGG AAC
 Y   K   V   E   T   V   G   D   A   Y   M   V   V   S   G   L   P   V   R   N 61
GGC ACC ACA CAC ACA CGG GAG ATC GCC AGG ATG TCC CTT GCG CTG CTA CAG GCG GTG GGT
 G   T   T   H   T   R   E   I   A   R   M   S   L   A   L   L   Q   A   V   G 121
ACC TTC AAA ATC CGC CAC CGG CCA AAA GAC ACG CTC AAG CTG AGG ATC GGG TTA CAC ACG
 T   F   K   I   R   H   R   P   K   D   T   L   K   L   R   I   G   L   H   T 181
GGT CCT TGC GTC GCC GGC GTG GTG GGA CTC AAG ATG CCG CGC TAC TGC CTG TTC G-3′
 G   P   C   V   A   G   V   V   G   L   K   M   P   R   Y   C   L   F
```

The partial cDNA sequence of soluble NO-insensitive guanylyl cyclase from land crab (Gl-GCIII). The cDNA (235 bp) encoded a protein of 79 amino acids, which contained GTP-binding domain.



Alignment of deduced amino acid sequences of arthropod guanylyl cyclase cDNAs. Three partial amino acid sequences from *Gecarcinus lateralis* (Gl-GCIß-1,Gl-GCII, and Gl-GCIII) and four full amino acid sequences from *Manduca sexta* (Ms-GC-IB-1, Ms-GC-II, Ms-GCIII, and Ms-GCIV) are aligned. Identical amino acids in all sequences are highlighted in black. Arrow indicates GTP-binding domain, which is highly conserved in all GC sequences. Genebank accession numbers: AAC61264 (Ms-GcIB-1), AAN16469 (Ms-GCII), AAC62238 (Ms-GCIII), and AAD09836 (Ms-GCIV).

Appendix Table 1. Other clones isolated from American lobster, *Homarus americanus*.

Gene	clone	sequence	source	comments
14-03-03 protein	Ha-14-3-3	partial	claw muscle	scaffold protein;signaling pathway activatin PKB
calpain B	ha-CalpB	partial	claw muscle	Dm-CalpB homologue
calpain T	Ha-CalpT	partial	claw muscle	TRA-3 homologue
calcineurin A	Ha-Can.	partial	claw muscle	Catalytic subunit :phosphatase domain
E75A	Ha-E75A	partial	claw muscle	ecdysone response gene
E75B	Ha-E75B	full	claw muscle	ecdysone response gene N-terminus variant
GABA transporter	Ha-GABAt	partial	deep abdominal muscle	GABA transporter
Mef2	Ha-Mef	partial	claw muscle	MADS transcription factor in muscle differenciation
Retinoic acid receptor	Ha-RXR	partial	claw muscle	retinoic acid receptor
Troponin-C3	Ha-TnC3	full	deep abdominal muscle	novel lobster troponin

REFERENCES

Averna, M., R. De Tullio, et al. (2001). "Changes in intracellular calpastatin localization are mediated by reversible phosphorylation." Biochemical Journal 354: 25-30.

Baghdassarian, D., N. De Bessé, et al. (1996). "Neuropeptide-induced inhibition of steroidogenesis in crab molting glands: Involvement of cGMP-dependent protein kinase." General and Comparative Endocrinology 104: 41-51.

Barnes, T. M. and J. Hodgkin (1996). "The tra-3 sex determination gene of Caenorhabditis elegans encodes a member of the calpain regulatory protease family." EMBO Journal 15: 4477-4484.

Beydon P, L. R. (1983). "Feedback inhibition of ecdysone production by 20 hydroxyecdysone in Pieris brassicae pupae." Journal Insect Physiology 29: 529-33.

Beyette, J. R., J.-S. Ma, et al. (1993). "Purification and autolytic degradation of a calpain-like calcium-dependent proteinase from lobster (Homarus americanus) striated muscle." Comparative Biochemistry and Physiology 104B: 95-99.

Beyette, J. R., Y. Emori, et al. (1997). "Immunological analysis of two calpain-like Ca2+-dependent proteinases from lobster striated muscles: Relationship to mammalian and Drosophila calpains." Archives of Biochemistry and Biophysics 337: 232-238.

Beyette, J. R. and D. L. Mykles (1997). "Autolysis and biochemical properties of a lobster muscle calpain-like proteinase." Journal of Experimental Zoology 277: 106-119.

Böcking, D. and D. Sedlmeier (1994). "Protein phosphorylation in the molting gand of the crayfish, Orconectes limosus - Role of cyclic nucleotides, calcium, and molt inhibiting hormone (MIH)." Invertebrate Reproduction & Development 26(3): 237-245.

Bredt, D. S. and S. H. Snyder (1994). "Nitric oxide: a physiologic messenger molecule." Annual Review of Biochemistry 63: 175-95.

Broderick, K. E., M. R. MacPherson, et al. (2003). "Interactions between epithelial nitric oxide signaling and phosphodiesterase activity in Drosophila." Am Journal Physiology Cell Physiology 285(5): C1207-18.

Buchholz, C. and D. Adelung (1980). "The ultrastructural basis of steroid production in the Y-organ and the mandibular organ of the crabs Hemigrapsus nudus (Dana) and Carcinus maenas L." Cell and Tissue Research 206(1): 83-94.

Carlisle, D. B. and P. F. R. Dohrn (1953). "Studies on Lysmata seticaudata Risso (Crustacea Decapoda). II. Experimental evidence for a growth- and moult-accelerating

factor obtainable from eyestalks." Pubblicazioni della Stazione Zoologica di Napoli 24: 69-83.

Chan, S. M., X. G. Chen, et al. (1998). "PCR cloning and expression of the molt-inhibiting hormone gene for the crab (Charybdis feriatus)." Gene 224: 23-33.

Chan, S. M. (1998). "Cloning of a shrimp (Metapenaeus ensis) cDNA encoding a nuclear receptor superfamily member: an insect homologue of E75 gene." FEBS Letter. 436: 395-400.

Chang, E. S. and M. J. Bruce (1980). "Ecdysteroid titers of juvenile lobsters following molt induction." Journal of Experimental Zoology 214: 157-160.

Chang, E. S. (1985). "Hormonal control of molting in decapod Crustacea." American Zoologist 25: 179-185.

Chang, E. S., M. J. Bruce, et al. (1987). "Purification and amino acid composition of a peptide with molt-inhibiting activity from the lobster, Homarus americanus." General and Comparative Endocrinology 65: 56-64.

Chang, E. S. (1993). "Comparative endocrinology of molting and reproduction: Insects and crustaceans." Annual Review of Entomology 38: 161-180.

Chung, J. S., M. C. Wilkinson, et al. (1996). "Determination of the amino acid sequence of the moult-inhibiting hormone from the edible crab, Cancer pagurus." Neuropeptides 30: 95-101.

Chung, A. C. K., D. S. Durica, et al. (1998). "Cloning of crustacean ecdysteroid receptor and retinoid-X receptor gene homologs and elevation of retinoid-X receptor mRNA by retinoic acid." Molecular and Cellular Endocrinology 139: 209-227.

Chung, A. C. K., D. S. Durica, et al. (1998). "Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X-receptor mRNA during the molt cycle of the fiddler crab, Uca pugilator." General and Comparative Endocrinology 109: 375-389.

Chung, J. S. and S. G. Webster (2003). "Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, Carcinus maenasi - From target to transcript." European Journal of Biochemistry 270(15): 3280-3288.

Davies, S. A. (2000). "Nitric oxide signalling in insects." Insect Biochemistry and Molecular Biology 30(12): 1123-1138.

Denison, S. H., M. Orejas, et al. (1995). "Signaling of ambient pH in Aspergillus involves

a cysteine protease." Journal of Biological Chemistry 270(48): 28519-22.

Drach, P. (1939). "Meu et cycle d'intermue chez les Crustaces Decapodes." Annales de l'Institut Oceanographique 19: 103-391.

Durica, D. S. and P. M. Hopkins (1996). "Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, Uca pugilator." Gene 171: 237-241.

Durica, D. S., X. H. Wu, et al. (2002). "Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation." Molecular and Cellular Endocrinology 189(1-2): 59-76.

El Haj, A. J., S. R. Clarke, et al. (1996). "In vivo muscle protein synthesis rates in the American lobster Homarus americanus during the moult cycle and in response to 20-hydroxyecdysone." Journal of Experimental Biology 199: 579-585.

El Haj, A. J., S. L. Tamone, et al. (1997). "An ecdysteroid-responsive gene in a lobster - a potential Crustacean member of the steroid hormone receptor superfamily." Gene 201: 127-135.

Feelish M., M. J. (1995). "The early role of nitric oxide in evolution." Trends in Ecology and Evolution 10: 496-499.

Futai, E., T. Maeda, et al. (1999). "The protease activity of a calpain-like cysteine protease in Saccharomyces cerevisiae is required for alkaline adaptation and sporulation." Molecular and General Genetics 260: 559-568.

Gilbert, L. I., R. Rybczynski, et al. (2002). "Control and biochemical nature of the ecdysteroidogenic pathway." Annual Review of Entomology 47: 883-916.

Goll, D. E., V. F. Thompson, et al. (2003). "The calpain system." Physiological Reviews 83(3): 731-801.

Goy, M. F., D. A. Mandelbrot, et al. (1987). "Identification and Characterization of a Polypeptide from a Lobster Neurosecretory Gland That Induces Cyclic-Gmp Accumulation in Lobster Neuromuscular Preparations." Journal of Neurochemistry 48(3): 954-966.

Hadzic, E., V. Desai-Yajnik, et al. (1995). "A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor alpha is essential for transcriptional activation and interaction with the general transcription factor TFIIB." Molecular and Cellular Biology 15(8): 4507-17.

Hata, S., K. Nishi, et al. (2001). "Both the conserved and the unique gene structure of

stomach-specific calpains reveal processes of calpain gene evolution." Journal of Molecular Evolution 53(3): 191-203.

Hoffman, D. L. (1967). "The structure of lymphogenous tissue of a caridean shrimp previously described as Y-organ (molting gland)." Canadian Journal of Zoology 45: 886-889.

Hoffmann, J. and M. Porchet (1984). Biosynthesis, metabolism, and mode of action of invertebrate hormones. Berlin ; New York, Springer-Verlag.

Hopkins, P. M. (1983). "Patterns of serum ecdysteroids during induced and uninduced proecdysis in the fiddler crab, Uca pugilator." General and Comparative Endocrinology 52(3): 350-6.

Hosfield, C. M., J. S. Elce, et al. (1999). "Crystal structure of calpain reveals the structural basis for Ca2+-dependent protease activity and a novel mode of enzyme activation." EMBO Journal 18: 6880-6889.

Hosfield, C. M., T. Moldoveanu, et al. (2001). "Calpain mutants with increased Ca2+ sensitivity and implications for the role of the C(2)-like domain." Journal of Biological Chemistry 276(10): 7404-7.

Huet, F., C. Ruiz, et al. (1995). "Sequential gene activation by ecdysone in Drosophila melanogaster: the hierarchical equivalence of early and early late genes." Development 121(4): 1195-204.

Hughes, J. T., and Matthiessen, G.C. (1962). "Observations on the biology of the American lobster, Homarus americanus." Limnology and Oceanography 7: 414-421.

Hughes, J. T., Sullivan,j. j., and Schleser (1972). "Enhancement of lobster growth." Science 177: 1110-1111.

Imamura, M., J. Yang, et al. (2002). "cDNA cloning, characterization and gene expression of nitric oxide synthase from the silkworm, Bombyx mori." Insect Molecular Biology 11(3): 257-65.

Ishiura, S., H. Murofushi, et al. (1978). "Studies of a calcium-activated neutral protease from chicken skeletal muscle. I. Purification and characterization." Journal of Biochemistry (Tokyo) 84(1): 225-30.

Ismail, S. Z. M. and D. L. Mykles (1992). "Differential molt-induced atrophy in the dimorphic claws of male fiddler crabs, Uca pugnax." Journal of Experimental Zoology 263: 18-31.

Jekely, G. and P. Friedrich (1999). "Characterization of two recombinant Drosophila

calpains. CALPA and a novel homolog, CALPB." Journal of Biological Chemistry 274(34): 23893-900.

Jenkin, P. M. (1966). "Apolysis and hormones in the moulting cycles of Arthropoda." Annales d'Endrocrinologie 27(Suppl. 3): bis:331-341.

Jindra, M., F. Sehnal, et al. (1994). "Isolation, characterization and developmental expression of the ecdysteroid-induced E75 gene of the wax moth Galleria mellonella." European Journal of Biochemistry 221(2): 665-75.

Jindra, M., J. Y. Huang, et al. (1997). "Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of Manduca sexta." Insect Molecular Biology 6(1): 41-53.

Johansson, K. U. and D. Mellon, Jr. (1998). "Nitric oxide as a putative messenger molecule in the crayfish olfactory midbrain." Brain Research 807(1-2): 237-42.

Kapitskaya, M., S. Wang, et al. (1996). "The mosquito ultraspiracle homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis." Molecular and Cellular Endocrinology 121(2): 119-32.

Katayama, H., K. Nagata, et al. (2003). "The solution structure of molt-inhibiting hormone from the kuruma prawn Marsupenaeus japonicus." Journal of Biological Chemistry 278(11): 9620-9623.

Keller, R. (1992). "Crustacean neuropeptides: Structures, functions and comparative aspects." Experientia 48: 439-448.

Klein, J. M., S. Mangerich, et al. (1993). "Molecular cloning of crustacean putative molt-inhibiting hormone (MIH) precursor." FEBS Letter 334: 139-142.

Kone, B. C., T. Kuncewicz, et al. (2003). "Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide." American Journal of Physiology-Renal Physiology 285(2): F178-F190.

Lachaise, F., M. Goudeau, et al. (1992). "Eyestalk ablation in female crabs: Effects on egg characteristics." Journal of Experimental Zoology 261: 86-96.

Lachaise, A., A. Le Roux, et al. (1993). "The molting gland of crustaceans: localization, activity, and endocrine control (a review)." Journal of Crustacean Biology 13: 198-234.

Lee, K. J., T. S. Elton, et al. (1995). "Molecular cloning of a cDNA encoding putative molt-inhibiting hormone from the blue crab, Callinectes sapidus." Biochemical and Biophysical Research Communications. 209: 1126-1131.

Lee, K. J., R. D. Watson, et al. (1998). "Molt-inhibiting hormone mRNA levels and ecdysteroid titer during a molt cycle of the blue crab, Callinectes sapidus " Biochemical and Biophysical Research Communications. 249: 624-627.

Lee, C. Y., H. S. Zou, et al. (2000). "Nitric oxide synthase activity and immunoreactivity in the crayfish Procambarus clarkii." Neuroreport 11(6): 1273-6.

Lee, K. J. and R. D. Watson (2002). "Antipeptide antibodies for detecting crab (Callinectes sapidus) molt-inhibiting hormone." Peptides 23(5): 853-862.

Lu, W. Q., G. Wainwright, et al. (2000). "Clustering of mandibular organ-inhibiting hormone and moult-inhibiting hormone genes in the crab, Cancer pagurus, and implications for regulation of expression." Gene 253: 197-207.

Luckhart, S., Y. Vodovotz, et al. (1998). "The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide." Proceedings of the National Academy of Sciences of the United States of America 95(10): 5700-5.

Luckhart, S. and K. Li (2001). "Transcriptional complexity of the Anopheles stephensi nitric oxide synthase gene." Insect Biochemistry and Molecular Biology 31(3): 249-56.

Maniere, G., E. Vanhems, et al. (2003). "Inhibition of ovarian steroidogenesis by cyclic GMP in a fly." Journal of Endocrinol 177(1): 35-44.

Mattson, J. M. and D. L. Mykles (1993). "Differential degradation of myofibrillar proteins by four calcium-dependent proteinase activities from lobster muscle." Journal of Experimental Zoology 265: 97-106.

Medler, S. and D. L. Mykles (2003). "Analysis of myofibrillar proteins and transcripts in skeletal muscles of the American lobster, Homarus americanus: variable expression of myosins, actin, and troponins in fast, slow-twitch, and slow-tonic fibres." Journal of Experimental Biology in press.

Minakuchi, C., Y. Nakagawa, et al. (2002). "Molecular cloning, expression analysis and functional confirmation of two ecdysone receptor isoforms from the rice stem borer Chilo suppressalis." Insect Biochemistry and Molecular Biology 32(9): 999-1008.

Mugita, N., Y. Kimura, et al. (1997). "Identification of a novel, tissue-specific calpain htra-3, a human homologue of the Caenorhabditis elegans sex determination gene." Biochemical and Biophysical Research Communications 239: 845-850.

Mykles, D. L. and D. M. Skinner (1981). "Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle." Journal of Ultrastructure Research 75: 314-325.

Mykles, D. L. and D. M. Skinner (1982). Crustacean muscles: atrophy and regeneration

during molting. Basic Biology of Muscles: A Comparative Approach. B. M. Twarog, R. J. C. Levine and M. M. Dewey. New York, Raven Press: 337-357.

Mykles, D. L. and D. M. Skinner (1982). "Molt cycle-associated changes in calcium-dependent proteinase activity that degrades actin and myosin in crustacean muscle." Developmental Biology 92: 386-397.

Mykles, D. L. and D. M. Skinner (1983). "Ca2+-dependent proteolytic activity in crab claw muscle. Effects of inhibitors and specificity for myofibrillar proteins." The Journal of Biological Chemistry 258: 10474-10480.

Mykles, D. L. and D. M. Skinner (1986). "Four Ca2+-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca2+ and inhibitors." The Journal of Biological Chemistry 261: 9865-9871.

Mykles, D. L. and D. M. Skinner (1990). "Atrophy of crustacean somatic muscle and the proteinases that do the job. A review." Journal of Crustacean Biology 10: 577-594.

Mykles, D. L. (1990). "Calcium-dependent proteolysis in crustacean claw closer muscle maintained in vitro." Journal of Experimental Zoology 256: 16-30.

Mykles, D. L. and M. F. Haire (1991). "Sodium dodecyl sulfate and heat induce two distinct forms of lobster muscle multicatalytic proteinase: the heat-activated form degrades myofibrillar proteins." Archives of Biochemistry and Biophysics 288: 543-551.

Mykles, D. L. (1997). "Crustacean muscle plasticity: Molecular mechanisms determining mass and contractile properties." Comparative Biochemistry and Physiology 117B: 367-378.

Mykles, D. L. (1999). "Proteolytic processes underlying molt-induced claw muscle atrophy in decapod crustaceans." American Zoologist 39: 541-551.

Nakatsuji, T. and H. Sonobe (2003). "Measurement of molt-inhibiting hormone titer in hemolymph of the American crayfish, Procambarus clarkii, by time-resolved fluoroimmunoassay." Zoological Science20(8): 999-1001.

Nathan, C. and Q. W. Xie (1994). "Nitric oxide synthases: roles, tolls, and controls." Cell 78(6): 915-8.

Nighorn, A., N. J. Gibson, et al. (1998). "The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of Manduca sexta." Journal of Neuroscience 18(18): 7244-7255.

Nishida, C. R. and P. R. de Montellano (2001). "Control of electron transfer in nitric-oxide synthases. Swapping of autoinhibitory elements among nitric-oxide synthase

isoforms." Journal of Biological Chemistry 276(23): 20116-24.

Ohira, T., T. Watanabe, et al. (1997). "Molecular cloning of a molt-inhibiting hormone cDNA from the kuruma prawn Penaeus japonicus." Zoological Science 14: 785-789.

Onate, S. A., V. Boonyaratanakornkit, et al. (1998). "The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors." Journal of Biological Chemistry 273(20): 12101-8.

Passano, L. M. (1953). "Neurosecretory control of molting in crabs by the X-organ sinus gland complex." Physiologia comparata et Eecolgia 3: 155-189.

Passano, L. M. (1960). "Low temperature blockage of molting in Uca pugnax." Biological Bulletin 118: 129-136.

Ranga Rao, K. (1965). "Isolation and partial characterization of the molt-inhibiting hormone of the crustacean eyestalk " Experientia 21: 593-594.

Renaud, J. P. and D. Moras (2000). "Structural studies on nuclear receptors." cellular and molecular life sciences 57(12): 1748-69.

Reverter, D., M. Braun, et al. (2002). "Flexibility analysis and structure comparison of two crystal forms of calcium-free human m-calpain." Biological Chemistry 383(9): 1415-1422.

Ribeiro, J. M. and R. H. Nussenzveig (1993). "Nitric oxide synthase activity from a hematophagous insect salivary gland." FEBS Letter 330(2): 165-8.

Roman, L. J., P. Martasek, et al. (2002). "Intrinsic and extrinsic modulation of nitric oxide synthase activity." Chemical Reviews 102(4): 1179-90.

Saïdi, B., N. De Bessé, et al. (1994). "Involvement of cAMP and cGMP in the mode of action of molt-inhibiting hormone (MIH) a neuropeptide which inhibits steroidogenesis in a crab." Molecular and Cellular Endocrinology 102: 53-61.

Sakurai, S. and C. M. Williams (1989). "Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, Manduca sexta." General and Comparative Endocrinology 75(2): 204-16.

Salerno, J. C., D. E. Harris, et al. (1997). "An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase." Journal of Biological Chemistry 272(47): 29769-77.

Schmiege, D. L., R. L. Ridgway, et al. (1992). "Ultrastructure of autotomy-induced

atrophy of muscles in the crab Carcinus maenas." Canadian Journal of Zoology. 70: 841-851.

Scholz, N. L., M. F. Goy, et al. (1996). "Nitric oxide and peptide neurohormones activate cGMP synthesis in the crab stomatogastric nervous system." Journal of Neuroscience 16(5): 1614-1622.

Scholz, N. L., E. S. Chang, et al. (1998). "The NO/cGMP pathway and the development of neural networks in postembryonic lobsters." Journal of Neurobiology 34(3): 208-226.

Scholz, N. L., J. de Vente, et al. (2001). "Neural network partitioning by NO and cGMP." Journal of Neuroscience 21(5): 1610-1618.

Scholz, N. L., J. S. Labenia, et al. (2002). "Expression of nitric oxide synthase and nitric oxide-sensitive guanylate cyclase in the crustacean cardiac ganglion." Journal of comparative neurology 454(2): 158-67.

Segraves, W. A. and D. S. Hogness (1990). "The E75 ecdysone-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily." Genes & Development 4(2): 204-19.

Segraves, W. A. and C. Woldin (1993). "The E75 gene of Manduca sexta and comparison with its Drosophila homolog." Insect Biochemistry and Molecular Biology 23(1): 91-7.

Shean, B. S. and D. L. Mykles (1995). "Polyubiquitin in crustacean striated muscle: Increased expression and conjugation during molt-induced claw muscle atrophy." Biochimica et Biophysica Acta Gene Structure and Expression 1264: 312-322.

Skinner, D. M. (1962). "The structure and metabolism of a crustacean integumentary tissue during a molt cycle." Biological Bulletin 123: 635-647.

Skinner, D. M. (1966). "Breakdown and reformation of somatic muscle during the molt cycle of the land crab, Gecarcinus lateralis." Journal of Experimental Zoology 163: 115-124.

Skinner, D. M. (1985). Molting and regeneration. The Biology of Crustacea. D. E. Bliss and L. H. Mantel. New York, Academic Press: 43-146.

Sokol, S. B. and P. E. Kuwabara (2000). "Proteolysis in Caenorhabditis elegans sex determination: cleavage of TRA-2A by TRA-3." Genes & Development 14: 901-906.

Sorimachi, H., S. Ishiura, et al. (1993). "A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca2+-binding domain." The Journal of Biological Chemistry 268: 19476-19482.

Sorimachi, H. and K. Suzuki (2001). "The structure of calpain." Journal of Biochemistry (Tokyo) 129: 653-664.

Soyez, D., and Kleinholz, L.H (1977). "Molt-inhibiting factor from the crustacean eyestalk." General and Comparative Endocrinology 31: 233-242.

Spadoni, C., A. Farkas, et al. (2003). "Molecular cloning and RNA expression of a novel Drosophila calpain, Calpain C." Biochemical and Biophysical Research Communications 303(1): 343-349.

Stasiv, Y., M. Regulski, et al. (2001). "The Drosophila nitric oxide synthase gene (dNOS) encodes a family of proteins that can modulate NOS activity by acting as dominant negative regulators." Journal of Biological Chemistry 276(45): 42241-42251.

stephens, G. C. (1955). "Induction of molting in the crayfish, Cambarus, by modification of daily photoperiod." Biological Bulletin 108: 235-241.

Strobl, S., C. Fernandez-Catalan, et al. (2000). "The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium." Proceedings of the National Academy of Sciences USA 97: 588-592.

Sultan, K. R., B. T. Dittrich, et al. (2001). "Fiber type-specific expression of major proteolytic systems in fast- to slow-transforming rabbit muscle." American Journal of Physiology: Cell Physiology 280: C239-C247.

Syntichaki, P., K. L. Xu, et al. (2002). "Specific aspartyl and calpain proteases are required for neurodegeneration in C-elegans." Nature 419(6910): 939-944.

Talbot, W. S., E. A. Swyryd, et al. (1993). "Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms." Cell 73(7): 1323-37.

Tang, H., W. M. Cheung, et al. (2000). "Identification and characterization of differentially expressed genes in denervated muscle." Molecular and Cellular Neuroscience 16(2): 127-40.

Theopold, U., M. Pinter, et al. (1995). "CalpA, a Drosophila calpain homolog specifically expressed in a small set of nerve, midgut, and blood cells." Molecular and Cellular Biology 15(2): 824-34.

Thummel, C. S. (1992). "Mechanisms of transcriptional timing in Drosophila." Science 255(5040): 39-40.

Thummel, C. S. (1996). "Files on steroids--Drosophila metamorphosis and the mechanisms of steroid hormone action." Trends in Genetics 12(8): 306-10.

Tompa, P., Y. Emori, et al. (2001). "Domain III of calpain is a Ca2+-regulated phospholipid-binding domain." Biochemical and Biophysical Research Communications. 280: 1333-1339.

Truman, J. W., J. De Vente, et al. (1996). "Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects." Development 122(12): 3949-58.

Umphrey, H. R., K. J. Lee, et al. (1998). "Molecular cloning of a cDNA encoding molt-inhibiting hormone (MIH) of the crab, Cancer magister." Molecular and Cellular Endocrinology 136: 145-149.

Wang, S. F., C. Li, et al. (2000). "Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms." Developmental Biology 218(1): 99-113.

Wang, S. F., C. Li, et al. (2002). "Differential expression and regulation by 20-hydroxyecdysone of mosquito ecdysteroid receptor isoforms A and B." Molecular and Cellular Endocrinology 196(1-2): 29-42.

Watson, R. D., K. J. Lee, et al. (2001). "Molecular cloning, expression, and tissue distribution of crustacean molt-inhibiting hormone." American Zoologist 41(3): 407-417.

Webster, S. G. (1991). "Amino acid sequence of putative moult-inhibiting hormone from the crab Carcinus maenas." Proceedings of the Royal Society of London. Series B: Biological Sciences 244: 247-252.

Weis, J. S. (1976). "Effects of environmental factors on regeneration and molting in fiddler crabs." Biological Bulletin 150(1): 152-62.

Weiske, J. and A. Wiesner (1999). "Stimulation of NO synthase activity in the immune-competent lepidopteran Estigmene acraea hemocyte line." Nitric Oxide 3(2): 123-31.

Wilson, T. E., R. E. Paulsen, et al. (1992). "Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors." Science 256(5053): 107-10.

Yao, T.-P., B. M. Forman, et al. (1993). "Functional ecdysone receptor is the product of EcR and Ultraspiracle genes." Nature 366: 476-479.

Yu, X. L. and D. L. Mykles (2003). "Cloning of a muscle-specific calpain from the American lobster (Homarus americanus): expression associated with muscle atrophy and restoration during moulting." Journal of Experimental Biology 206: 561-575.

Chan, S. M. (1998). "Cloning of a shrimp (*Metapenaeus ensis*) cDNA encoding a nuclear receptor superfamily member: an insect homologue of E75 gene." FEBS Letter **436**: 395-400.