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**Molecular analysis of protein kinase C in the sea urchin**  
*Lytechinus pictus*

Rakow, Terese Louise, Ph.D.

Iowa State University, 1993

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Molecular analysis of protein kinase C in the sea urchin *Lytechinus pictus*

by

Terese Louise Rakow

A Dissertation Submitted to the  
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## INTRODUCTION

### Protein Kinase C history and description

Protein kinase C (PKC) is a  $\text{Ca}^{2+}$ , phosphatidylserine (PS) and diacylglycerol (DAG) dependent kinase which phosphorylates serine and threonine residues on target proteins (Inoue *et al.*, 1977; Takai *et al.*, 1977). These substrates have within their sequence a series of amino acid residues which represent the conserved recognition sequence for phosphorylation by PKC. Examination of substrates has determined the consensus of the recognition sequence to be  $\text{K/RX}_{1-2}\text{S}^*/\text{T}^*\text{XK/R}$  (Hunter, 1991) where X is any amino acid. Protein kinase C was originally described as a brain tissue-derived kinase activity requiring the above mentioned cofactors. This activity has now grown to consist of a family of related enzymes with nine known members including some  $\text{Ca}^{2+}$ -independent kinase activities. The isozymes have been designated as,  $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$  (or L), and  $\theta$ . The  $\text{Ca}^{2+}$ -dependent members of the family are  $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$  and  $\gamma$  (Coussens *et al.*, 1986; Parker *et al.*, 1986) and the remaining isozymes are the  $\text{Ca}^{2+}$ -independent members (Bacher *et al.*, 1991; Koide *et al.*, 1992; Ogita *et al.*, 1992; Ono *et al.*, 1988; Osada *et al.*, 1990; Osada *et al.*, 1992; Wetsel *et al.*, 1992).

The isozymes of PKC are widely distributed, but it has been shown that certain isozymes are predominantly expressed in specific cell types. PKC- $\eta$ , or -L, is mainly found in lung and skin (Bacher *et al.*, 1991; Osada *et al.*, 1990),  $\theta$ , the newest member of the kinase family, is concentrated in muscle (Osada *et al.*, 1992) and  $\gamma$ , one of the originally described activities of PKC, seems to have expression limited to the brain (Nishizuka, 1988; Wetsel *et al.*, 1992). Although the members of the PKC family are very close in sequence, evidence points to the fact that the isozymes have varying substrate specificities (Liyanage *et al.*, 1992; Marais *et al.*, 1990; Parker *et al.*, 1989), activation requirements (Ryves *et al.*, 1991) and as mentioned above, tissue distributions (Nishizuka, 1988; Wetsel *et al.*, 1992). Together these lead to the

theory that the existence of so many members of a family is not mere redundancy (Kikkawa *et al.*, 1989; Nishizuka, 1989).

Protein kinase C consists of a single polypeptide unit, and the physical structure is broken up into highly conserved functional domains interspaced by variable regions. Figure 1 shows a cartoon illustration of the structures of the  $\text{Ca}^{2+}$ -dependent and -independent groups of PKC. The conserved domains are numbered C1-C4 from the N-terminal end to the C-terminus of the protein. The first structure in the PKC polypeptide is the pseudosubstrate domain of the protein. The consensus sequence of the pseudosubstrate domain is Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys (House & Kemp, 1987) with very little variation between different isozymes (Parker *et al.*, 1989). The pseudosubstrate domain is a negative regulatory sequence within the protein, which mimics the recognition consensus for phosphorylation by the kinase, with the serine or threonine being replaced by an alanine. This autoinhibitory domain most likely interacts with the catalytic portion of the kinase and holds it in an inactive state (House & Kemp, 1987; Soderling, 1990). This suggests that the protein must undergo some conformational shift for activity (Gschwendt *et al.*, 1991; Orr *et al.*, 1992). In support of the function of the pseudosubstrate domain are experiments using an antibody generated against the pseudosubstrate domain and partially purified PKC. Incubation of PKC with the pseudosubstrate antisera caused activation of PKC in the absence of  $\text{Ca}^{2+}$ , PS and DAG (Makowske & Rosen, 1989). The activation of PKC by this antibody leads to the hypothesis that the pseudosubstrate domain becomes occupied by the antibody and releases the intrinsic inhibition of the kinase. The rest of the C1 domain consists of two cysteine repeat structures, except in the case of  $\zeta$  which has one cysteine repeat (Ono *et al.*, 1989). These repeats are similar to those in DNA binding proteins (Mitchell & Tjian, 1989), and it is proposed that the cysteine repeats aid in binding diacylglycerol or phorbol esters (Gschwendt *et al.*, 1991). The sequence of the cysteine repeat is Cys-X<sub>2</sub>-Cys-X<sub>13-14</sub>-Cys-X<sub>2</sub>-Cys-X<sub>7</sub>-Cys-X<sub>7</sub>-Cys where X represents any amino acid.

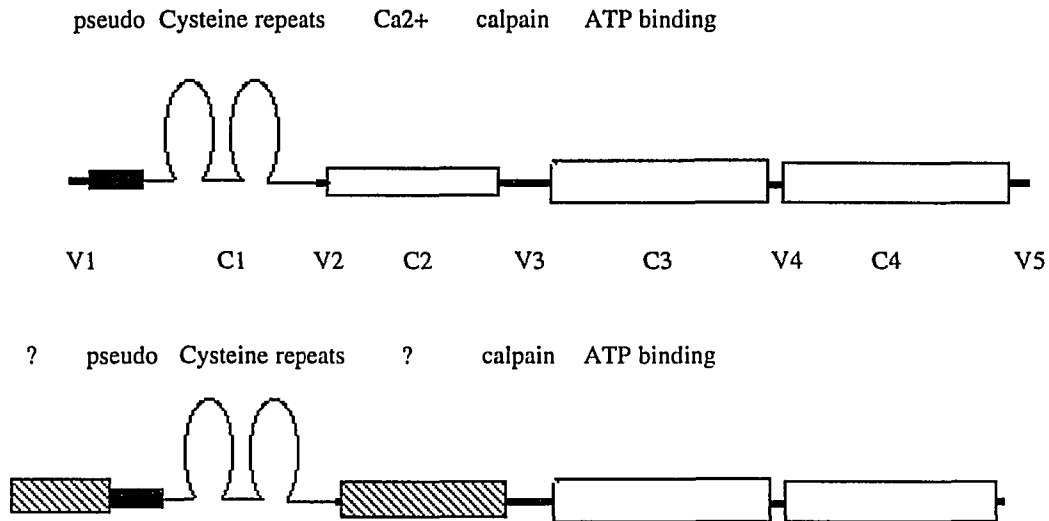


Figure 1. Structure of PKC isozymes. Cartoon illustrations of the two PKC groups are shown with the  $\text{Ca}^{2+}$ -dependent isozymes represented at the top and the  $\text{Ca}^{2+}$ -independent isozymes below. The variable and conserved domains of PKC are listed between the two as V1-V5 and C1-C4 respectively. Above each of the isozymes, are the functions of the regions as discussed in the text. The hatched areas of the  $\text{Ca}^{2+}$ -independent isozymes are the regions in which the two groups differ. The function of these two regions is not yet known.

Following the C1 cysteine repeat domain is the C2 region which has been proposed to be the  $\text{Ca}^{2+}$  binding region of the kinase (Nishizuka, 1988). This proposal is based on the fact that the isozymes with  $\text{Ca}^{2+}$  independent activation properties do not contain the C2 region. The C2 domain may also bind other factors required for the function of PKC. Synaptotagmin or p65, a synaptic vesicle protein, also contains two C2-like domains (Perin *et al.*, 1990). Studies on p65, and fragments of the p65 C2 domains, have shown the C2 has the ability to bind proteins called RACKS or Receptors for Activated C-Kinase (Mochly-Rosen *et al.*, 1992). These RACKS are part of the triton-insoluble fraction of cells, indicating cytoskeletal elements or proteins associated with the cytoskeleton. The binding requires PS, DAG and  $\text{Ca}^{2+}$ , suggesting that C2 domain binding of RACKS mediates association of activated PKC to plasma membrane and cytoskeletal elements (Mochly-Rosen *et al.*, 1992). It is also known that PKC can phosphorylate these RACKS *in vitro* and *in vivo*, and the phosphorylated RACKS no longer bind PKC (Smith & Mochly-Rosen, 1992). The C2 domain may not be responsible for the translocation of the enzyme to the membrane or cytoskeletal elements, as there is evidence for membrane bound  $\text{Ca}^{2+}$ -independent isozymes (Khalil *et al.*, 1992; Ozawa *et al.*, 1993). Alternatively, these isozymes may have other elements within their structure which function to direct translocation to the membrane or cytoskeleton.

There is a variable V3 region following the C2 domain. It is referred to as the hinge region and has been shown to be cleaved *in vitro* by the  $\text{Ca}^{2+}$  activated protease calpain (Kishimoto *et al.*, 1983). The cleavage produces two fragments, one consisting of the regulatory half of the kinase and the other the catalytic region. This proteolysis, however, does not occur equally for all isozymes. Early studies with  $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$  showed that  $\gamma$  is cleaved at a much higher rate than  $\beta$ I-II and  $\alpha$ , with both calpain I and II (Kishimoto *et al.*, 1989). Specific aspects of the cleavage may be a function of the isozyme studied, or the method that is used to determine or define down-regulation. For example, it has been suggested that the regulatory domain is cleaved from the catalytic portion with a resulting loss of phorbol ester

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binding, presumably from degradation of that half of the protein, but there is a persisting kinase activity that is cofactor-independent (Chida *et al.*, 1986).

The C3 region C-terminal to the hinge region is the beginning of the catalytic portion of the enzyme. The C3 includes the hallmark of a kinase, the ATP binding site, which includes the residues involved in binding the ATP molecule used as the phosphate group donor. The C4 region is also part of the catalytic portion of the kinase. This region contains highly conserved residues found in the catalytic domains of all known serine/threonine protein kinases (Hunter, 1991). Much less is known about how the C3 and C4 regions function to bind and phosphorylate substrate molecules.

#### Activation properties of PKC

PKC activation is a result of stimulation of a cell by an external signal responsible for cellular activation, growth or differentiation. Growth factors can bind to transmembrane receptors activating a phospholipid hydrolyzing enzyme, a phospholipase. The activation is either direct, as in the case of receptor tyrosine kinases, or indirect, as in G-protein coupled receptors. The phospholipases are a large heterogeneous family of enzymes with different activation properties and different substrate specificities. This characteristic allows for generation of many different products. The phospholipases cleave membrane phospholipids resulting in the generation of DAG, DAG precursors, as well as other molecules (Liscovitch, 1992). DAG is one of the cofactors responsible for activation of PKC. Other products of phospholipid hydrolysis are cis-unsaturated fatty acids such as arachidonic acid (AA) and lysophosphatidylcholine, which can also act to increase activity of some PKC isozymes (Koide *et al.*, 1992; Nishizuka, 1988; Shinomura *et al.*, 1991). Another messenger derived from phospholipid hydrolysis is inositol 1,4,5-tris phosphate (IP<sub>3</sub>). IP<sub>3</sub> binds to an intracellular receptor to cause Ca<sup>2+</sup> release into the cytoplasm from internal stores (Berridge, 1993). Some members of the phospholipase family can be activated by Ca<sup>2+</sup> (Liscovitch, 1992), allowing a

method of bypassing receptor tyrosine kinase and G-protein linked receptors by the opening of  $\text{Ca}^{2+}$  channels. Many phospholipases are  $\text{Ca}^{2+}$  activated and can generate a second wave of DAG after intracellular  $\text{Ca}^{2+}$  release via  $\text{IP}_3$ . This produces other AA-like molecules to potentiate a sustained PKC activation (Liscovitch, 1992; Nishizuka, 1992). So it has been concluded that a variety of cellular activating mechanisms converge to activate PKC as a key signaling molecule within the cell.

PKC not only phosphorylates other substrate molecules, it also undergoes autophosphorylation (Kikkawa *et al.*, 1982; Newton & Koshland, 1987). This autophosphorylation has been well characterized for the  $\beta\text{II}$  isozyme cloned from rat and expressed in insect cells (Flint *et al.*, 1990), however, other isozymes are also known to be autophosphorylated (Huang *et al.*, 1986b; Ohno *et al.*, 1990). The  $\beta\text{II}$  autophosphorylation event takes place in the presence of PS, DAG,  $\text{Ca}^{2+}$  and ATP. It is estimated that each PKC- $\beta\text{II}$  molecule undergoes an intramolecular phosphorylation on one of six sites located throughout the molecule (Flint *et al.*, 1990). These autophosphorylated sites do not reflect recognition sites of the kinase for substrate phosphorylation. It is assumed the residues are phosphorylated as a function of proximity and accessibility to the catalytic region of the kinase. Some of these phosphorylated residues are conserved in other isozymes of the PKC family. The function of this autophosphorylation has not been assigned but theories suggest alteration of the activation properties of the kinase (Huang *et al.*, 1986a; Mochly-Rosen & Koshland Jr, 1987), down-regulation of the enzyme (Ohno *et al.*, 1990), or cellular localization of the protein (Wolf *et al.*, 1985).

The cellular localization of PKC has been a topic of study since the kinase was first discovered. Nishizuka and colleagues found that the kinase activity becomes associated with the membrane fraction and was assumed to be associated with the plasma membrane (Kraft & Anderson, 1983). This phenomenon is most often detected biochemically or immunologically by tracing the kinase in cellular fractions before and after activators of the kinase are applied

(Borner *et al.*, 1992; Ganesan *et al.*, 1992; Kikkawa *et al.*, 1982; Wetsel *et al.*, 1992). The translocation from cytosolic to membrane fractions seems to be isozyme and/or cell type specific (Borner *et al.*, 1992; Crabos *et al.*, 1991). The localization may not be restricted to the plasma membrane as generally thought but may also include cytoplasmic aggregations that are maintained by the cytoskeletal framework of the cell (Gregorio *et al.*, 1992; Mochly-Rosen *et al.*, 1990). This agrees with more recent data on possible PKC involvement in the rearrangement of cytoskeletal elements as discussed below.

### Functions for PKC

The function of PKC has been examined through the use of phorbol esters and membrane permeable synthetic DAG analogs. Phorbol esters, a class of tumor promoters, are the most important of the artificial activators of PKC. In most cases, upon prolonged exposure to phorbol esters, the kinase activity is down-regulated (Collins & Rozengurt, 1984; Phillips & Jaken, 1983). This down-regulation of PKC is not observed with PKC activation by DAG, the *in vivo* activator. One study (Chida *et al.*, 1986) examined this down-regulation and found that the regulatory domain was quickly degraded. There is a persisting catalytic domain which continues to be active in the absence of PKC activators. Also, this phorbol ester-induced down-regulation has differential effects on the various isozymes of PKC. The differences have no correlation with the translocation properties of the isozyme or its affinity for  $\text{Ca}^{2+}$ , which suggests that the isoforms of PKC have varying sensitivities to the mechanism of down-regulation induced by phorbol esters (Olivier & Parker, 1992).

Used in conjunction with phorbol esters and DAG derivatives, inhibitors of PKC have also proven useful in functional studies. There are three classes of inhibitor of PKC: synthetic inhibitors, microbe derived compounds and cellular or native inhibitors. Synthetic inhibitors include isoquinolinesulfonamides, structurally related compounds such as HA1004, W-7 and H-7 which target the ATP binding domain, inhibiting PKC, cAMP-dependent kinase (PKA)

and  $\text{Ca}^{2+}$ -CaM-kinase to varying degrees (Hidaka *et al.*, 1984; Kawamoto & Hidaka, 1984). Other synthetic inhibitors of PKC are peptides. The initial peptide inhibitor was PKC 19-36 which is the sequence of the pseudosubstrate domain (House & Kemp, 1987). An improvement of this is myr- $\psi$ PKC a myristoylated form of 19-36 which can be used as a membrane permeant, very specific inhibitor of PKC (Eichholtz *et al.*, 1993). Microbial inhibitors staurosporine and derivatives UCN-01 and -02, K252a, and other indolocarbazole compounds also target the catalytic domain, but most likely not the ATP binding site (Tamaoki & Nakano, 1990), and inhibit kinases at much lower concentrations. In the case of staurosporine, the concentration used can preferentially inhibit PKC over PKA. Microbial inhibitors that target the regulatory domain are the perylenequinone-containing compounds, such as calphostin C and cercosporin. These compounds are also more specific for PKC than those that target the catalytic portion. Calphostin C and cercosporin have been shown to compete with phorbol ester binding indicating the site of action of these inhibitors (Tamaoki & Nakano, 1990).

In addition to synthetic and microbial agents, there are cellular constituents that inhibit PKC. Lysosphingolipids such as sphingosine are inhibitors of PKC which may have implications in some sphingolipid metabolic disorders (Hannun & Bell, 1987). Recently, natural protein inhibitors of PKC have been discovered (McDonald *et al.*, 1987; Toker *et al.*, 1992), however, the mechanism of inhibition is unknown. One inhibitor protein consists of a widely expressed group of isoforms, the 14-3-3 proteins, which have been found in many species ranging from plants to yeast to humans (Aitkin, Collinge, van Heuseden, Isobe, Roseboom, Rosenfeld, et al., 1992). These proteins were originally characterized as activators of tyrosine and tryptophan hydroxylases which are involved in neurotransmitter synthesis. Within this class, the protein sequences show a possible pseudosubstrate-like domain and also possible PKC phosphorylation sites which are shown to be phosphorylated *in vitro* (Toker *et al.*, 1992) and may be involved in inhibition. In *in vivo* experiments using the 14-3-3 proteins,

translocation of PKC to the membrane and binding to cytoskeletal elements are obstructed. These proteins may also play a role repressing  $\text{Ca}^{2+}$ -dependent exocytosis (Aitkin *et al.*, 1992). Another protein inhibitor of PKC termed PKC-I has been isolated from human neutrophils and enriched at the membrane and granules (Balazovich *et al.*, 1992). PKC-I inhibits purified PKC histone phosphorylation *in vitro* and also PKC phosphorylation of endogenous substrates in neutrophil homogenates (Balazovich *et al.*, 1992).

Since its discovery in the late 1970s, work on PKC has been concerned with discovering functions and substrates for the PKC family of enzymes, using a variety of means. Some methods involve experimenting with a purified native or recombinant protein, creating a cell line that overexpresses a particular isozyme, or by adding activators and inhibitors of the kinase to cells or tissues.

One group of molecules PKC has been shown to influence are various ion channels. PKC activators applied to isolated neurons (Doerner & Alger, 1992), or oocytes expressing a particular channel (Singer-Lahat *et al.*, 1992), can reduce current of N-type  $\text{Ca}^{2+}$  channels, and have a biphasic effect (increase initially followed by decrease) on the L-type  $\text{Ca}^{2+}$  channels. Similarly PKC has been shown to phosphorylate voltage-gated  $\text{Na}^+$  channels (Murphy & Catterall, 1992; West *et al.*, 1991), which slows inactivation of the channel and reduces peak  $\text{Na}^+$  current.

In addition to ion channels, PKC is thought to modulate other types of transporters such as the  $\text{Na}^+/\text{H}^+$  antiporter (Grinstein & Rothstein, 1986). The antiporter is present in a wide range of cells and can be activated by many physiological signals such as growth factors, hormones and osmotic stress. The mechanism of PKC modulation is not entirely known. (Clark & Limbird, 1991).

One cell system in which PKC has been well studied is in blood platelets. In platelets, PKC is activated when the cells are stimulated by extracellular factors such as thrombin, which stimulate aggregation of the platelet cells to form the precursor to a clot and also result in

serotonin secretion (Kaibuchi *et al.*, 1983; Murphy & Westwick, 1992). Early work by Nishizuka and colleagues (Kaibuchi *et al.*, 1983) showed that addition of synthetic DAG to platelets contributed to maximum serotonin release when used with  $\text{Ca}^{2+}$  ionophore A23187. More recent research investigating PKC inhibitors modeled after staurosporine corroborates PKC activity in platelet activation by Platelet Activating Factor (Murphy & Westwick, 1992) and thrombin (Walker & Watson, 1993). However, the manner in which PKC functions to generate these cellular responses to activating factors remains elusive.

In addition to functions in these already differentiated cells, PKC isozymes have been shown to play a role in the differentiation process of cells. This differentiation is mainly observed as a change in morphology of a cell which is often accompanied by a change in expression of various genes. PKC may function in both of these aspects of cell differentiation (GuptaRoy & Cohen, 1992; Leli *et al.*, 1992). Phorbol esters and DAG derivatives activate transcription of certain genes that have regulatory sequences called the TPA (Tumor Promoting Agent) Response Elements or TREs. Transcription factors bind the TREs and regulate transcription of TRE-containing genes (Angel *et al.*, 1987; Mitchell & Tjian, 1989). Genes that have been found to have regulatory elements resembling the TRE include the cystic fibrosis transmembrane conductance regulator (Chou *et al.*, 1991; Yoshimura *et al.*, 1991), which is transcriptionally down-regulated by PKC activators and the human collagenase gene (Angel *et al.*, 1987). A component of the AP-1 transcription factor, c-jun, is phosphorylated in response to phorbol ester activation of PKC (Adler *et al.*, 1992). PKC plays a role in activating another transcription factor, NF- $\kappa$ B, which becomes active and localized to the nucleus after phorbol ester treatment. Presumably PKC causes release of NF- $\kappa$ B from an inhibitory cytosolic complex so it can be translocated to the nucleus (Baeuerle & Baltimore, 1988)). Mahoney and colleagues (1992) have shown that PKC phosphorylates the CCAAT enhancer binding protein (C/EBP) in cell extracts. The phosphorylation occurs in the DNA binding domain of C/EBP and attenuates protein binding to the CCAAT enhancer element. Conversely preincubation of

C/EBP with the CCAAT enhancer element inhibits the phosphorylation of C/EBP. Although this phosphorylation has yet to be shown *in vivo*, the possibility of it occurring proposes a potential control of transcription by PKC (Mahoney *et al.*, 1992).

PKC may also act posttranscriptionally to stabilize some mRNAs involved in maintaining cell differentiation, such as the muscle cell specific genes  $\alpha$ -cardiac and  $\alpha$ -skeletal actin, myosin light chain 1f and cardiac troponin C, whose messages have shorter half life when PKC is inhibited (Zhu *et al.*, 1991).

Morphological modifications accompany changes in gene expression during cell differentiation. These changes are in part a result of cytoskeletal rearrangements within the cell. PKC has been shown to associate with cytoskeletal elements (Cadrin *et al.*, 1992; Gregorio *et al.*, 1992; Jaken *et al.*, 1989; Mochly-Rosen *et al.*, 1990; Omary *et al.*, 1992), which leads to cytoskeletal changes (Downey *et al.*, 1992; Jaken *et al.*, 1989; Omary *et al.*, 1992; Werth & Pastan, 1984). It has been shown that the  $\epsilon$ -isozyme of PKC physically associates with and phosphorylates cytokeratins expressed in single layer epithelial cells but the purpose of the PKC- $\epsilon$  mediated phosphorylation is unknown (Omary *et al.*, 1992). PKC- $\alpha$ , a widely expressed isozyme, associates with the cytoskeleton at focal points in some cells and upon addition of phorbol ester those focal points are lost, becoming diffuse and disorganized as observed by antibody staining. In addition, this change in pattern was also observed in staining of vinculin (Jaken *et al.*, 1989). Vinculin belongs to a group of attachment proteins involved in the adhesion of actin filaments to the cell surface (Werth & Pastan, 1984). Inhibition of PKC can also disrupt talin distribution in cells, and disruption of these two combined inhibits focal adhesion of fibroblast cells to a fibronectin substrate (Woods & Couchman, 1992). Actin assembly is also involved in cell differentiation and cell activation, and PKC can induce actin assembly leading to membrane ruffling in what seems to be a  $\text{Ca}^{2+}$ -independent manner (Downey *et al.*, 1992).

Just how the activation of PKC can induce or contribute to changes in membrane shape has recently been addressed. One mechanism of PKC action at the membrane is via myristoylated alanine rich C kinase substrate (MARKS) the first well studied PKC substrate. The myristoylation causes MARKS to become a membrane associated protein (Hartwig *et al.*, 1992). MARKS contains two sites for binding of either actin or  $\text{Ca}^{2+}$ -calmodulin, this region also contains three sites of phosphorylation by PKC (Heemskerk *et al.*, 1993). In the unphosphorylated form, MARKS is membrane bound and can bind the sides of actin filaments, which will crosslink them near the membrane. Also in the unphosphorylated form MARKS can bind to  $\text{Ca}^{2+}$ -calmodulin and one actin filament. Upon phosphorylation MARKS is able to bind only one actin filament, and becomes dissociated from the membrane. The phosphorylation then can allow flexibility in the region directly on the cytoplasmic side of the membrane and cytoskeletal remodeling (Aderem, 1992; Thelen *et al.*, 1991). Another MARKS-like protein, F52 has been cloned from mouse and studied for MARKS-like function. F52 may play a role similar to that of MARKS since its abilities to be phosphorylated and bind  $\text{Ca}^{2+}$ -calmodulin are similar to MARKS, however, F52 has not been used for actin binding studies (Blackshear *et al.*, 1992).

Another function proposed for MARKS is to be a reservoir for calmodulin or a means of regulating cytoplasmic free calmodulin levels. Studies using calmodulin antibodies have shown an increase in cytosolic immunoreactivity along with a concomitant decrease of membrane associated calmodulin upon activation of PKC (Mangels & Gnegy, 1990). In addition to MARKS are calmodulin binding, neural specific, proteins, GAP43 also called neuromodulin, B50 or F1 (Baudier *et al.*, 1991; Sheu *et al.*, 1990), and RC3 or neurogranin (Watson *et al.*, 1992). Like MARKS, these proteins do not bind calmodulin when phosphorylated by PKC (Alexander *et al.*, 1987). This free calmodulin generated by PKC activation can lead to activation of  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinases (CaM kinase) if accompanied by an increase in  $\text{Ca}^{2+}$ . CaM kinase phosphorylates proteins associated with synaptic vesicle release,

modulating trafficking and fusion of vesicles (Greengard *et al.*, 1993). It is possible that this may be an interesting example of PKC regulation of another kinase family, the CaM kinases, within the cell (Alexander *et al.*, 1987; Watson *et al.*, 1992).

Either of the functions proposed for MARKS may aid in secretion or exocytosis. During secretion, like membrane ruffling and cell growth, there may be some necessary rearrangements of the cytoskeleton. Secretion of serotonin from platelets (Kaibuchi *et al.*, 1983) and interleukin 2 from T-cells (Modiano *et al.*, 1991), have been shown to be regulated in part by PKC activation; however, the mechanism of the release is unknown.

A process in which activation of kinases and secretion are thought to be important is Long Term Potentiation (LTP). LTP is thought to be the cellular basis of learning and memory in vertebrates, and is described as a potentiation or strengthening of synapses within the brain. One form of LTP is mediated by activation of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. One of the glutamate receptors in the postsynaptic cell is one which may be activated secondarily to NMDA. These metabotropic glutamate receptors can couple through G-proteins to different phospholipases which generate both arachidonic acid and DAG to activate PKC. The location of this PKC activity has not been determined; it may be both pre- and postsynaptically localized (Bliss & Collingridge, 1993).

One role for PKC that has been uncovered by work in the field of yeast molecular genetics is control of a point in the cell cycle. Genetic depletion studies resulting in cells having no endogenous PKC protein showed that these yeast cells arrest at the G2-M transition. The arrest occurs at a point after DNA replication has taken place but before cytokinesis, and arrested cells halt growth at small bud stage. The cells also had not formed spindles but appeared to have replicated their spindle pole bodies. Cloning and sequencing of the *S. cerevisiae* PKC1 gene revealed a PKC-like sequence that resembles the Ca<sup>2+</sup>-dependent isozymes (Levin *et al.*, 1990). Recently, a similar cell cycle arrest with a 4n nucleus at G2-M

transition was obtained by down-regulating PKC in tissue culture cells overexpressing PKC-  $\delta$  (Watanabe *et al.*, 1992).

Despite the vast amount of work on PKC in mammalian systems, exact substrates and functions of members of the kinase family are not well defined. One reason for the elusive nature of studying these kinases is the complexity of signaling pathways in cells. Multiple kinase families tend to be activated during cellular events, so the function or substrate of a particular kinase becomes minimized by the total phosphorylation events. Studies in our lab consist of the ultimate goal of assigning a particular substrate to a single isozyme of PKC.

#### PKC in lower vertebrates and invertebrates

Despite having been widely studied in mammalian systems, PKC in lower vertebrate and invertebrate model systems such as *Xenopus*, *Drosophila*, *Caenorhabditis elegans* and sea urchin have only recently been investigated. Of particular importance is the role of PKC in development of the organism, and these systems lend themselves well to understanding development. There is biochemical evidence for PKC activity in sea urchin (Heinecke *et al.*, 1990; Shen & Rieke, 1989), *Aplysia* (Saktor & Schwartz, 1990), *C.elegans* (Sassa & Miwa, 1992), *Drosophila* (Choi *et al.*, 1991), *Xenopus* (Otte *et al.*, 1990), and yeast (Ogita *et al.*, 1990). Clones have been recovered and sequenced from *Xenopus* (Chen *et al.*, 1989), *Drosophila* (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989), *C.elegans* (Tabuse *et al.*, 1989), yeast *Sacchromyces cerevisiae* (Levin *et al.*, 1990), *Aplysia* (Kreuger *et al.*, 1991), and *Dictyostelium* (Ravid & Spudich, 1992). In all cases the clones from these organisms do not fall into any one isozymic subgroup, but bear homology to the PKC family by containing the previously described elements. There is fairly strong homology in these sequences; for example, AplII the C2 containing isoform in *Aplysia*, is 65-73% homologous to mammalian PKCs (Kreuger *et al.*, 1991). It is unknown whether all of these organisms have multiple isozymes of PKC, but the fact that there are three isozymes cloned from *Drosophila* ; and two in

both *Aplysia* and *Xenopus* contends that expression of multiple isozymes, albeit on a smaller scale, exists in these species.

Interestingly, some of the cloning of lower vertebrate and invertebrate PKCs has shown that there are isozymes that are missing the C2 domain in some of these organisms. The *Drosophila* PKC98F gene contains the C1 domain cysteine repeats but diverges at the C2 domain only to have fairly good homology throughout the rest of the protein to the PKC family (Schaeffer *et al.*, 1989). *Aplysia* also has a C2 deficient clone, AplII (Kreuger *et al.*, 1991), and the *C.elegans* tpa-1 gene encodes a C2 deficient PKC (Tabuse *et al.*, 1989). Since these organisms have PKC clones that contain the C2 and PKCs that do not, it indicates that both classes of PKC isozymes play a unique role basic to cellular and organismal functions.

Studies on PKC in lower vertebrates and invertebrates generally have shown functions in development similar to the functions addressed in mammalian systems, however, some developmental roles have been investigated. One aspect that has been well studied in *Xenopus* is the induction of cells to adopt a particular tissue phenotype. In the developing frog embryo, it has been discovered that growth factor-like agents are responsible for mesoderm directed inductions (Kimelman & Kirschner, 1987). Dorsal and ventral ectoderm can be induced toward a neural phenotype, however, dorsal ectoderm shows stronger expression of neural markers. The inductions are due to a dorsal mesoderm factor and mimicked by phorbol esters (Kimelman & Kirschner, 1987; Otte *et al.*, 1988). Further work on the induction process has shown that two different isozymes of PKC are thought to be involved in formation of neural tissue. Overexpression of the two isozymes suggested that one isozyme regulates local competence of tissue to be induced and the other mediates the changes after induction (Otte & Moon, 1992).

A decrease in PKC activity may be necessary for another important developmental event in *Xenopus*. Progesterone naturally induces oocyte maturation in *Xenopus* oocytes, which is basically a resumption of the cell cycle at the metaphase to anaphase transition of meiosis I. One group has shown that after addition of progesterone to oocytes, there is a large decrease of

endogenous DAG. Also, when eggs are exposed to DAG analogs or microinjected with PKC, there is a concentration dependent delay or inhibition of maturation of the oocytes (Varnold & Smith, 1990).

In mouse embryos, PKC induces two linked events. At the eight cell stage the cells of the embryo become more adhesive, upon compaction of the embryo, and the nuclei migrate towards the center of the embryo (Reeve & Kelly, 1983). It is at this stage that cell polarity becomes evident, with respect to cytoskeletal elements and some organelles (Ducibella, Ukena, Karnovsky, & Anderson, 1977). Activators of PKC can mimic these events in embryos as early as two and four cell stages. Inhibitors block them when they are added to two cell embryos. This indicates PKC plays a role in cytoskeletal rearrangements in early embryos (Ohsugi *et al.*, 1993).

## PROJECT BACKGROUND

### Protein kinase C in the sea urchin

The interest in PKC activity in the sea urchin egg originally arose from studies involving the piecing together of the cellular events that occur during fertilization. Two ionic events during fertilization have been found to be necessary and sufficient for activation of the egg (Whitaker & Steinhardt, 1982). Once the egg has experienced these it can proceed into development (Brandriff *et al.*, 1975). The first of these events consists of a transient increase in intracellular  $\text{Ca}^{2+}$  that rises from resting levels of  $\sim 100$  nM and peaks at about  $1\text{--}2$   $\mu\text{M}$  within 1.5 minutes of sperm-egg binding and falls to near resting levels in minutes (Chambers *et al.*, 1974; Steinhardt & Epel, 1974; Steinhardt *et al.*, 1977). The second ionic event is a sustained increase in intracellular pH ( $\text{pH}_i$ ), which in the unfertilized egg is about 6.8 and by 5 minutes postfertilization is at about pH 7.2 (Johnson *et al.*, 1976; Shen & Steinhardt, 1978). These ionic changes in the egg cytoplasm are not unlike those reported in mammalian cells stimulated by mitogens (Moolenaar *et al.*, 1986).

The shift in  $\text{pH}_i$  in the egg has been shown to result from enhanced activity of the  $\text{Na}^+/\text{H}^+$  antiporter (Johnson *et al.*, 1976; Shen & Steinhardt, 1979). Studies using phorbol esters and DAG analogs showed that these agents can mimic the  $\text{pH}_i$  shift that occurs during fertilization (Shen & Burgart, 1986; Swann & Whitaker, 1985). Concurrent with the work on the  $\text{pH}_i$  shift was investigation of the respiratory burst at fertilization. Studies revealed that PKC was responsible for activating the NADPH dependent oxidase involved during fertilization (Heinecke *et al.*, 1990). This confers yet another role for PKC in early development of an invertebrate system. In addition to these early events, PKC plays a role later in tissue induction. Application of phorbol esters to early embryos shifted cell fates later in development away from ectoderm and toward endoderm and mesoderm (Livingston & Wilt, 1992).

Independent experiments were undertaken to biochemically purify PKC activity from sea urchin eggs. Unfortunately, unlike the mammalian PKC, the sea urchin kinase activity was lost after initial stages of purification. An anion exchange fraction was found to have kinase activity dependent upon PS, DAG and  $\text{Ca}^{2+}$  (Heinecke *et al.*, 1990; Shen & Ricke, 1989), but all activity was lost during subsequent purification procedures. Due to the fact that the PKC activity from sea urchin eggs was not amenable to biochemical purification, a molecular approach was used to investigate PKCs expressed in the sea urchin system. This dissertation describes work on using molecular cloning techniques to isolate and analyze PKC from the sea urchin *Lytechinus pictus*.

## MATERIALS AND METHODS

### Library screen

A cDNA library was constructed by Ambion (Austin, TX) in the expression vector lambda gt11. The cDNA was constructed using RNA isolated from whole ovary tissue primed with poly dT to generate the first strand of DNA. To screen the library, a cDNA clone of bovine PKC- $\alpha$  was obtained from Dr. Peter Parker of Imperial Cancer Research (London). To amplify regions of the bovine PKC- $\alpha$ , oligonucleotides to be used as primers in the Polymerase Chain Reaction (PCR) were generated by the nucleic acid facility (Iowa State University) on an Applied Biosystems Inc. 391 synthesizer. These primers are shown in Figure 2. The primers consist of two sense primers and one antisense primer. They are designed degenerate in order to be used in PCR reactions with any clones recovered from screening the cDNA library as template. The positions of the primers in the protein sequence are shown in Figure 3. The primer pair of 291296-589595 will generate a fragment of 950 basepairs (bp) spanning from the C2 region to the C4 and 441448-589595 primer pair will result in a 450 bp fragment covering the C3 to the C4. The primers were designed to regions of the kinase where the protein sequence is conserved. Sequences reported from bovine and *Drosophila* were used to identify regions that may be conserved between vertebrates and invertebrates. Figure 4 shows the amino acid sequences of bovine, *Drosophila* and sea urchin for the primers.

The primers were used in pairs of either 291296-589595 or 441448-589595 or with all three in the same reaction. Radioactive probes were generated by PCR which results in high specific activity probes. PCR was performed using 20 ng bovine clone DNA, 1  $\mu$ M each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C), 2 mM MgCl<sub>2</sub>, 5 units of Taq polymerase (Promega Madison, WI), 200  $\mu$ M each dTTP, dGTP and dCTP, and 150  $\mu$ Ci <sup>32</sup>P dATP (New England Nuclear or Amersham 3000-6000 Ci/mmol). The probe was passed over a spin column before adding to prehybridized filters at 42° C in 35% formamide,

sense primer 291296:

5' ACTTAAGCTTCTCGAGGA (AG) GTITGGGA (CT) TGGGA<sup>3'</sup>

sense primer 441448:

5' ACTTAAGCTTCTCGAGGA (CT) GA (CT) GTIGA (AG) TG (CT) AG (ACTG) AT<sup>3'</sup>

antisense primer 589595:

5' ACTTGGATCCGCGGCCGCA (AG) IACICC (AG) AAIGCCCACCA<sup>3'</sup>

Figure 2. Primers synthesized for PCR. The primers were designed to amplify two different fragments of PKC, using the antisense with each of the sense. The primers consist of a four nucleotide spacer at the 5' end to ensure restriction digestion. Next to the spacer are two different restriction sites, one set for the two sense primers and one set for the antisense primer. The restriction sites are followed by the PKC recognition sequence. The recognition sequences are degenerate, with inosine substituted at variable positions near the 5' ends. The numbers of the primers represent the amino acid numbers spanned by the recognition sequence in the *Drosophila* PKC clone reported by Schaeffer *et al.* (1989).

**I-**

1 MSNSTILEMKG FARRGALRQK NVHEIKNHKF IPRFFKQPTF CSHCKDFIWG  
**C1**

51 FGKQGFQCKV CSFVVKRCH EFVTFQCPGL DPGVDSDDPR NKHKFKVHSY

101 NSPTFCDHCG SLLYGLYHQG MKCGACDMNV HKRCQKSVPN LCGADHTERR  
**-I** **I-**

151 GRIKVKAEVI GNKLQVIVAE AKNLIPMDPN GLSDPFVKLK LIPDQKRETK  
**C2**

201 KKTRTIKGS LNPWGESFDF NLEDTRNRR LLVEVWDWR ATRNDFMGAL  
**-I** 291296

251 SFGISELMKA GVDWYKLLG QEEGEYYNVP AIAETESIDE LTSNIKKLPM  
**I-**

301 PTQEHVKPQN SNSMSGMGVV RASDFNFLSV LGKGSFGKVM LAEKKGTDEL  
**C3** **-I** **I-**

351 YAIKILKDV IIQDDVECT MIEKRVGLP SKPAFLTALH SCFQIMDRLF  
441448

401 FVMEFVNGGD LMFQIQKVGK FREPHAVFYA AETAVGLFYL HSQGVITYRDL  
**C4**

451 KLINVLVDAE GHIKTADFGM CKEHMNEGDT TRTFCGTPDY IAPETIVAYQP

501 YGKAVDWAF GVLLEYMLAG OPPFDGEDED ELFQSIMEHV PSYPKSMSRE  
589595 **C4**

551 SVIMCKGFLT KHPGKRLGSG PTGEQDIREH QFFRRIDWEK LANREIQPPF  
**-I**

601 VPSVRNPRAA ENFDPFYTKI PCALTPTDKL IIMNIQDEFQ GFTFVNEVFD

651 GYVRS LPS\*

Figure 3. Positions of the PCR primers in the sea urchin PKC. Each of the three primers are indicated by number and an underlined region. The conserved regions of PKC are indicated above the sequence as C1-C4. Primer pair 291296-589595 will yield a fragment of ~950 bp long spanning from the C2 to the C4. Primer pair 441448-589595 results in a fragment of 450 bp that spans from the C3 to the C4.

	291296	441448	589595
dPKC53E(ey)	EVWDWD	TDDMELPM	WWSFGVL
dPKC53E(br)	EVWDWD	DDDVECTM	WWAYGVL
bPKC-a	EIWDWD	DDDVECTM	WWAYGVL
bPKC-b	EIWDWD	DDDVECTM	WWAFGVL
bPKC-g	EVWDWD	DDDVDCTL	WWSFGVL
su	EVWDWD	DDDVECTM	WWAFGVL
consen	e.wdwd	dddvectm	WW. .GVL

Figure 4. Sequences of the regions used for generating primers for PCR. The *Drosophila* sequences are from Schaeffer *et al.* (1989). The bovine sequences are from Parker *et al.* (1986), and Coussins *et al.* (1986). The sea urchin sequence is reported here. The sequences are; dPKC53E(ey), eye specific *Drosophila* clone; dPKC53E(br) *Drosophila* brain specific clone; bPKC-a, bovine PKC- $\alpha$ ; bPKC-b, bovine PKC- $\beta$ ; bPKC-g, bovine PKC- $\gamma$ ; su, su PKC1 sequence; consen, consensus line. The line-up of the sequences used for the primers shows conservation in the sequences used between invertebrate and mammalian PKCs.

5X SSC (20X SSC consists of 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 5X Denhardt's (50X Denhardt's consists of 1 mg/ml each: ficoll, polyvinylpyrrolidone and bovine serum albumin (BSA)), 50 mM sodium phosphate (pH 7.0), and 250 µg/ml denatured salmon sperm DNA. The filters were hybridized 12-18 hours and washed three times at low stringency in 0.5X SSC, 0.1% sodium dodecylsulfate (SDS) at 37° C for 15 minutes each and exposed to X-ray film for 48 hours at -80° C.

Approximately  $1 \times 10^6$  plaques were screened, which resulted in the recovery of a clone. After passing secondary and tertiary screening, the clone was purified by the juicy method. For juicy purification, a tertiary plate was used to pick a well isolated positive plaque using a sterile toothpick. The end of which was clipped off into 1 milliliter of lambda dilution media (100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris pH 7.5) and incubated overnight at 4° C. A loop was used to streak out the diffused phage in lambda dilution media onto a small agar plate. The plate was overlaid with phage competent cells in top agarose. Phage competent cells are those that have been grown overnight and then diluted 1:100 in media and grown for 2-2.5 hours in the presence of 0.2% maltose. The cells were then pelleted at 3000 rpm for 10 minutes at 4° C and resuspended in 0.1X original volume of 10 mM MgSO<sub>4</sub>. The plates with the overlay are incubated at 37° C just until plaques appear. At this point, using a sterile toothpick, several of the plaques were transferred to a new plate that had a fresh overlay of competent cells in top agarose poured on it. Each of the plaques was transferred in closely arranged spots. A plate consisted of 4-5 individual plaques that were well spaced. The plates were incubated at 37° C for 8-12 hours until plaques appear fairly confluent in the spotted area. Filter hybridization was performed on the plates. Only those plates with all of the large plaques coming up positive are considered pure. A plug is pulled from the center of one of these juicy areas of plaque using the wide end of a disposable pasteur pipet and put into dilution medium. This plug yields a high titer phage stock ( $5 \times 10^5$  pfu/ml).

### Southern blotting

Agarose electrophoresis was performed to separate DNA fragments, then the gel was denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (0.5 M Tris pH 7.5, 1.5 M NaCl) and equilibrated in 10X SSC. DNA was transferred to nitrocellulose (Schleicher and Schuell) by capillary action using 20X SSC for 12-18 hours. Membranes were baked in a vacuum oven at  $\sim 15\text{lb/in}^2$  pressure at 80° C for 90-120 minutes. The baked membranes were prehybridized for 2 hours and then hybridized 12-16 hours with the addition of probe and washed as described above. High stringency hybridizations with homologous probes were at 55° C and washes were 0.2X SSC 0.1% SDS at 25-45° C. Membranes were exposed to X-ray film at -80° C or exposed to a Phospho Imager screen (Molecular Dynamics).

### Sequencing

The 3.1Kb fragment recovered from the library screen was used in PCR reaction with the primers. These fragments as well as the parent clone were used as templates in sequencing reactions. The sequencing was performed on an Applied Biosystems Inc. sequencer (model 373A), using dye terminators by the nucleic acid facility (Iowa State University). Sequencing was also done manually using the Sequenase kit (United States Biochemicals). To obtain the complete sequence for 2474 basepairs, nested serial deletions were generated (Erase-a-Base, Promega). In some cases restriction enzymes used singly or in pairs taking advantage of the restriction map of the clone produced small fragments of the clone that were subcloned for sequencing. Finally, primers were designed using the Oligo program (version 3.4 National Biosciences, Medprobe, Eurogentec) to obtain sequence for areas not accessed by the previously described methods. These primers were synthesized by Genset (Clifton N J).

### RNA isolation and ribonuclease protection assay

RNA was isolated from ovary, egg or embryo tissue. For every 1 ml of packed tissue, 6 ml of guanidinium digestion solution was added (4 M guanidinium thiocyanate, 0.04% 8-hydroxyquinoline, with 1:1:0.1 of 25 mM sodium citrate pH 7.0 : water saturated phenol : 2 M sodium acetate pH 2.5, and 750  $\mu$ l  $\beta$ -mercaptoethanol/100 ml phenol). Tissue was vortexed and 0.1 volume of chloroform was added. The digestion was incubated at 4° C for 20 minutes and centrifuged 12000 xg 15-20 minutes. The aqueous layer was extracted with an equal volume of chloroform and ethanol precipitated. RNA was pelleted at 12000 xg for 20 minutes and resuspended in water.

Total RNA (20  $\mu$ g) was used for the Ribonuclease Protection Assay (RPA) (Ambion, Austin TX). Probes for the assay were products of an *in vitro* transcription reaction. A DNA subclone was digested with an appropriate enzyme downstream of the transcriptional promoter and PKC fragment to be used as probe. *In vitro* transcription produced an antisense transcript of the sequence used as probe described in Figures 5 and 6. Figure 5 is the sequence of the probe used for the RPA and the position of the fragment in the suPKC1 clone protein sequence is in figure 6. The reaction conditions were, 40 mM Tris pH 7.5, 6 mM  $MgCl_2$ , 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol 20 units RNasin (Promega) 500 mM ATP, UTP and GTP, 2.5 mM CTP, 50  $\mu$ Ci P32-a CTP (800 Ci/mmol, Amersham), 0.5 mg template DNA and 40 units T7 or T3 RNA polymerase (Ambion and Promega, respectively). Approximately  $1 \times 10^5$  cpm of probe and 20  $\mu$ g of total RNA from ovary, egg or embryo tissue were coprecipitated with 100% ethanol. The RPA was performed essentially according to manufacturers instructions. The RNA-probe mixture was heated to 95° C and hybridized overnight at 42° C. The unduplexed RNA was digested by RNAase T1 at a 1:50 dilution, at 37° C for 60 minutes. The undegraded RNA was precipitated at -20° C. The precipitate was resuspended in loading buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol 2mM EDTA) and separated on an 8 M urea 5% acrylamide gel (38%

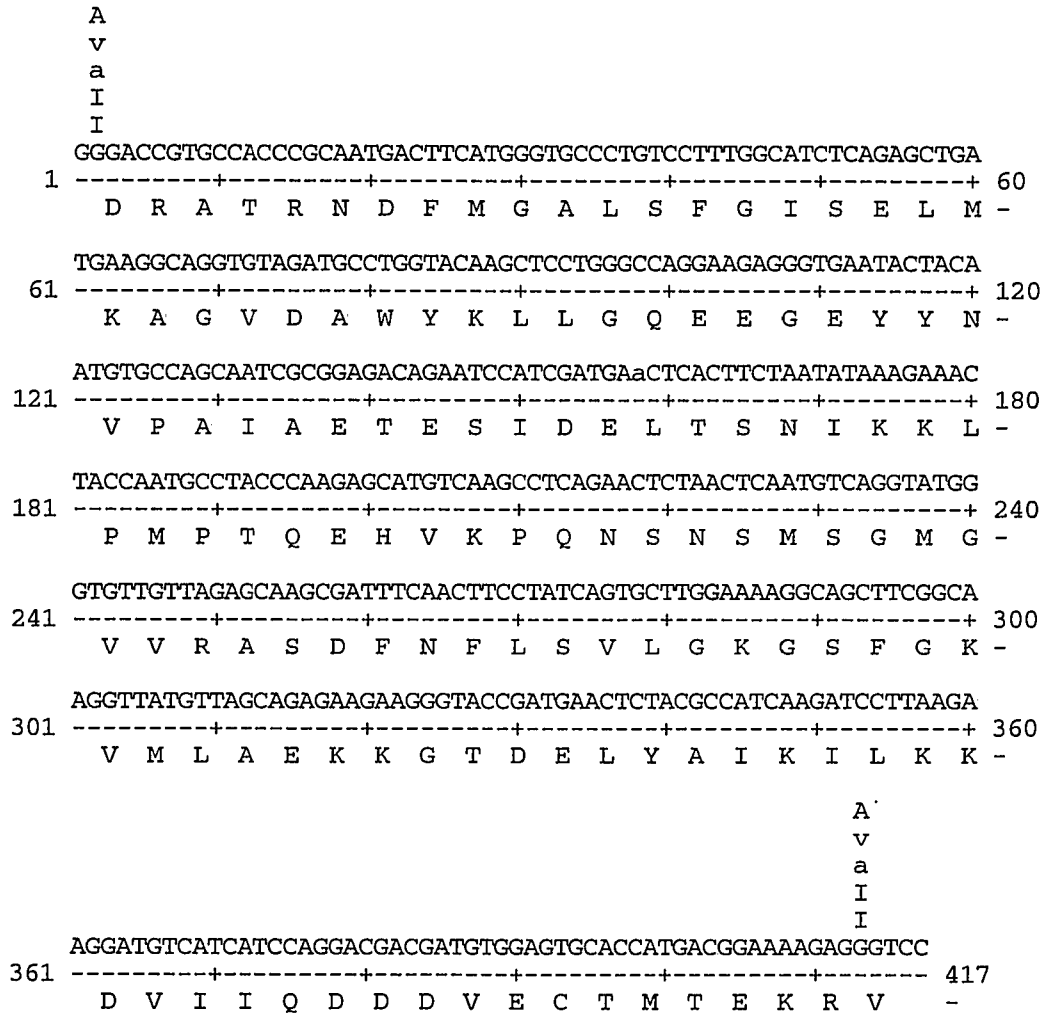


Figure 5. Map of the *Ava*II fragment used for the RPA probe. *In vitro* transcription of the subcloned fragment generates a fragment of approximately 450 bp long, 50 bp consist of vector sequence that follows the transcriptional promoter and the remaining sequence code for part of the *suPKC1* gene.

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      I-
1  MSNSTLEMKG FARRGALRQK NVHEIKNHKF IPRFFKQPTF CSHCKDFIWG
      C1
51 FGKQGFQCKV CSFVVHKRCH EFVTFQCPGL DPGVDSDDPR NKHKFKVHSY

101 NSPTFCDHCG SLLYGLYHQG MKCGACDMNV HKRCQKSVPN LCGADHTERR
      -I      I-      C2
151 GRIKVKAIVI GNKLQVTVAE AKNLIPMDEN GLSDPFVKLK LIPDQKRETK

201 KKTRTIKGS L NPTWGESFDF NLEDTRNRR LLVEVWDWDR ATRNDFMGAL
      -I
251 SFGISELMKA GVDWYKLLG OEEGEYINV ATAETESIDE LTSNIKKLPM
      I-      C3
301 PTOEHVKPON SNSMSGMGVV RASDFNFLSV LGKGSFGKVM LAEKKGTDEL
      -I      I-
351 YAIKILKDV IIODDVECT MTEKRVLGLP SKPAFLTALH SCFQIMDRLF
      C4
401 FVMEFVNGGD IMFQIQKVGK FREPHAVFYA AETAVGLFYL HSQGVITYRDL

451 KLINVLVDAE GHIKIADFGM CKEHMNEGDT TRTFCGTPDY IAPETVAYQP
      C4
501 YGKAVDWAF GVLLYEMLAG QPPFDGEDED ELFQSIMEHV PSYPKSMSRE

551 SVIMCKGFLT KHPGKRLGSG PTGEQDIREH QFFRRIDWEK LANREIQPPF
      -I
601 VPSVRNPRAA ENFDPYFTKI PCALTPIDKL IIMNIQDEFQ GFTFVNEVFD

651 GYVRS LPS*

```

Figure 6. The peptide sequence of suPKC1 showing the region spanned by the RPA probe. The underlined portions is the sequence of the *ava*II fragment used for making the RPA probe. The conserved portions of PKC are indicated above the sequence as C1-C4. The probe consists of part of the C2 through the C3 regions including the V3 which lies between the two conserved regions.

acrylamide 2% N'N' methylene-bis-acrylamide). Dried gels were exposed to X-ray film at -80° C with an intensifying screen. Densitometry was performed on the RPA results after exposing the gels to a Phospho Imager screen (Molecular Dynamics) following the manufacturer's instructions. Two control samples included probe incubated with yeast total RNA, one of which received RNAase treatment and the other did not. To determine that the same amount of RNA was utilized for each sample, the samples were also subjected to RPA with 18S rRNA probe as manufacturers instructions (Ambion). The 18S rRNA probe was hybridized to 5 µg of total RNA to ensure probe excess. In performing the RPA, we occasionally observed signal remaining in the wells of the gel after electrophoresis and autoradiography. This signal did not correspond with any particular treatment or condition, and control experiments performed to eliminate the aberrant signal did not have an effect. Discussions with the manufacturer led to the conclusion that the signal was due to unreacted probe (Dr. M. M. Winkler, Ambion, personal communication).

#### Polyclonal antibody production

Three peptides were synthesized (Molecular Biology Resource Facility University of Oklahoma Health Sciences Center) for antibody production. The peptides are designed from N-terminal, V3 and C-terminal regions in sea urchin PKC and are shown in Figure 7 as underlined portions of the sea urchin peptide sequence. The peptides are in regions of PKC that have low homology among isozymes which is displayed in Figure 8 which lists the regions surrounding the peptide in the three Ca<sup>2+</sup>-dependent rat PKCs and the sea urchin PKC. The peptides were conjugated to thyroglobulin used as a carrier protein. Sixteen milligrams of thyroglobulin was suspended in 10 mM sodium phosphate pH 7.2. Five milligrams of activator m-maleimido-benzoyl-N-hydroxysuccinimide ester (Sigma) was dissolved into 150 µl dimethyl formamide and added dropwise to the thyroglobulin with gentle stirring. The mixture was stirred for 45 minutes at 25° C and then at 4° C overnight.

Activated thyroglobulin was passed over a spin column equilibrated with 50 mM sodium phosphate (pH 6.0). The activated thyroglobulin was divided into three aliquots and approximately 5 mg of peptide (at 6mg/ml) suspended in 1X 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub> (PBS) was added to each. The thyroglobulin and peptide were stirred for 3 hours at 25° C. The conjugates were divided into 0.6 mg aliquots and stored at -80° C.

Two rabbits were inoculated per peptide and were given an initial subcutaneous injection two weeks after a preimmune bleed. The injection consisted of 0.6 mg peptide-thyroglobulin conjugate mixed 1:1 (v:v) into an emulsion with complete Freund's adjuvant (Difco). Boosts consisted of injection of 0.3-0.6 mg peptide-thyroglobulin conjugate in a 50:50 emulsion with incomplete Freund's adjuvant (Difco) and were given two weeks past initial inoculation. Sera were tested after boosts for immunoreactivity to thyroglobulin, and subsequently to peptide-ovalbumin conjugations. Ovalbumin (15 mg) was activated as thyroglobulin, with 6 mg N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Pierce) in 100 mM NaPO<sub>4</sub> pH 6.0. Activated Ovalbumin was passed over a spin column and 4 mg of peptide in 100 mM NaPO<sub>4</sub> pH 6.0 was added to each of three equal aliquots. The peptide-ovalbumin conjugation was incubated overnight at 4° C, then stored at -20° C.

#### Total protein preparation

Sea urchins (*Marinus*) were induced to spawn gametes by intracoelomic injection of 0.5 M KCl. Eggs were shed into artificial sea water (ASW) (470 mM NaCl, 10 mM KCl, 11 mM CaCl<sub>2</sub>, 29 mM MgSO<sub>4</sub>, 27 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub> pH 8.0), and dejellied by passage through a fine silk mesh. The eggs were allowed to settle twice in ~400 ml of ASW to remove excess debris. Eggs were then washed in 0 Ca<sup>2+</sup>-0 Mg<sup>2+</sup> ASW (510 mM NaCl, 10 mM KCl, 10 mM EGTA, 2.5 mM NaHCO<sub>3</sub>, pH 8.0) and twice in intracellular buffer (220 mM K-acetate, 500 mM glycine, 40 mM NaCl, 5.9 mM MgCl<sub>2</sub>, 4.3 mM CaCl<sub>2</sub>, 10 mM

```

      I-
1  MSNSTLEMKG FARRGALRQK NVHEIKNHKF IPRFFKQPTF CSHCKDFIWG
                                     C1
51 FGKQGFQCKV CSFVVHKRCH EFVTFQCPGL DPGVDSDDPR NKHKFKVHSY
101 NSPTFCDHCG SLLYGLYHQG MKCGACDMNV HKRCQKSVFN LCGADHTERR
      -I      I-
151 GRIKVKAIVI GNKLQVIVAE AKNLIPMDPN GLSDPFVKLK LIPDQKRETK
                                     C2
201 KKTRTIKGS L NPTWGESFDF NLEDTDRNR LLVEVWDWDR ATRNDFMGAL
                                     -I
251 SFGISEIMKA GVDAWYKLLG QEEGEYYNVP AIAETESIDE LTSNIKKLIM
      I-
301 PTOEHVKPQN SNSMSGMGVV RASDFNFLSV LGKGSFGKVM LAEKKGTDEL
      C3      -I      I-
351 YAIKILKKDV IIQDDDVECT MTEKRVGLGP SKPAFLTALH SCFQIMDRLF
                                     C4
401 FVMEFVNGGD LMFQIQKVGK FREPHAVFYA AEIAGVGLFYL HSQGVIVYRDL
451 KLDNVLVDAE GHIKIADFGM CKEHMNEGDT TRITFCGTPDY IAPFIVAYQP
      C4
501 YGKAVIDWAF GVLLYEMLAG QPPFDGEDED ELFQSIMEHV PSYPKSMSRE
551 SVTMCKGFLT KHPGKRLGSG PTGEQDIREH QFFRRIDWEK LANREIQPPF
      -I
601 VPSVRNPRAA ENFDPYFTKI PCALTPTDKL IIMNIQDEFQ GFTFVNEVFD
651 GYVRS LPS*

```

Figure 7. Location of peptides generated for polyclonal antibody production. The three peptides are indicated by an underline on the deduced peptide sequence of the sea urchin PKC clone. The peptides are in order of N-term, V3 and C-term. The conserved regions of the PKC family are indicated above the sequence as C1-C4.

**N-TERM PEPTIDE**

```

RAT-a  MADVYPANDS TASQDVANRF ARK
RAT-bI MADPAAGPPP SEGEESTVRF ARK
RAT-g  MAGLGPGGGD SEGGPRPL.F CRK
SU     .....M SNSTLEMKGF ARR
consen ma..... s..... f ark

```

**V3 PEPTIDE**

```

RAT-a  IPEGDEEGNV ELRQKF.... .....EKAK LGP...AGNK VISPSEDRKQ P..SNNLDRV
RAT-bI VPPEESEGNE ELRQKF.... .....ERAK IGQGTKAPEE KTANTISKFD N..NGNRDRM
RAT-g  VADAD...NC SLLQKFEACN YPLELYERVR MGPSSSPIPS PSPSPDTSKR CFFGASPGRL
SU     AIAETESIDE ..... LTSNIKKLPM PTOEHVKPQN SNSMSGMGVV
consen ..... .l.qkf.... .....e... .g.....

```

**C-TERM PEPTIDE**

```

RAT-a  PPFKPKVC GK.GAENFDK FFTRGQPVLT PPDQLVIANI DQSDFEFGFSY VNPQFVHPIL
RAT-bI PPYKPKAR DKRDTSNFDK EFTRQPVLT PTDKLFIMNL DQNEFAGFSY TNPEFVINV.
RAT-g  PPFRPRPC GRSG.ENFDK FFTRAAPALT PPDRLVLASI DQADFQGFY VNPDFVHPDA
SU     PPFFVPSVR NPRAAENFDP YFTKIPCALT PTDKLIIMNI .QDEFQGFY VNEVFDGYVR
consen ppf.p... .....enfdk .ft.....lt p.d.l...ni dq..f.gf.y vnp.fv....

```

Figure 8. The three rat PKC sequences are shown with the sea urchin PKC clone deduced amino acid sequence in the regions selected for designing peptides. The peptide sequences are indicated by an underline in the sea urchin sequence. The N-term peptide (top) is at the very N-terminus of the protein sequence and contains the first two amino acids of the pseudosubstrate domain. The V3 peptide (middle) is in the V3 region of the protein. The C-term peptide (bottom) is approximately 50 amino acids from the end of the protein, and contains three amino acids from the C4 region. The peptides were selected to be in non-conserved areas of the PKC family.

EGTA, 20 mM Tris pH 7.0 ) and resuspended in intracellular buffer with protease inhibitors added (5 mg/ml aprotinin, 1 mg/ml soybean trypsin inhibitor). The eggs were homogenized with 5 strokes of a Dounce homogenizer, and the homogenate was centrifuged at 100,000  $\times g$  for one hour. The supernatant was retained as the cytosolic protein fraction.

#### Separation of proteins

The cytosolic fraction was dialyzed in 20 mM Tris pH 7.0 (at 4° C), 2 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF). The dialyzed fraction (15-25 ml) was loaded onto a 12 x 1.5 cm column of DE-52 equilibrated in dialysis buffer at 4° C, and washed with 150-300 ml of the same buffer. The bound proteins were eluted with a linear gradient of NaCl in dialysis buffer, and fractions of approximately 6 ml were collected. Conductivity measurements and Bradford assays (Bradford, 1976) were done on the fractions to determine salt concentrations and protein concentrations respectively. Fractions were used directly for kinase assays or concentrated approximately 10 fold in Centricon 10 sample concentrators (Amicon, Beverly, MA).

#### Protein kinase assay

Kinase assays were performed at 20° C with calf thymus histone H1 as the phosphate acceptor. The assays (250  $\mu$ l) consisted of 20 mM Tris pH 7.0, 50  $\mu$ g histone H1, 5.5 mM Mg-acetate, 10 mM ATP, 1  $\mu$ Ci  $\gamma$ -<sup>32</sup>P ATP, and 0.5 mM EGTA, or 0.5 mM free Ca<sup>2+</sup>, with 20  $\mu$ g/ml of phosphatidylserine and 2  $\mu$ g/ml of diolein. The assay reactions were initiated by addition of 25  $\mu$ l of each fraction, incubated for 5 minutes and then terminated by addition of 1 ml ice cold 25% (w/v) trichloroacetic acid (TCA) and 50  $\mu$ l 6 mg/ml BSA. Precipitated proteins were collected on GA-6 filters (Whatman), and washed with ice-cold 5% TCA and 1% Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>. Filters were then counted in a scintillation counter (model 1217, LKB, Stockholm). Lipids were dissolved in chloroform, mixed, dried in a vacuum and

reconstituted into Tris pH 7.0 by sonication for approximately 1 minute with a sonicator (model 300, Artek System). Sonicated samples were prepared fresh for each experiment.

#### PAGE and immunoblotting

The concentrated DE52 samples were separated on 9% acrylamide gels (30% w/v acrylamide and 0.8% w/v N'N' methylene-bis-acrylamide, Amresco, Solon, OH). The proteins were transferred to Hybond-C (Amersham) nitrocellulose membranes which were Ponceau S (Fisher) stained for total protein detection. The membranes were blocked for one hour in 150 mM NaCl, 10 mM Tris pH 7.5 (TBS)-blotto (5% non-fat dry milk) and washed 3X for 5 minutes in TBS. The membranes were then incubated with sera diluted in TBS-blotto for 2-3 hours at 25° C or overnight at 4° C. Membranes were washed as before and incubated in HRP conjugated secondary antibody (goat anti-rabbit Gibco-BRL) in TBS-0.5% Tween at room temperature. Development of the HRP color reactivity utilized 4-chloro-1-naphthol (Gibco BRL) at 30 mg, 20 % methanol, and 1% H<sub>2</sub>O<sub>2</sub> in TBS.

## RESULTS

### Cloning and sequencing of a sea urchin protein kinase C

Screening the sea urchin ovary cDNA library yielded one clone that persisted through three rounds of screening. This clone was purified for analysis. Figure 9A is a representation of the cloned insert and vector, denoting the restriction sites used for cloning and digestion, in reference to the position of the insert. The Not1 restriction fragment from the clone was found to be approximately 3.1 Kb in length when digested from the lambda gt11 library vector. A Southern blot of the digested clone in Figure 9B shows the apparent size of the fragment. The blot also demonstrates that the clone has internal Eco R1 restriction sites as indicated by the smaller size fragment in the EcoR1 digested lane. The results from this suggested that the clone was full length or very near full length, with the assumption that most PKC mRNAs have been found to be 2.5-3.5 Kb.

Sequence analysis of the clone was performed and the nucleotide sequence is shown in Figure 10, with the deduced amino acid sequence immediately below. In all, 2474 nucleotides were sequenced. The sequence information spans the 5' untranslated sequence, the coding region and part of the 3' untranslated sequence. The last 130 nucleotides of the sequence shown are data from only one or two sequencing reactions. Other sequencing from the 3' end towards the 5' did not reveal a poly A+ tail, nor a sequence resembling a polyadenylation signal. This may be an artifact of the subcloning of the cDNA into the library vector, for example, there may be a Not I site upstream of these sequences in the gene. None of the N-terminal methionines meet the Kozak (Kozak, 1987) requirement for an initiation AUG, so the first AUG after the last stop codon is taken to be the initiating methionine. A more extensive explanation of the sequencing information compiled to generate the sequence of the clone and figures pertaining to the sequencing are found in Appendix 1.

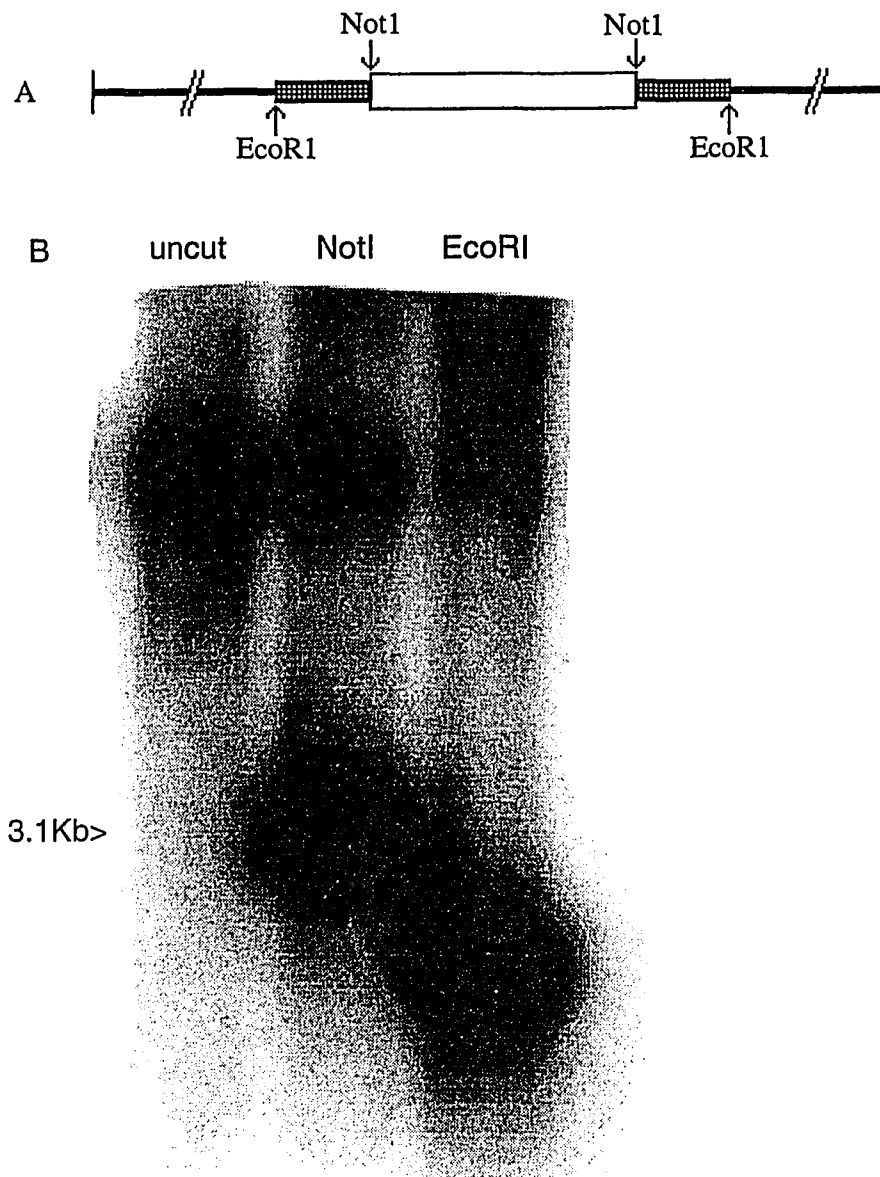


Figure 9. Library construct and clone size. (A) Construct of the ovary tissue cDNA in the lambda phage vector gt11. The cDNA was inserted in the unique EcoRI cloning site of gt11 using adaptors that contain an internal NotI restriction site. This construction allows excision of inserts with either EcoRI or NotI. (B) Southern blot of the positive clone isolated from the cDNA library probed with bovine PKC- $\alpha$ . Lane 1, undigested clone; lane 2, NotI; and lane 3, EcoRI digested DNA. The size of the clone is approximately 3.1Kb.

Figure 10. Sequence of the sea urchin PKC cDNA clone. Nucleotide sequence of the sea urchin PKC cDNA clone, and deduced amino acid sequence in single-letter code. The cDNA was cloned and sequenced as in materials and methods, and all information was obtained from the 3.1Kb clone in one open reading frame. The pseudosubstrate domain of the protein is underlined, the conserved cysteines are circled and the residues involved in ATP binding are indicated by arrowheads. Peptides generated for polyclonal antibody production are indicated by boxes. The conserved domains are indicated as C1-C4 below the protein sequence.

1 ACGAACATTTAATCGAAGGCGGATATTCTCCAGTCTTATGTGCAAGAACGAAGCACATTAAACATTGGTTAAATGTGCCCGTCGTAATTAAGTACT 100  
 101 GTATCGCTATCATTTGGGAGTAGTACATGTATACGGTAGCGGAGGCGGAGGTCACAGCTTGTACTTCCATTCCTAAAGTGGATATTACATTTGGTGTGG 200  
 201 CCGAGACAGTGGATATAAATGTCGAATTCGACACTTGAGATGAAGGGATTTCGGAGGAGGGAGCTTTGCGTCAGAAAGACGTCATGAGATAAAAAAT 300  
 H S N S T L E H K G F A H R G A L R Q K N V H E I K N  
 301 CACAAATTCATCCGAGGTTTTCAGCAGCCGACATTTGTAGCCATTGCAAGGATTTCATTGGGGATTGGCAAGCAAGGGTTTCAGTCCAAAGTGT 400  
 H K F I P R F P K Q P T F C S H C K D F I W G F G K Q G F Q C K V C  
 401 GCAGTTTGTAGTTCATAAGCGCTGCCATGAATTTGTCACTTCCAGTGCCTGGACTCGACCCAGGAGTCGATTCTGACGATCCTAGGAACAAGCATAA 500  
 S F V V H K R C H E F V T F Q C P G L D P G V D S D D P R N K H R  
 501 GTTCAAAGTTCACAGTTATAACAGTCCACATTCGTGACCACTGTGGGCTCTCTCTATATGGGCTCTATCACCAGGCAATGAATGTGGAGCATGTGAC 600  
 F K V H S Y N S P T F C D H C G S L L Y G L Y H Q G M K C G A C D  
 601 ATGAATGTACACAAACGATGTACAGAGTCAGTACCAAAATTTATGTGGAGCAGACCATACAGAGAGACGAGGTCGTATCAAGGTCAAAGCTGAGGTCAATTG 700  
 M N V H K R C Q K S V P N L C G A D H T E R R G R I K V K A E V I G  
 701 GAAACAACTCCAGGTTCAGTGTCTGAAGCCAAAGAACTAATTCGATGGACCCCTAATGGATTGTGAGATCCCTTTGTGAACTCAAACCTATTCAGCA 800  
 N K L Q V T V A E A K N L I P M D P N G L S D P F V K L K L I P D  
 801 TCAGAAACGAGAGACTAAGAAGAGACTAGGACTATTAAAGGAAGCCTAAATCCAACTCGGGAGAAATCATTTGATTTTAACTTAGAAGACACGGACAGG 900  
 I K K R E T K K K T R T I K G S L N P T W G E S P D P N L E D T D  
 901 AACCGGAGGCTTCTGGTTGAGGTGTGGGACTGGGACCGTGCACCCGCAATGACTTCATGGGTGCCCTGTCTCTTTGGCATCTCAGAGCTGATGAAGGCAG 1000  
 N R R L L V E V W D W D R A T R N D F M G A L S F G I S E L M K A G  
 1001 GTGTAGATGCTGTGTAAGCTCTGGGCCAGGAAGAGGGTGAATATACAAATGTGCCAGCAATCGCGGAGACAGAATCCATCGATGAAGTCACTTCTAA 1100  
 V D A W Y K L L G Q E E G E Y Y N V P A I A E T E S I D E L T S N  
 1101 TATAAAGAACTACCAATGCTTACCCCAAGAGCATGTCAAGCCTCAGAATCTAATCAATGTGAGGTATGGGTGTTGTTAGCAAGCGATTTCACCTTC 1200  
 I K K L P M P T O E H V K P L P S N S M S G M G V V R A S D F N  
 1201 CTATCAGTCTTGGAAAAGGCGAGCTTCGGCAAGGTTATGTAGCAGAGAAGAAGGATACCGATGAATCTACGCCATCAAGATCCTTAAGAAGGATGTCA 1300  
 L S V L G K G S F G K V M L A E K K G T D E L Y A I K I L K K D V I  
 1301 TCATCCAGAGCAGCATGTGGAGTGCACCATGACGGAAGAGGGTCTGGGCGCTGCCAGCAACCCGCTTTCTTGACGGCGCTTCACTCATGCTTCCA 1400  
 I Q D D D V E C T M T E K R V L G L P S K P A F L T A L H S C F Q  
 1401 GACAAATGACAGACTCTCTTTGTGATGAGTTCGTCAATGGGGAGATCTCATGTTCCAGATCCAGAAAGTCGGCAAGTTCAGGGAACCTCATGCTGTA 1500  
 T M D R L L F F V M E F V N G G D L M F Q I Q K V G K F R E P H A V  
 1501 TTCTATGCAGCTGAGATAGCAGTAGGCTTATTCTACCTCATTACAGGTTGTGATATACAGAGATCTCAAACCTAGATAATGTACTAGTAGATGCAGAG 1600  
 F Y A A E I A V G L F Y L H S Q G V I Y R D L K L D N V L V D A E G  
 1601 GTCACATCAAGATTGCTGACTTTGGTATGTGCAAGGAACACATGAACGAGGGCGACACAACGAGAAGCTTCTCGGGAACACAGACTACATCGCTCCCGA 1700  
 H I K I A D F G M C K E H M N E G D T T R T F C G T P D Y I A P E  
 1701 GATTGTAGCATATCAGCCATATGCGAAGGCTGTGACTGGTGGGCGCTTTGGTGTMTTACTGTATGAAATGTAGCAGGACAGCCTCCATTTGATGGTGA 1800  
 I V A Y Q P Y G K A V D W W A F G V L L Y E M L A G Q P P F D G E  
 1801 GATGAAGATGAATTTTCAATCTATCATGGAACATGTACCCCTCATATCCCAATCAATGTCAAGAGAATCTGTGACTATGTGCAAGGGTTTGTGACAA 1900  
 D E D E L F Q S I M E H V P S Y P K S M S R E S V T M C K G F L T K  
 1901 AGCATCTCTGTAAGCGGTAGGAAGTGGACCTACAGGGGAACAGGACATCAGGGAGCATCAATCTTCCTGCGAATCGACTGGGAGAACTAGCTAACAG 2000  
 H P G K R L G S G P T G E Q D I R E H Q F F R R I D W E K L A N R  
 2001 AGAAATACAGCCTCGTTTGTGCGCTCAGTGAGAAACCCAGAGCTGTGAAACTTTGACCCCTATTTTCACAAAAATCCCGTGTGCGCTTACCCCGACC 2100  
 E I Q P P F V P S V R N P R A A E N E D P Y F T K I P C A L T P T  
 2101 GACAAATCATTTATCATGAATATTCCAGGCAATTCAGGCTTTTACCTTCGTGAACGAGGTCCTTCGATGGCTATGTGCGTAGTTTGCCAGCTAGTGCC 2200  
 D K L I I H N I Q D E F Q G F T F V N E V F D G Y V R S L P S \*  
 2201 AAGTTTGTAGCTCCACCGTCTGTCTTACGCTACTTGGTGTCCCAAGCTATCCAAATGTACAGGTTTATACATGTGCTGTCTTTTCTTTCT 2300  
 2301 TTTTGTACTTGTAGTGTACATAGTACATGTAGGTAGTAGAAGTTGGATGTAGTCTGAGTGTGCTGAGAGAGTGAATCGGTACATTAAATTTGTTGAAG 2400  
 2401 TTTTAGCATACAGAGGATAGTGAATGAATAGTAGCATATAATGGATCTGTGATTAGTCAAGAAATGTCGTAC 2474

The deduced amino acid sequence codes for 658 amino acids to yield a protein of estimated 72.4 KDa. The clone was found to have strong homology to the PKC family of kinases; but like other PKC clones from lower vertebrates and invertebrates, this PKC does not fall into any one subtype of mammalian PKC isozymes. In the  $\text{Ca}^{2+}$ -dependent mammalian PKCs the V3 region of the isozymes contains the isozyme specific sequence (Makowske *et al.*, 1988). This region is used to make isozyme specific antibodies, which can be used against a particular isozyme in other species. The V3 regions of the  $\text{Ca}^{2+}$ -dependent mammalian PKCs are shown in Figure 11 with the V3 sequence of the sea urchin PKC and the *Aplysia* PKC1 sequence. This shows there is no homology between the V3 region of the mammalian PKCs and the suPKC1 V3 region, but suPKC1 V3 region shares some residues in common with the *Aplysia* V3. The amino acid sequence contains all the indicators of a PKC family member. There is a pseudosubstrate domain at amino acids 13-25 nearly identical to those of the mammalian enzymes. The C1 cysteine repeats follow the pseudosubstrate domain, there are two repeats, like the majority of the PKC isozymes. Following the C1 is a C2 domain suggesting that the enzyme would have a  $\text{Ca}^{2+}$ -dependent kinase activity. The C3 and C4 regions are also similar to the mammalian PKCs having the ATP binding site and regions of homology (Hunter, 1991).

#### Homology comparisons of sea urchin PKC

The proposed protein sequence of the sea urchin clone is shown in an alignment with the three rat  $\text{Ca}^{2+}$ -dependent isozymes in Figure 12. The highest regions of homology between the sequences are within the conserved domains of the protein. Individual comparisons between the sea urchin clone and the rat sequences yields approximately 70% identity at the amino acid level for each of the mammalian isozymes. All of the elements that

```

suPKC1  AIAE.TESID ELTSNIKKLP MPTQEH.VKP QNSNSMSGMG VVRAS
APLPKC1 VTDDITESIQ EIKSKMHRSS ISSEKRYPEP DKVQNMSKQD IVRAS

rat-a   AAGNKVISPS EDRRQ

rat-b   GPKTPEEKTA ANTISSEKFD

rat-g   NYPLELYERV RTTG

```

Figure 11. V3 regions of the  $\text{Ca}^{2+}$ -dependent mammalian PKCs (Housey *et al.*, 1987; Ono *et al.*, 1988) the *Aplysia* PKC1 (Kreuger *et al.*, 1991) and the sea urchin PKC. The V3 peptide sequences that are conserved in the different  $\text{Ca}^{2+}$ -dependent isozyme families are listed, with the V3 region of the *Aplysia* PKC1 sequence and the su PKC1 clone. The sea urchin V3 does not contain a sequence resembling any of the mammalian V3s, consistent with sequence information from other lower vertebrates and invertebrates. It does, however share some residues in common with the *Aplysia* V3 mainly at the termini of the region.

	1				50					100	
RAT-a	MADVPVANDS	TASQDVANRF	ARRGALRQKN	VHEVKDHKFI	ARFFKQPTFC	SHCTDFIWGP	GKQGFQCCVC	CFVVHRCHE	FVTFSCPGAD	KGPDITDDPRS	
RAT-bi	MADPAAGPPP	SEGEESTVRF	ARRGALRQKN	VHEVKNHQFT	ARFFKQPTFC	SHCTDFIWGP	GKQGFQCCVC	CFVVHRCHE	FVTFSCPGAD	KGPDITDDPRS	
RAT-g	MAGLPGGGD	SEGGPRFL.P	ARRGALRQKN	VHEVKSHKFT	ARFFKQPTFC	SHCTDFIWGI	GKQGLQCCVC	SFVVHRCHE	FVTFPCPGAG	KGPDITDDPRN	
SU	.....M	SNSTLEMKGF	ARRGALRQKN	VHEIKNHKFI	PRFFKQPTFC	SHCKDFIWGP	GKQGFQCCVC	SFVVHRCHE	FVTFPCRGLD	RGVSDSDPRN	
consen	na.....f	a.....f	arkgalrqkn	vhevkhkfi	arffkqptfc	shctdfiwgf	gkqgfqccvc	.fvvhrcche	fvtf.cpgad	kyp...ddpr.	
			I-		C1						
	101				150					200	
RAT-a	KHGFKIHYS	SPTFCDHGGS	LLYGLIHQGN	KCDTCMNVH	KQCQVNVPSL	CGDHTEKRG	RIYLKAEV.T	DEKLHVTVRD	AKNLIPHPDN	GLSDPYVKLK	
RAT-bi	KHGFKIHYS	SPTFCDHGGS	LLYGLIHQGN	KCDTCMNVH	KRCVMNVPSL	CGDHTEKRG	RIYIQAH.I.D	REVLIVVVRD	AKNLIPHPDN	GLSDPYVKLK	
RAT-g	KHGFRLHSYS	SPTFCDHGGS	LLYGLIHQGN	KSCCENNVH	KRCVRSVPSL	CGDHTEKRG	RLQLEIRAPT	SDEIHTVGE	AKNLIPHPDN	GLSDPYVKLK	
SU	KHGFRLHSYN	SPTFCDHGGS	LLYGLIHQGN	KGACACMNVH	KRCQKSVPSL	CGADHTEKRG	RIYKVAEIVG	.NKLQVTVAE	AKNLIPHPDN	GLSDPYVKLK	
consen	khkfk.h.y.	sptfcdhgs	llygl.hqgn	kc..c.nmvh	k.cv..vpsl	cg.dhterrg	xi.....	....vtv..	aknlipdpn	glsdpyvklk	
					C1						
	201				250					300	
RAT-a	LIPDPKESK	QRTKTRISTL	NPQNESFTF	KLKPSDKDRR	LSVEINDWDR	TTRNDFMGSL	SFGVSELHGM	PASGWYKLLN	QEEGEYVNPV	IPGDEBGNV	
RAT-bi	LIPDPKESK	QRTKTRISTL	NPQNESFTF	KLKPSDKDRR	LSVEINDWDR	TTRNDFMGSL	SFGVSELHGM	PASGWYKLLN	QEEGEYVNPV	IPGDEBGNV	
RAT-g	LIPDPKESK	QRTKTRISTL	NPQNESFTF	KLKPSDKDRR	LSVEINDWDR	TTRNDFMGSL	SFGVSELHGM	PASGWYKLLN	QEEGEYVNPV	IPGDEBGNV	
SU	LIPDPKESK	QRTKTRISTL	NPQNESFTF	KLKPSDKDRR	LSVEINDWDR	TTRNDFMGSL	SFGVSELHGM	PASGWYKLLN	QEEGEYVNPV	IPGDEBGNV	
consen	lipdpk.e.k	qrtktrik..l	np.wne.f.f	.lk..d..rr	lsve..wdwr	t.trndfng..l	sfg.sel..ka	.vdgwykll.	qeegeyynvp	.....	
					C1						
	301				350					400	
RAT-a	ELRQKF....	.....ERAK	LGP...AGNK	VLSPEDRKQ	P..SNNLDRV	KLTDENFLMV	LKGSFSGKVM	LADRKGTDEL	YAIKILKKDV	IVQDDOJECT	
RAT-bi	ELRQKF....	.....ERAK	LGP...AGNK	VLSPEDRKQ	P..SNNLDRV	KLTDENFLMV	LKGSFSGKVM	LADRKGTDEL	YAIKILKKDV	IVQDDOJECT	
RAT-g	ELRQKF....	.....ERAK	LGP...AGNK	VLSPEDRKQ	P..SNNLDRV	KLTDENFLMV	LKGSFSGKVM	LADRKGTDEL	YAIKILKKDV	IVQDDOJECT	
SU	ELRQKF....	.....ERAK	LGP...AGNK	VLSPEDRKQ	P..SNNLDRV	KLTDENFLMV	LKGSFSGKVM	LADRKGTDEL	YAIKILKKDV	IVQDDOJECT	
consen	.l.qkf....	.....e...	.g.....	.....	.....	...dnlflmv	lgkgsfsgkvm	laerkgtdel	yaikilkkdv	.iqddvect	
						I-					
	401				450					500	
RAT-a	MVEKRVLALL	DK	PPF	LTQLHSCFQT	MDRLYFVMEY	VNGGDLMYHI	QVVGKFKEPH	AVFYAAEISI	GLFFLHKGRI	IYRDLKLDNV	MLDSEGHKI
RAT-bi	MVEKRVLALL	DK	PPF	LTQLHSCFQT	MDRLYFVMEY	VNGGDLMYHI	QVVGKFKEPH	AVFYAAEISI	GLFFLHKGRI	IYRDLKLDNV	MLDSEGHKI
RAT-g	MVEKRVLALL	DK	PPF	LTQLHSCFQT	MDRLYFVMEY	VNGGDLMYHI	QVVGKFKEPH	AVFYAAEISI	GLFFLHKGRI	IYRDLKLDNV	MLDSEGHKI
SU	MVEKRVLALL	DK	PPF	LTQLHSCFQT	MDRLYFVMEY	VNGGDLMYHI	QVVGKFKEPH	AVFYAAEISI	GLFFLHKGRI	IYRDLKLDNV	MLDSEGHKI
consen	mvekrvial.	.....p.f	lqlhscfqt	.drlyfvme	vnggdmyhi	qvgvkfkph	avfyaaeia	glfflh..gi	iyrdklldnv	ml.d.eghiki	
	501				550					600	
RAT-a	ADFGMKCHH	MDGVTRTFEC	GTFDYIAPEI	IAYQPYGKSV	DWAFGVLLY	EMLAGQPFED	GEDEDELFSQ	IMEHNVSYPK	SLSKAEVASIC	KGLMTRHPAK	
RAT-bi	ADFGMKCHH	MDGVTRTFEC	GTFDYIAPEI	IAYQPYGKSV	DWAFGVLLY	EMLAGQPFED	GEDEDELFSQ	IMEHNVSYPK	SLSKAEVASIC	KGLMTRHPAK	
RAT-g	ADFGMKCHH	MDGVTRTFEC	GTFDYIAPEI	IAYQPYGKSV	DWAFGVLLY	EMLAGQPFED	GEDEDELFSQ	IMEHNVSYPK	SLSKAEVASIC	KGLMTRHPAK	
SU	ADFGMKCHH	MDGVTRTFEC	GTFDYIAPEI	IAYQPYGKSV	DWAFGVLLY	EMLAGQPFED	GEDEDELFSQ	IMEHNVSYPK	SLSKAEVASIC	KGLMTRHPAK	
consen	adfgmcke..	.g.trtrf	gtpdyiap	iaypqygs	dwafgvll	emlagqpf	gededelq	imeh.v.ypk	s.s.eav.ic	kg..tkhpgk	
	601				650					700	
RAT-a	RLGCGPGEER	DIREHAFPR	IDWEKLENRE	IQPPFKPKVC	GK.GAENFDK	FTTRGQPVLT	PPDQLVIANI	DQSEDFGFSY	VNPQFVHPIL	QSAV.....	
RAT-bi	RLGCGPGEER	DIREHAFPR	IDWEKLENRE	IQPPFKPKVC	GK.GAENFDK	FTTRGQPVLT	PPDQLVIANI	DQSEDFGFSY	VNPQFVHPIL	QSAV.....	
RAT-g	RLGCGPGEER	DIREHAFPR	IDWEKLENRE	IQPPFKPKVC	GK.GAENFDK	FTTRGQPVLT	PPDQLVIANI	DQSEDFGFSY	VNPQFVHPIL	QSAV.....	
SU	RLGCGPGEER	DIREHAFPR	IDWEKLENRE	IQPPFKPKVC	GK.GAENFDK	FTTRGQPVLT	PPDQLVIANI	DQSEDFGFSY	VNPQFVHPIL	QSAV.....	
consen	rlg.gp.ge.	direh.fpr.	idwekle..e	iqppf.p..	.....enfdk	.ft.....lt	p.d.i...ni	dq..f.gf.y	vnp.fv....	.....	
	701										
RAT-a	..										
RAT-bi	..										
RAT-g	..										
SU	..										
consen	..										

Figure 12. Homology alignment of sea urchin PKC with mammalian isoforms

The sequences used in this comparison are rat PKC- $\alpha$  (RAT-a), rat PKC- $\beta$ I (RAT-b) and Rat PKC- $\gamma$  (RAT-g) (Housey *et al.*, 1987; Ono *et al.*, 1988) and sea urchin PKC1 (su). Gaps were introduced in the sequences to allow for optimal alignment. A consensus line (consen) is at the bottom of the lineup. The conserved regions of PKC are indicated below the sequences as C1-C4.

define PKC are very highly conserved between the different isozymes. The highest identity is observed when comparing sea urchin PKC with rat PKC- $\alpha$  at 71% identity and the lowest with rat PKC- $\gamma$  with 68%. Reasons for this conservation pattern are addressed in the discussion.

In Figure 13 the sea urchin PKC is compared to other lower vertebrate and invertebrate PKC sequences. All of the sequences are the  $\text{Ca}^{2+}$ -dependent members cloned from these species. Comparison between the sea urchin PKC and the protein sequence for the *Drosophila* eye specific PKC, shows that there is 53% identity. There is less conservation in the N-terminal half of the protein, however, the pseudosubstrate domain and the conserved cysteines are apparent, and the homology increases at the C3. Both XPKCI and XPKCII have about 70-71% identity to the sea urchin PKC, with conservation low only in the V3 region. The *Aplysia* PKC is 67% identical to sea urchin PKC, and unlike other species compared, there is more identity in the V3 region, and the pseudosubstrate domains are identical. It is evident from the consensus line in Figure 13 that the homology between the lower vertebrates and the invertebrates sequences tends to lie within the conserved regions of PKC, and is low at the termini and at the V3 region. This observation is consistent with the comparisons with the mammalian sequences for PKC which also vary at the V3 region and at the N-terminus and C-terminus. When examining the individual comparisons between sea urchin PKC and the other previously mentioned PKCs it was noted that the sequences tended to have more identity in the catalytic or C-terminal half of the protein. The identities of the protein comparisons are shown in Figure 14, the first column containing the identity value of the entire protein comparison and the second column representing the comparison from the start of the V3 to the end of the protein sequence. This difference may be due to lower conservation of the amino acids that lie between the conserved cysteines in the C1 domain and lower conservation in the C2 domain or higher identity in the catalytic portion may reflect similar substrate preferences in the isozymes.

	1				50				100	
XPKCII	.....	.....	.....	.....	SDSEACEPGD	DTTTRFARKG	ALRQGNVHEV	KERKFTARFF	KQPTFCSHCT	DFWNGFGKQG
XPKCI	.....	.....	.....	.....	MADVQFQND	STSSSSSSSQ	EVAQRFAKRG	KNHKFIARFF	KQPTFCSHCT	DFWNGFGKQG
SU	.....	.....	.....	.....	.....	.....	LEHKGFPARRG	ALRQGNVHEI	KNHKFIARFF	KQPTFCSHCT
APLPKCI	.....	.....	.....	.....	.....	.....	ME.KRVARRG	ALRQGNVHEV	KNHKFIARFF	KQPTFCSHCK
dPKC53E	.....	MAAAAVATP	GATVLPSPVP	SAAFGAKAPA	AGAGKGPGLN	LEITGEANIV	NYMKNLRKRG	AKHKGKLEMV	NGHHRGVNFF	KNPTYCGHCK
consen	.....	.....	.....	.....	.....	.....	.....ar.g	alrqgnvhev	k.hkf.arff	kqptfcshck
										dfwiwfgqg
	101									
XPKCII	FQCCVCCFVV	HKRCHEVTF	SCPGADNGPA	SDDPRSGHKF	RIHTYSSPTF	CDHCGSLLYG	LIHQGHKCEI	CMNVHKRCV	MNVPSLOGTD	HTERRGRTHI
XPKCI	FQCCVCCFVV	HKRCHEVTF	SCPGADNGPD	TDDPRSGHKF	KIHTYSSPTF	CDHCGSLLYG	LIHQGHKCEI	CMNVHKRCV	INVPSLKQGD	HTRKGRGRTV
SU	FQCCVCCFVV	HKRCHEVTF	SCPGADNGPD	SDDPRSGHKF	KIHTYSSPTF	CDHCGSLLYG	LIHQGHKCEI	CMNVHKRCV	KSVNPLQOGD	HTERRGRGTV
APLPKCI	FQCCVCCFVV	HKRCHEVTF	SCPGADNGPD	SDDPRSGHKF	KIHTYSSPTF	CDHCGSLLYG	LIHQGHKCEI	CMNVHKRCV	KSVNPLQOGD	HTERRGRGTV
dPKC53E	FQCCVCCFVV	HKRCHEVTF	SCPGADNGPD	SDDPRSGHKF	KIHTYSSPTF	CDHCGSLLYG	LIHQGHKCEI	CMNVHKRCV	KSVNPLQOGD	HTERRGRGTV
consen	fqcqvccfvv	hkrchefvtf	scpgadngpd	sddprsghkf	rihtyssptf	cdhcgallyg	lihgkce	cmnvhkrc	..vp.lcg.d	hte.xgri..
										-I
	201									
XPKCII	KAELKEEVMT	VTVDARNLV	PMDFNGLSDP	YVKLKLIPDP	KSETKQRTKT	IKCSLNPWN	ETFKFQKES	DKDRRLSVEI	WDWELTSRND	FMGSLSFSGIS
XPKCI	KAELKEEVMT	VTVDARNLV	PMDFNGLSDP	YVKLKLIPDP	KSETKQRTKT	IKCSLNPWN	ETFKFQKES	DKDRRLSVEI	WDWELTSRND	FMGSLSFSGIS
SU	KAELKEEVMT	VTVDARNLV	PMDFNGLSDP	YVKLKLIPDP	KSETKQRTKT	IKCSLNPWN	ETFKFQKES	DKDRRLSVEI	WDWELTSRND	FMGSLSFSGIS
APLPKCI	KAELKEEVMT	VTVDARNLV	PMDFNGLSDP	YVKLKLIPDP	KSETKQRTKT	IKCSLNPWN	ETFKFQKES	DKDRRLSVEI	WDWELTSRND	FMGSLSFSGIS
dPKC53E	KAELKEEVMT	VTVDARNLV	PMDFNGLSDP	YVKLKLIPDP	KSETKQRTKT	IKCSLNPWN	ETFKFQKES	DKDRRLSVEI	WDWELTSRND	FMGSLSFSGIS
consen	.....kg	.....v	.....akml	.....pmdnglscdp	.....yvkklipdp	.....k	.....ktkt	.....l	.....l	fmgsfsfgis
	301									
XPKCII	ELKAGVGDW	FKLLSQEAGE	YFNVPPPEG	EEGNEELRQK	FERAKIPG	G	NKAAGREGGK	PSVQCGQQGN	RDMKVSDFN	FLKVLGKGSF
XPKCI	ELKAGVGDW	FKLLSQEAGE	YFNVPPPEG	EEGNEELRQK	FERAKIPG	G	NKAAGREGGK	PSVQCGQQGN	RDMKVSDFN	FLKVLGKGSF
SU	ELKAGVGDW	FKLLSQEAGE	YFNVPPPEG	EEGNEELRQK	FERAKIPG	G	NKAAGREGGK	PSVQCGQQGN	RDMKVSDFN	FLKVLGKGSF
APLPKCI	ELKAGVGDW	FKLLSQEAGE	YFNVPPPEG	EEGNEELRQK	FERAKIPG	G	NKAAGREGGK	PSVQCGQQGN	RDMKVSDFN	FLKVLGKGSF
dPKC53E	ELKAGVGDW	FKLLSQEAGE	YFNVPPPEG	EEGNEELRQK	FERAKIPG	G	NKAAGREGGK	PSVQCGQQGN	RDMKVSDFN	FLKVLGKGSF
consen	el.k	.....gw	.....k.l	.....qeage	.....ynvp	.....e	.....el	.....	.....	fl.vlghgsaf
										gkv.laerky
	401									
XPKCII	TDELYAIIKL	KKDVIQDD	VECTHIEKRV	LALSGKPPFL	TLHSCFQTM	DRLYPVMEFV	NGGDLHYOIQ	QVGRFKPEHA	VFYAAEIRVG	LLFLHSGQVI
XPKCI	TDELYAIIKL	KKDVIQDD	VECTHIEKRV	LALSGKPPFL	TLHSCFQTM	DRLYPVMEFV	NGGDLHYOIQ	QVGRFKPEHA	VFYAAEIRVG	LLFLHSGQVI
SU	TDELYAIIKL	KKDVIQDD	VECTHIEKRV	LALSGKPPFL	TLHSCFQTM	DRLYPVMEFV	NGGDLHYOIQ	QVGRFKPEHA	VFYAAEIRVG	LLFLHSGQVI
APLPKCI	TDELYAIIKL	KKDVIQDD	VECTHIEKRV	LALSGKPPFL	TLHSCFQTM	DRLYPVMEFV	NGGDLHYOIQ	QVGRFKPEHA	VFYAAEIRVG	LLFLHSGQVI
dPKC53E	TDELYAIIKL	KKDVIQDD	VECTHIEKRV	LALSGKPPFL	TLHSCFQTM	DRLYPVMEFV	NGGDLHYOIQ	QVGRFKPEHA	VFYAAEIRVG	LLFLHSGQVI
consen	tdelyaikkil	kkdviiqdd	vectm.ekrv	lal..kppfl	tl..hscfqtm	drl.fvmev	nggdilmy.iq	qvg.fkep.a	.fyaaei.g	lflfh..gi.
	501									
XPKCII	YRDLKLENV	LDSEGHKIA	DFGCKENM	EGVITRTFCG	TPDYIARZII	RYQFYAKSVD	WAAFGILLYE	MLAGVFFPDG	EDEDELFSOI	MEHNVAYPKS
XPKCI	YRDLKLENV	LDSEGHKIA	DFGCKENM	EGVITRTFCG	TPDYIARZII	RYQFYAKSVD	WAAFGILLYE	MLAGVFFPDG	EDEDELFSOI	MEHNVAYPKS
SU	YRDLKLENV	LDSEGHKIA	DFGCKENM	EGVITRTFCG	TPDYIARZII	RYQFYAKSVD	WAAFGILLYE	MLAGVFFPDG	EDEDELFSOI	MEHNVAYPKS
APLPKCI	YRDLKLENV	LDSEGHKIA	DFGCKENM	EGVITRTFCG	TPDYIARZII	RYQFYAKSVD	WAAFGILLYE	MLAGVFFPDG	EDEDELFSOI	MEHNVAYPKS
dPKC53E	YRDLKLENV	LDSEGHKIA	DFGCKENM	EGVITRTFCG	TPDYIARZII	RYQFYAKSVD	WAAFGILLYE	MLAGVFFPDG	EDEDELFSOI	MEHNVAYPKS
consen	yrdlklenv	ld.eghikia	dfgckene	.....egvitrftcg	tpdyiarzii	ryqfy...	wda.gvllye	mlagvffdg	edeel.fsi	mehnvaypk
	601									
XPKCII	MSKEAVALCK	GLMTKHGKR	LOCCPEGERD	IKDRAFFRRI	DWEKLENEI	QPPFYKPKA.C	GRNAENFDKF	FTRHPPVLTF	PDHEVIRNID	QSEFEGFSV
XPKCI	MSKEAVALCK	GLMTKHGKR	LOCCPEGERD	IKDRAFFRRI	DWEKLENEI	QPPFYKPKA.C	GRNAENFDKF	FTRHPPVLTF	PDHEVIRNID	QSEFEGFSV
SU	MSKEAVALCK	GLMTKHGKR	LOCCPEGERD	IKDRAFFRRI	DWEKLENEI	QPPFYKPKA.C	GRNAENFDKF	FTRHPPVLTF	PDHEVIRNID	QSEFEGFSV
APLPKCI	MSKEAVALCK	GLMTKHGKR	LOCCPEGERD	IKDRAFFRRI	DWEKLENEI	QPPFYKPKA.C	GRNAENFDKF	FTRHPPVLTF	PDHEVIRNID	QSEFEGFSV
dPKC53E	MSKEAVALCK	GLMTKHGKR	LOCCPEGERD	IKDRAFFRRI	DWEKLENEI	QPPFYKPKA.C	GRNAENFDKF	FTRHPPVLTF	PDHEVIRNID	QSEFEGFSV
consen	mskeavalc	g.ltk.p.kr	lgcg..ge.d	i..h.frr.i	dw.kle.rei	qpp.kp....	..kdaenfd.k	ftke....ltp	d.l.i.i.mid	q.ef.gfs.v
	701									
XPKCII	NSDPSKEEEK	KD..								
XPKCI	NSDPSKEEEK	KD..								
SU	NSDPSKEEEK	KD..								
APLPKCI	NSDPSKEEEK	KD..								
dPKC53E	NSDPSKEEEK	KD..								
consen	n..f	.....								

Figure 13. Sequence alignment of sea urchin clone with lower vertebrates and invertebrates. The sequences used in this alignment are, *Xenopus* PKC-I and -II (XPKCII and XPKCI), *Aplysia* PKCI (APLPKCI), and *Drosophila* (dPKC53E, eye specific) (Chen *et al.*, 1989; Kreuger *et al.*, 1991; Schaeffer *et al.*, 1989) and sea urchin (su). Gaps are introduced to permit alignment of the sequences. A consensus line (consen) is located on the bottom line. The conserved regions of PKC are indicated as C1-C4 below the sequence alignment.

	Protein	C3-V5
Rat PKC-a	71.2%	76.1%
Rat PKC-b	70.0%	75.8%
Rat PKC-g	67.5%	72.2%
<i>Xenopus</i> PKCII	70.5%	74.9%
<i>Xenopus</i> PKCI	69.5%	73.8%
<i>Aplysia</i> PKCI	68.9%	70.6%
<i>Drosophila</i> PKC53e	51.8%	55.5%

Figure 14. Identities of the sea urchin PKC with the mammalian, lower vertebrate and invertebrate sequences. The first column is the identity values for the whole protein sequence and the second column is the values for the C3 through the V5 regions or the catalytic half of the proteins. The catalytic half has slightly higher identity values indicating higher conservation in this half of the protein.

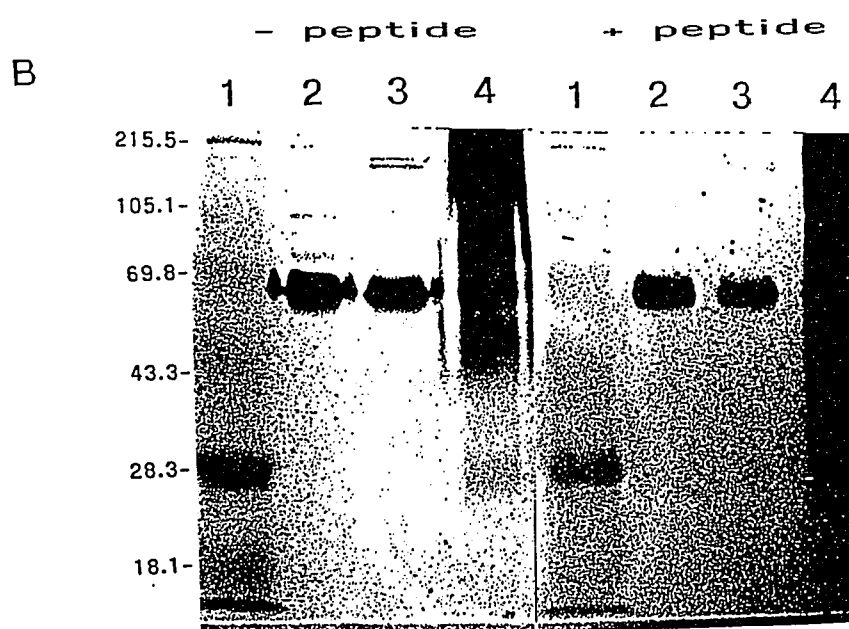
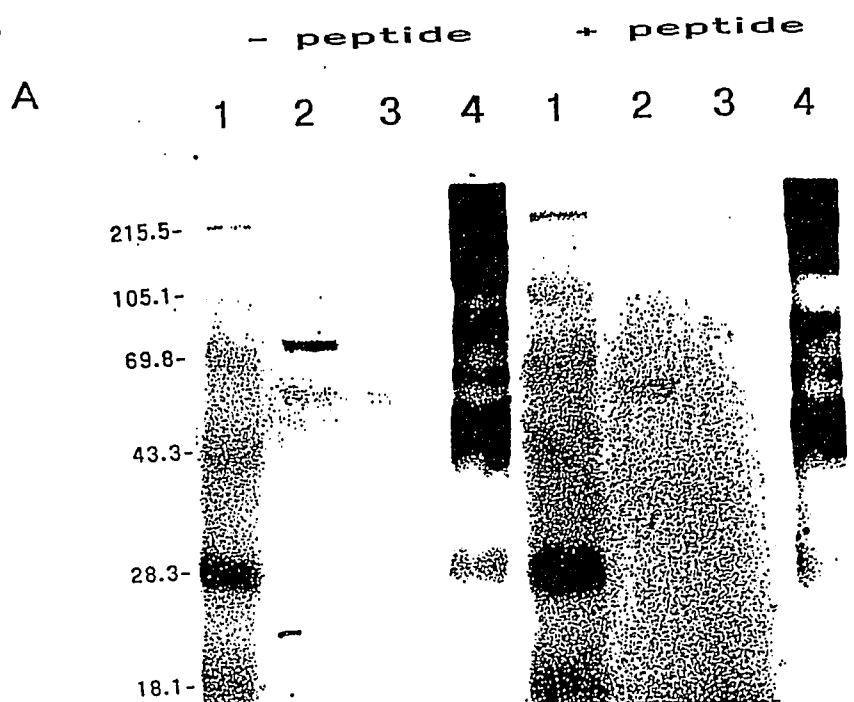
### Antibody recognition of PKC in DE52 fractions

To examine whether the PKC cloned from the sea urchin is related to the PKC activity previously identified in partially purified egg fractions, column separation of egg homogenates was performed. Protein kinase C activity was prepared as previously described (Shen & Rieke, 1989). Sea urchin egg homogenates were loaded onto a DE52 anion exchange column and eluted with a salt gradient. Fractions collected were tested for PKC activity using histone H1 as the phosphate acceptor. Table 1 gives the salt concentration, the protein content and the relative PKC kinase activity of the fractions used for Western blotting. The activity represents the capability of the fraction to phosphorylate histone in the presence of EGTA or phosphatidylserine,  $\text{Ca}^{2+}$ , and diolien. The fractions that were chosen either demonstrated relatively high kinase activity (fraction 6) or had low activity (fraction 9). The values for the dialysate used for column loading are also included. The selected fractions were challenged with polyclonal antisera raised against peptide antigens synthesized to the proposed translation product of the PKC clone. To determine specificity, duplicate blots were probed with sera that had been preincubated with peptide antigen. The Western blot probed with the N-terminal peptide antigen is shown in Figure 15. Western analysis shows that fraction 6, which had PKC activity, has an immunoreactive band at ~71 KDa (lane 2) that is eliminated when the sera is incubated with peptide antigen. Fraction 9 does not contain this antigen at 71 KDa (lane 3), supporting that the kinase contributes to the activity predominantly observed in fraction 6. There is also a band at approximately 58 KDa, but the band appears in both fractions 6 and 9 (lanes 2 and 3), and is present when the sera is preincubated with the peptide antigen (+-peptide). Panel B shows blots probed with the V3 antisera. The V3 sera also has an immunoreactive band at 71 KDa in the high kinase fraction (lane 2) and like the N-terminus antisera the band does not appear in fraction 9 (lane 3) and is not present in fractions 6 or 9 of the +-peptide blot. The V3 antisera also recognizes a set of diffuse bands in the 65-67 KDa

Table 1. Fractions used for testing antisera. Fractions collected from a DE52 column were tested for histone H1 phosphorylation in the absence (EGTA) and presence of (PS/Ca<sup>2+</sup>/DAG) activators of PKC as well as for protein content and conductivity. The relative protein content, salt concentration and kinase activity are given for the two fractions that were chosen to probe with the polyclonal anti-peptide antibodies. The dialysate is also included which is total cytosolic proteins before column separation.

<u>fraction</u>	<u>NaCl</u>	<u>Protein</u>	<u>EGTA</u>	<u>PS/Ca<sup>2+</sup>/DAG</u>
Dialysate	6 mM	5.8 mg/ml	1836 cpm	3256 cpm
6	38	0.68	-17	4735
9	88	0.58	267	926

Figure 14. Western analysis of fractions from a DE52 separation of sea urchin egg cytosolic proteins. Two fractions were separated by PAGE and electrophoretically transferred, and probed with polyclonal antisera. Duplicate blots were probed with antisera preincubated with peptide antigen (+-peptide). Two blot sets are shown one probed with the N-terminal peptide antisera (A) and one with V3 antisera (B). For each blot the lanes are as follows; lane 1, prestained molecular weight markers; lane 2, fraction 6 with high kinase activity; lane 3, fraction 9 with low kinase activity. The blots include thyroglobulin (lane 4) as a control for sera antibody binding.

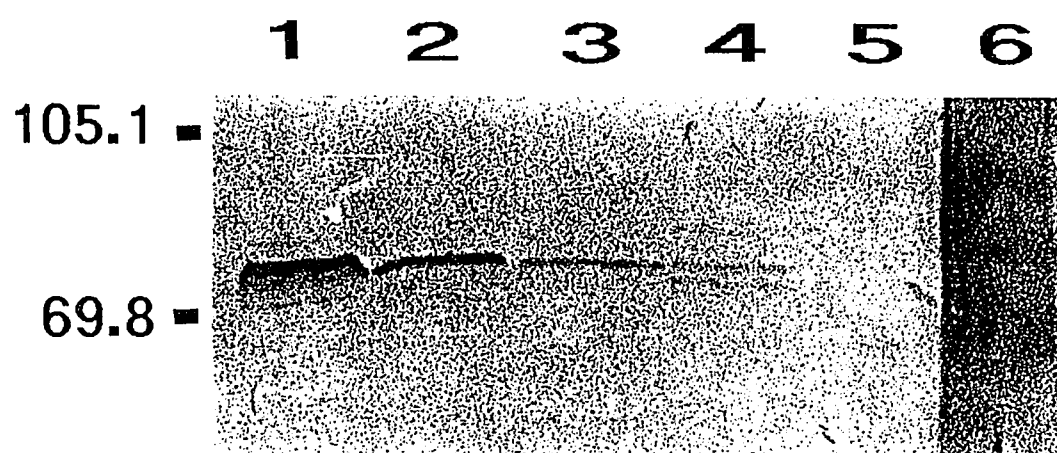


range, these bands are apparent in both fractions and in the blot probed with sera preincubated with peptide. The Western for the C-terminal peptide was too light to be photographed, so the data is not shown. The results of the Westerns support that there is PKC present in sea urchin eggs. It also is shown that the PKC cloned from the sea urchin is present in a fraction that contains PKC activity. The Western analysis was repeated with fractions that contained higher protein content in order to determine if the fraction with the lower kinase activity has reactivity with the N terminal antisera. The kinase activity, protein content and salt concentration of each fraction used for this Western are given in Table 2. The protein content of the three fractions, 6, 8 and 10 is similar. The  $\text{Ca}^{2+}$ , PS, and DAG dependent kinase activity is highest in fraction 6 relative to values of kinase activity in the presence of EGTA. The  $\text{Ca}^{2+}$ -dependent kinase activity in fraction 8 is approximately 30% that of fraction 6, and fraction 10 has no  $\text{Ca}^{2+}$ -dependent kinase activity (Table 2). Probing these fractions also shows a reactivity in fractions having kinase activity. The antisera recognizes a protein of approximately 71 kDa as shown in Figure 16 lane 1 in which fraction 6 is concentrated ten fold. The strongest reactivity is in fraction 6 when used for analysis at the concentration collected from the column (lane 2), and when fraction 6 is diluted to the activity of that found in fraction 8 the reactivities of the sera are similar (lanes 3 & 4). Fraction 10 which had no kinase activity also does not have reactivity at 71 kDa (lane 5). The reactivity if the 71 kDa antigen is specific as it can be eliminated when competed with excess peptide antigen (data not shown). As is common with many polyclonal antibodies, there are immuno crossreactive species, therefore, in addition to the 71 kDa band, there are additional bands in the ten fold concentration of fraction 6 (lane 1), but they persist when sera is preincubated with peptide antigen. There is a weak band at 80 kDa in fractions 8 and 10 (lanes 4 & 5) which is absent when sera is preincubated with peptide, the identity of the reactivity is unknown. There is also a lower molecular weight band in fraction 8 that is present when sera is preincubated with peptide. However, the fact that the intensity of the reactivity at

Table 2. Fractions containing various levels of kinase activity for Western analysis. Sea urchin egg homogenate was fractionated and the fractions collected from a DE52 column were examined for histone H1 phosphorylation, as well as for protein content and conductivity. Kinase activity was either in the presence of EGTA (column 4) or in the presence of PKC activators  $\text{Ca}^{2+}$ , PS and DAG (column 5). The relative protein content, salt concentration and kinase activity are given for the three fractions that were chosen to probe with the polyclonal anti-peptide antibodies. The dialysate is also included which is total cytosolic proteins before column separation

<u>fraction</u>	<u>NaCl</u>	<u>Protein</u>	<u>EGTA</u>	<u>PS/<math>\text{Ca}^{2+}</math>/DAG</u>
Dialysate	6 mM	14.2 mg/ml	6193 cpm	4240 cpm
6	44	1.2	1314	5776
8	110	1.1	805	1849
10	128	1.2	1330	1514

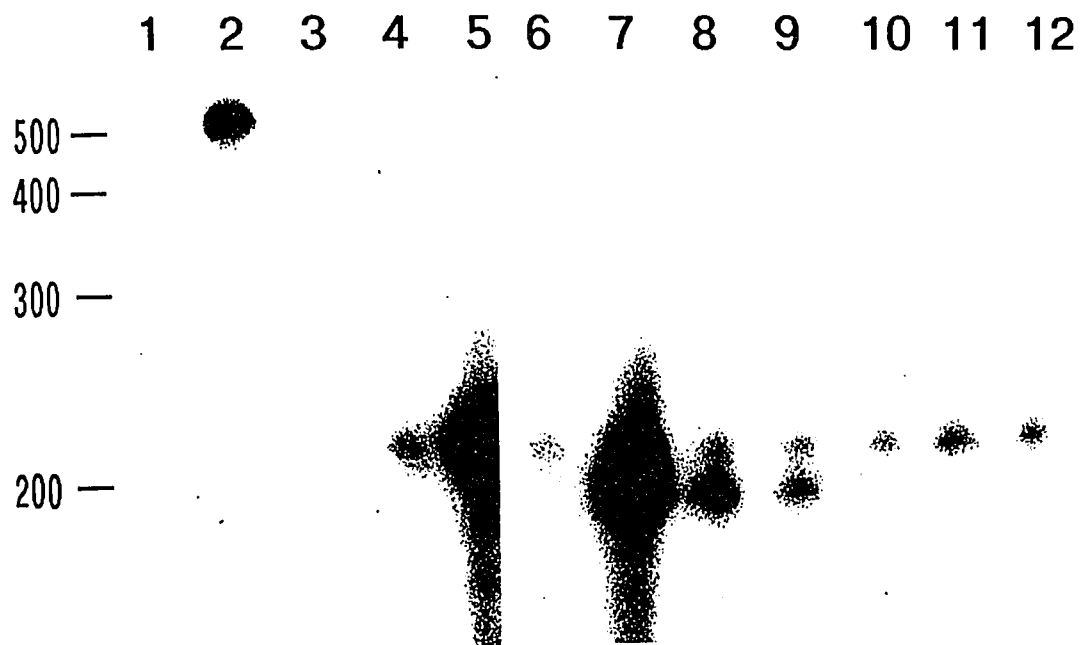
Figure 15. Western analysis of fractions that lack or contain PKC activity. Fractions from a DE52 column for partial purification of PKC were collected, tested for kinase activity and subjected to polyacrylamide gel electrophoresis and protein transfer. The Western was probed with the N-terminal antibody, representing the first 13 amino acids of the deduced protein sequence. The fractions containing PKC activity are 6 and 8. Fraction 6 was concentrated ten fold (lane 1), used as collected (lane 2), or diluted 70 % or to the PKC activity of that in fraction 8 (lane 3). Fractions 8 (lane 4) and 10 (lane 5) are used as collected, without dilution or concentration. The Western shows specific reactivity to an antigen at 71 kDa only in fractions that contain PKC activity.



71 kDa in fractions 6 and 8 corresponds with the kinase activity in those fractions supports that the antisera is recognizing the suPKC1 protein. This does not indicate that it is the only PKC in this fraction or that it is active, since in mammalian systems, the three  $\text{Ca}^{2+}$ -dependent PKCs  $\alpha$ ,  $\beta$  and  $\gamma$  elute off the DE52 anion exchange column at the same point and further manipulations must be performed in order to separate them (Marais and Parker 1989). The antisera was tested against rat brain PKC- $\alpha$ , - $\beta$  and - $\gamma$  and had no reactivities to these PKCs at kinase levels similar to kinase levels in the fractions of sea urchin proteins (Rakow and Shen, data not shown).

In order to determine if the PKC cloned from the ovary tissue library is expressed in other stages of development, a Ribonuclease Protection Assay (RPA) was performed. RNA from ovary and eggs was used, in addition to five post-fertilization stages. Embryos were collected at early and late 16 cell stage and early and late gastrulation and RNA was prepared for the RPA. A 0.4kb *Ava*II subcloned fragment of the suPKC1 cDNA was used to transcribe an antisense probe. In addition to egg, the developmental stages used for the assay were, 8-16 cells, 16-32 cells, very early gastrula, late gastrula and hatched pluteus. The full length probe is approximately 560 bp with 160 bp consisting of vector sequence. The expected size of the protected fragment is approximately 400 bp, however, RPA experiments consistently produced a protected fragment of approximately 220 bp. This 220 bp fragment is protected in control experiments when the opposite strand from *in vitro* transcription is included as the target RNA with the antisense probe in the RPA. Furthermore, the intensity of the 220 bp signal increases corresponding with increasing amounts of opposite strand added to the reaction (Fig. 17 lanes 2-4). At least two explanations can be given for the shorter than expected protected fragment these are intramolecular stemloop formation or RNase nicking within the protected region. Examination of the probe sequence in the fold program in UWGCG (GCG 1991 program

Figure 15. Western analysis of fractions that lack or contain PKC activity. Fractions from a DE52 column for partial purification of PKC were collected, tested for kinase activity and subjected to polyacrylamide gel electrophoresis and protein transfer. The Western was probed with the N-terminal antibody, representing the first 13 amino acids of the deduced protein sequence. The fractions containing PKC activity are 6 and 8. Fraction 6 was concentrated ten fold (lane 1), used as collected (lane 2), or diluted 70 % or to the PKC activity of that in fraction 8 (lane 3). Fractions 8 (lane 4) and 10 (lane 5) are used as collected, without dilution or concentration. The Western shows specific reactivity to an antigen at 71 kDa only in fractions that contain PKC activity.



manual for the GCG package, Version 7, April 1991, 575 Science Drive, Madison, WI 53711) indicated possible fold structures (positions 945-980 base pairing with positions 1237-1265, and positions 1016-1056 base pairing with 1092-1136 Fig. 1). Target RNA hybridizing to these structures would yield smaller than expected protected fragments (~250 bp and ~200 bp respectively); however, the presence of a fold structure in the RPA reaction has not been determined. Since basepairing at AU rich sites can be less stable it is possible that the RNase T1 used had nicking activity in an AU rich region of the protected sequence. There is an AU rich region (9 As or Us in succession) at basepairs 1098-1106. This nicking, however, would result in two fragments, one at ~170 basepairs and one ~230 basepairs. the 230 basepair size is near that observed, but there is no evidence of an equal intensity fragment at 170 basepairs. Despite the fact that the protected fragment is smaller than expected, control experiments with the opposite strand transcript hybridizing to the antisense probe resulting in a 220 basepair fragment demonstrate that this is the target. When total RNA is probed with the suPKC1 antisense probe, the intensity of the 220 bp protected band shows some variation in the different developmental stages. Densitometry readings indicate that the egg (lane 7) and 8-16 cell stage (lane 8) have the highest intensities with 8-16 cell being the maximum. Intensity decreases in the 16-32 (lane 9) cell and early gastrula (lane 10) and increases slightly in the late gastrula and pluteus stages (lanes 11 & 12). In addition to the 220 bp band there is a second band at 190 bp. This band does not appear in the sense strand control and its identity is unknown. It is possible, however, that this protected fragment is another PKC isozyme, as PKC is a multigene family (Nishizuka 1986) and other lower vertebrates and invertebrates have been found to have more than one isozyme (Rosenthal *et al.*, 1987; Chen *et al.*, 1989; Schaeffer *et al.*, 1989; Kreuger *et al.*, 1991). Comparison of the two fragments in each tissue sample shows the 190 bp band appears in the egg (lane 7) at a high intensity, roughly three fold higher than the 220 bp band. The signal decreases to two fold greater than the 220 bp band in the 8-16 cell stage (lane 8), and is roughly equal in the 16-32 cell stage (lane 9). In

early and late gastrula (lanes 10 & 11), the 190 bp fragment decreases again to just above background levels, then reappears in pluteus (lane 12), but at an intensity almost three fold lower than the 220 bp fragment. The presence of the suPKC1 message in these different stages of development suggests that it may be involved in the various processes that the cells of the developping sea urchin undergo, such as determination or cell migration.

## DISCUSSION

Protein kinase C is an intracellular serine-threonine kinase that plays roles in many cellular events. Cells stimulated by mitogens or other factors initiate a signal transduction cascade that often includes PKC activity. Fertilization is a cellular activating event that displays similarities to mitogenic stimulation. In order to dissect the molecular events of fertilization, identification of members of the various pathways is necessary. This dissertation discusses the identification of protein kinase C from sea urchin eggs. It has been previously determined that sea urchin eggs have a calcium dependent PKC activity, which may play a role during fertilization (Heinecke *et al.*, 1990; Shen & Rieke, 1989). This activity was not amenable to purification, hindering any possible manipulations of the kinase *in vitro* or *in vivo*. A molecular approach was taken to clone PKC from the sea urchin and information from this will allow for investigating the role of PKC during fertilization and early development.

The cloning of a PKC isozyme from the sea urchin yielded a C2 domain-containing PKC. In addition to the C2 domain the sea urchin PKC has all the conserved regions of the PKC family, including two cysteine repeats, the pseudosubstrate domain and the catalytic domain with an ATP binding site. The isozyme is predicted to encode for a 72.4 kDa protein, which is slightly smaller than the  $\text{Ca}^{2+}$ -dependent isozymes from mammalian systems (Kikkawa *et al.*, 1989). The size falls within the range of estimated molecular weights of the lower vertebrates and invertebrates utilized in the homology comparisons, which are all below 80 kDa. This isozyme has high homology to the *Aplysia*  $\text{Ca}^{2+}$ -dependent isozyme as well as *Xenopus* and rat isozymes.

Figure 13 shows a cartoon structure of C2 containing PKCs as discussed below. The pseudosubstrate domain as mentioned previously is the negative regulatory element of protein kinase C. The sequence of the pseudosubstrate domain resembles the recognition sequence for phosphorylation by the kinase with the serine or threonine replaced by alanine. The pseudosubstrate domain of the sea urchin PKC is identical to the pseudosubstrate domain of

the *Aplysia* PKCI protein. However the rat and *Xenopus* pseudosubstrate domains all have the second arginine replaced by lysine. Synthetic peptides of the pseudosubstrate domain have been reported to block PKC activity during fertilization events (Sheri & Buck, 1990).

The C1 domain of the sea urchin PKC clone contains two cysteine repeats, like all PKC isozymes, except  $\alpha$  (Ono *et al.*, 1988). The spacing of the cysteines is the prominent feature of this domain, as all isozymes compared have invariant spacing of the cysteine residues, with some homology between the cysteines. The invariant spacing most likely indicates secondary structure, considering related elements in "zinc finger" DNA binding proteins. The cysteine repeat structures are thought to complex with a metal ion such as zinc to form finger like structures that may allow for association with other molecules. (Mitchell & Tjian, 1989). The function of the C1 domain is to bind DAG or phorbol esters. It is thought that in this case the cysteine residues do not complex with a metal ion, however, the DAG or phorbol ester are hydrogen-bonded to the sulfhydryl group of the cysteine. This binding may cause the conformational shift necessary for kinase activity (Gschwendt *et al.*, 1991).

The remainder of the regulatory half of PKC consists of the C2 domain. Other proteins contain C2-like domains (Clark *et al.*, 1991; Maruyama & Brenner, 1991; Perin *et al.*, 1990) and one of these, p65 has been used to study the role of the C2 domain of PKC. In addition to binding  $\text{Ca}^{2+}$ , it is possible that the domain is involved in translocation of PKC, or maintaining the protein at the membrane (Mochly-Rosen *et al.*, 1991). The C2 domain consists of stretches of homology among the PKC isozymes. When comparing the C2 of PKC with the C2 from different proteins, such as p65, two regions of homology remain. The functions of these different regions are unknown, but experiments with the C2 domain of p65 point to the ability to bind proteins at the cytoskeleton in a  $\text{Ca}^{2+}$ -dependent manner (Mochly-Rosen *et al.*, 1992). The sea urchin PKC has the sequences common to all of the PKC isozymes, including the two stretches from C2 containing proteins. The presence of the C2 domain suggests that the sea urchin PKC has the capability to be translocated to the membrane and/or to bind

membrane associated proteins. The C2 also indicates that the sea urchin PKC has a  $\text{Ca}^{2+}$ -dependent kinase activity.

Dividing the regulatory and catalytic halves of PKC is the V3 or hinge region. The V3 contains sequences that distinguish the three  $\text{Ca}^{2+}$ -dependent isozymes from one another in the mammalian PKC family. These sequences have been used to make isozyme specific monoclonal and polyclonal antibodies (Makowske *et al.*, 1988). In lower vertebrates and invertebrates, the PKC isozymes do not have these sequences to assign them into any one isozyme type. XPKCI has within its V3 a sequence resembling part of the PKC- $\alpha$  specific sequence, however it is unknown whether XPKCI has any reactivity to the PKC- $\alpha$  antibodies. Like the PKCs from lower vertebrates and invertebrates, the sea urchin PKC does not contain any of these determining sequences as is observed in Figure 7 of the results section. Previous observations with one of the V3-specific monoclonal antibodies to mammalian PKC showed no reactivity to sea urchin proteins (Shen & Ricke, 1989). Two of the V3-specific polyclonal antibodies, anti-PKC- $\alpha$  and anti-PKC- $\beta$ , have been tested against sea urchin egg DE52 fractions. Both antibodies recognized proteins which could be blocked by peptide antigen, with anti-PKC- $\alpha$  recognizing a protein of 67 kDa and anti-PKC- $\beta$  staining a protein at 77 kDa. These immunoreactive bands are present in both fractions with relatively high and in fractions with low PKC activity (VanDePol and Shen unpublished observations). The reason for this lack of antibody recognition and/or specificity is apparent from the V3 region sequence of the sea urchin clone and from the V3 region sequences of the other lower vertebrates and invertebrates. The PKCs of these species vary at the V3 region, the portion of the protein used to generate these antibodies. Since antibodies generated to the sea urchin PKC clone react with a protein of 72 kDa, it seems that the commercial, isozyme specific antibodies are not recognizing the same protein.

Comparisons of homology of the catalytic domain following the V3 region of all the PKC clones show very strong homology. The homologies for the catalytic domain are often

higher than those for comparison of the entire protein. Mammalian C3 through C4 domains are 72% (rat PKC- $\gamma$ ) and 76% (rat PKC- $\alpha$  and - $\beta$ ) identical to the sea urchin PKC clone. The *Xenopus* XPKCI and XPKCII are 74% and 75% identical, the *Aplysia* PKC is 70% identical and the *Drosophila* PKC has 56% identity. One finding in comparing these PKCs is that rat PKC- $\gamma$  catalytic region has a comparison value much closer to the entire polypeptide comparison value than do rat PKC- $\beta$ I and - $\alpha$ . The differences in the identities for the catalytic region may reflect substrate preferences for the different isozymes. The three mammalian isozymes all utilize histone as a phosphate acceptor in *in vitro* assays (Nishizuka, 1988), however, peptide substrate studies have indicated that preferences may vary, particularly for PKC- $\gamma$  (Marais *et al.*, 1990). The sea urchin PKC has been reported to have a lower histone kinase activity than mammalian PKC (Heinecke *et al.*, 1990), however the reason for this difference has not been identified.

To determine if the PKC cloned contributes to the biochemical activity assayed in *Lytechinus pictus*, polyclonal antibodies were generated to the isozyme. The antibodies reacted to a protein of 72 kDa in fractions that contain PKC activity and the reactivity is competed by preincubation of the sera with the peptide antigen. The antibodies did not recognize the 72 kDa protein in fractions that have no kinase activity. This does not indicate however, that this is the only PKC isozyme in the sea urchin, since in mammalian preparations all three  $\text{Ca}^{2+}$ -dependent isozymes elute off the DE52 anion exchange column at the same point (Marais & Parker 1989). The antibodies do, however, provide a useful tool for investigating PKC in the sea urchin, since they are the first antibodies tested to show specific reactivity only in fractions containing kinase activity.

The Western blots show that the PKC clone isolated from the cDNA library is present in the egg. An RPA was done to look for the PKC message at other stages of development. The RPA shows that not only is the message present in the ovary and in eggs, as expected, but the message is also present in the later developmental stages such as gastrula and pluteus. This

is not surprising considering that PKC is involved in many cellular processes that occur during development such as changes in transcription rates and changes in cell shape.

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## APPENDIX

In this appendix, more detailed information on the sequencing of the sea urchin PKC clone is given. The clone was sequenced at least three times in any one region. A consensus is drawn from the sequence data by those nucleotides which are represented in the majority of cases in the sequencing data. This consensus sequence is then reported as the nucleotide sequence, which is used for generating the amino acid sequence. In total, fifty six pieces of sequence data from individual sequencing reactions are included in a "contig", and this contig spans the entire sequenced region.

The contig is represented in figure A1 of this appendix it is the output of the pretty program in GCG (Genetics Computer Group (1991), Program Manual for the GCG package, version 7, April 1991, 575 Science Drive, Madison, WI 53711). The individual sequences are arranged in order of appearance in the clone from 5' to 3' with a consensus at the bottom. The capital letters in the consensus line indicate that all members of data making up the consensus are in agreement, lowercase letters in the consensus indicate that there is disparity among the members representing that particular nucleotide. The case of the letters within the individual sequences is irrelevant.

In the majority of the sequencing data the consensus is very easily assigned; in very few regions it is not as clear. What was done to call these nucleotides of contention (refer to region of nucleotides 1520 and 1523) was to determine if there was disparity in the template sequence by comparing to sequence from different templates. In the region indicated it appears that the sequences designated f, e and dd, may have intrinsic mistakes due to the fact that in many instances the three individuals diverge from the majority of the sequences at the same place; note nucleotides 1535, 1543, 1584, 1589, for example. These three originate from the same template.

Figure A2 is an arrow representation of Figure A1, the diagram is generated by the BigPic program in GCG. Each arrow represents a sequence entered which then span the sequenced region. The direction of the arrowhead does not indicate direction of sequencing. This figure allows for examination of overlapping fragments in the sequence data.

Figure A3 is a restriction map analysis of the clone. The Map program displays the nucleotide sequence along with requested restriction enzyme cutting sites. The restriction map was determined by restriction enzyme digestion of the clone and verified through the sequencing. The restriction map is shown including sites of some of the more commonly used restriction enzymes. The information in this figure provides a reference for future manipulations of the cDNA clone that require restriction digestion.

```

kpn34-1    acgaacattttaatcgaaggcgggatattctcgcagtcctattgtgcaagaacgaagcacat 60
bb         ACGAACATTTAATCGAAGGGGGATATTCTCGCAGTCCTATTGTaCAAGAACGAAGCACAT 60
pkc4       acgaacattttaatcgaaggcgggatattctcgcagtcctattgtgcaagaacgaagcacat 60
o          ACGAACATTTAATCGAAGGGGGATATTCTCGCAGTCCTATTGTGCAAGAACGAAGCACAT 60
CONSENSUS  ACGAACATTTAATCGAAGGcGGATATTCTCGCAGTCCTATTGTgCAAGAACGAAGCACAT 60

kpn34-1    taacattgggttaaagtgtgcccgctcgtaattactgattactgtatcgctatcatttgggg 120
bb         TAACATTGGTTATCTGTCCCCGGCGCAATCACTGATTACTGTCTCGCTATCATGTGGGG 120
pkc4       taacattgggttaaagtgtgcccgctcgtaattactgattactgtatcgctatcatttgggg 120
o          TAACATTGGTTAAATGTGCCCGtCGTAATTACTGATTACTGTATCGCTATCATtTGGGGT 120
CONSENSUS  TAACATTGGTTAaaTGTgCCCCGtCGtAATtACTGATTACTGTaTTCGCTATCATtTGGGGt 120

kpn34-1    agtacatgtatacggtagcggaggccgaggtccaacggttgacttccattccaaaagtgg 180
bb         AGtACATATATACGGGAGCGGAGGGCGAGGCCCAACGGTGCaCTTCCACTCCAAAAGTGG 180
pkc4       agtacatgtatacggtagcggaggccgaggtccaacggttgacttccattccaaaagtgg 180
o          AGtACATGTATACGGtAGCGGAGGCCGAGGTCCAACGTTGTaCTTCCATTCCAAAAGTGG 180
CONSENSUS  AGTACATgTATACGGtAGCGGAGGCCGAGGTCCAACGtTGtACTTCCATtCCAAAAGTGG 180

2bsr1-1    aatacgacacttgag 15
2br1-uni1  gaattcgacacttgag 16
nr1-uni    gaattcgacacttgag 16
2br1-uni2  cgaattcgacacttgag 17
kpn34-1    atattttacatt 191
bb         ATATTTTACATGTGGTGTGCgCCCACACAGCAGTTATAAAATGTGCAATTTCGACACTTGA 239
pkc4       atatttttcatattgggtgtgcccgcgacagtggatataaaaatgtcgaattcgacacttgag 240
o          ATATTTTACATtTGGTGTGCgCCGAgACAGtGGATATAAAATGTGCAATTTCGACACTTGAT 240
CONSENSUS  ATATTTTaCATtTGGTGTGCGCCgagACAGtgGaTATAAAATGTGCAATTtCGACACTTGAg 240

2bsr1-1    ataaagggatttgtgacgaggggagcttttgggggagaagaacgtgcattagataaacaat 75
2br1-uni1  atgaagggatttgcgaggaggggagctttgcgtcagaagaacgtgcatgagataaaaaat 76
nr1-uni    atgaagggatttgcgaggaggggagctttgcgtcagaagaacgtgcatgagataaaaaat 76
2br1-uni2  atgaagggatttgcgaggaggggagctttgcgtcagaagaacgtgcatgagataaaaaat 77
pkc4       atgaagggatttgcgaggaggggagctttgcgtcagaagaacgtgcatgagataaaaaat 300
o          ATGAAGGGATTGTGCGAAGAGGGGAGCTTTGCGtCAGAAGAACGT 284
CONSENSUS  ATgAAGGGATTGTGcGAgGAGGGGAGCTTTGcGtcAGAAGAACGTGCATgAGATAAAaAAAT 300

2bsr1-1    cacaaattcatcccagagggtttttcaagcagacgacattttgtagccattgcaaggatttc 135
2br1-uni1  cacaaattcatcccagagggtttttcaagcagccgacattttgtagccattgcaaggatttc 136
nr1-uni    cacaaattcatcccagagggtttttcaagcagccgacattttgtagccattgcaaggatttc 136
2br1-uni2  cacaaattcatcccagagggtttttcaagcagccgacattttgtagccattgcaaggatttc 137
pkc4       cacaaattcatcccagagggtttttcaagcagccgacattttgtagccattgcaa 353
CONSENSUS  CACAAATTcATCCCCGAGGTTTTTCAAGCAGcCGACATTTTGTAGCCATTGCAAGGATTTc 360

a          AAGCAAGGGTTTCAGTGCAAAGTGTGCAGTTGTGTAGTTCATAAG 45
b          AAGCAAGGGTTTCAGTGCAAAGTGTGCAGTTTGTAGTTCATAAG 45
2bsr1-1    atttgggtgatttggcaagcaagagtttcagtgc aaagtgtgcagttttgtggttcataag 195
2br1-uni1  atttggggatttggcaagcaagggttttcagtgc aaagtgtgcagttttgtagttcataag 196
nr1-uni    atttggggatttggcaagcaagggttttcagtgc aaagtgtgcagttttgtcggttcataag 196
2br1-uni2  atttggggatttggcaagcaagggttttcagtgc aaagtgtgcagttttgtagttcataag 197
CONSENSUS  ATTTGGgGATTtGGCAAGCAAGgGTTTCAGTGCAAAGTGTGCAGTTtTGTaGTTTCATAAG 420

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**Figure A1: Pretty output of sequencing data**

a	CGCTGCCATGAATTTGTCACTTTCCAGTGCCCTGGACTCGACCCAGGAGTCGATTCTTGAC	105
b	CGCTGCCATGAATTTGTCACTTTCCAGTGCCCTGGACTCGACCCAGGAGTCGATTCTTGAC	105
2bsr1-1	cgctgccatgaatttgtcactttccagtgccctggactcgaccca	240
2br1-uni1	cgctgccatgaatttgtcactttccagtgccctggactcgacccaggagtcgattcttgac	256
nr1-uni	cgctgccatgaatttgtcactttccagtgacctcgactcgacccaggagtcgattctttac	256
2br1-uni2	cgctgccatgaatttgtcactttccagtgccctggactcgacccaggagtcgattcttgac	257
CONSENSUS	CGCTGCCATGAATTTGTCACTTTCCAGTGcCCTgGACTCGACCCAGGAGTCGATTCTgAC	480
a	GATCCTAGGAACAAGCATAAGTTCAAAGTTCACAGTTATAACAGTCCCACATTCTGTGAC	165
b	GATCCTAGGAACAAGCATAAGTTCAAAGTTCACAGTTATAACAGTCCCACATTCTGTGAC	165
2br1-uni1	gacccataggaacaagcataaagttcaaagttcacagttataacagtccccacattctgtgac	316
nr1-uni	gcacccataggaacacatacagttcaaagttcacagttataacagtccccacattctgtcac	316
2br1-uni2	gacccataggaacaagcataaagttcaaagttcacagttataacagtccccacattctgtgac	317
CONSENSUS	GatCCTAGGAACaAgCATAaAGTTCaAGTTCACAGTTAtaACAGTCCCACATTCTGTgAC	540
2404-2r		tgtgac 6
a	CACTGTGGGTCTCTCCTATATGGGCTCTATCACCAGGGTATGAAATGTGGAGCATGTGAC	225
b	CACTGTGGGTCTCTCCTATATGGGCTCTATCACCAGGGCATGAAATGTGGAGCATGTGAC	225
2br1-uni1	cactgtgggtctctcctatatatgggtctatcaccagggcatgaaatgtggagcatgtgac	376
nr1-uni	cactgtgggtctctcct	333
2br1-uni2	cactgtgggtctctcctatatatgggtctatcaccagggcatgacatgtggggcatgtgac	377
CONSENSUS	CACTGTGGGTCTCTCCTATATGGGCTCTATCACCAGGGcATGAaATGTGGaGCATGTGAC	600
2404-4r	tgtacacaaacgatgtcagaagtcagttaccaaatttatgtggagcagaccataca	55
2404-2r	.tgaatgtacacaagc.atgtcagaagtcagttaccaaatttatgtggagcagaccataca	66
a	ATGAATGTACACAAACGATGTGAGAAGTCAGTCCCAaATTATGTGGAGCAGCCATACA	285
b	ATGAATGTACACAAACGATGTGAGAAGTCAGTCCCAaATTATGTGGAGCAGCCATACA	285
2br1-uni1	atgaatgttcacaaacgatgtcagaagtcagttaccaaatttatgtggagcagaccataca	436
2br1-uni2	atg	380
CONSENSUS	aTGAATGTaCACAAaCgATGTGAGAAGTCAGTaCCAAATTtATGTGGAGCAGaCCATACA	660
2404-3r		aagctgaggtcatttgaaacaaactccaggtcaca 35
2404-4r	gagagacgaggtcgtatcaaggtcaaagctgaggtcatttgaaacaaactccaggtcaca	115
2404-2r	gagagacgaggtcgtatcaaggtcaaagctgaggtcatttgaaacaaactccaggtcaca	126
a	GAGAGACGAGGTCGTCTCAAGGTCAAAGCTGAGGTCATTGGAAACAAaCTCCAGGTCACA	345
b	GAGAGACGAGGTCGTCTCAAGGTCAAAGCTGAGGTCATTGGAAACAAaCTCCAGGTCACA	345
2br1-uni1	gagagacgaggtcgt..caaggtcaaagctgaggtcatttgaaacaaactccaggtcaca	496
CONSENSUS	GAGAGACGAGGTCGTatCAAGGTCAAAGCTGAGGTCATTGGAAACAAaCTCCAGGTCACA	720
2404-3r	gttgctgaagccaagaacctaattccgatggaccctaattggattgtcagAtcctttttgtg	95
2404-4r	gttgctgaagccaagaacctaattccgatggaccctaattggattgtcagatcctttttgtg	175
2404-2r	gttgctgaagccaagaacctaattccgatggaccctaattggattgtcagatcctttttgtg	186
a	GTTGGTGAAGcCaaGAaCCTAATTCcG	372
b	GTTGcTGAAGCCaagAaCCTAATTCcGATGGACCCTAATGGATTGtCAGatcctttttgtg	405
2br1-uni1	gttggtgaagccaagaacctaattccgatg	526
CONSENSUS	GTTGcTGAAGCCAAGAACCTAATTCcGATGGACCCTAATGGATTGTcAGATCCTTTTGTG	780

Figure A1: (continued)

ss15	taagaagaagactaggactattaaa	25
2404-3r	aaactcaaactcattccagatcagaaacgagagactaagaagaagactaggactattaaa	155
2404-4r	aaactcaaactcattccagatcagaaacgagagactaagaagaagactaggactattaaa	235
2404-2r	aaactcaaactcattccagatcagaaacgagagactaagaagaagactaggactattaaa	246
b	aaactcaaa	414
CONSENSUS	AAACTCAAAC TCATTCCAGATCAGAAACGAGAGACTAAGAAGAAGACTAGGACTATTAAA	840
ss15	ggaagcctaaatccaacctggggagaatcatttgattttaacttagaagacacggacagg	85
2404-3r	ggaagcctaaatccaacctggggagaatcatttgattttaacttagaagacacggacagg	215
2404-4r	ggaagcctaaatccaacctggggagaatcatttgattttaacttagaagacacggacagg	295
2404-2r	ggaagcctaaatccaacctggggagaatcatttgattttaacttagaagacacggacagg	306
CONSENSUS	GGAAGCCTAAATCCAACCTGGGGAGAATCATTTGATTTTAACTTAGAAGACACGGACAGG	900
d	CcGCAATGACTTCATG	16
c	CcGCAATGACTTCATG	16
ava9-1	gaccgtgccacccgcaatgacttcatg	27
ava6-1	gaccgtgccacccgcaatgacttcatg	27
ss15-2	gggactgggacgtgccacccgcaatgacttcatg	35
pkc1	gaggtgtgggactgggacgtgccacccgcaatgacttcatg	42
ss15	aaccggagggtcttctggttgaggtgtgggactgggacgtgccacccgcaatgacttcatg	145
2404-3r	aaccggagggtcttctggttgaggtgtgggactgggacgtgccacccgc	263
2404-4r	aaccggagggtcttctggttgaggtgtgggactgggacgtgccacccgcaat	346
2404-2r	aaccggagggtcttctggttgaggtgtgggactgggacgtgccacccgcaatgac	360
CONSENSUS	AACCGGAGGCTTCTGGTTGAGGTGTGGGACTGGGACCGTGCCACCCGCAATGACTTCATG	960
cc	GTCCTTTGGAATCTCAGAGCTGATGAAGGCAGGTGTAGATGCCTGGTACAAG	52
d	GGTGCCCTGTCTTtTGGCATCTCAGAGCTGATGAaGGCAGGTGTAGATGCCTAGTACAAG	76
c	GGTGCCCTGTCTTtTGGCATCTCAGAGCTGATGAAGGCAGGTGTAGATGCCTGGTACAAG	76
ava9-1	ggt.ccctgtccttttggcatctcagagctgatgaaggcaggtgtagatgcctggttacaag	87
ava6-1	ggt.ccctgtccttttggcatctcagagctgatgaaggcaggtgtagatgcctggttacaag	87
ss15-2	ggtgccctgtccttttggcatctcagagctgatgaaggcaggtgtagatgcctggttacaag	95
pkc1	ggtgccctgtccttttggcatctcagagctgatgaaggcaggtgtagatgcctggttacaag	102
ss15	ggtgccctgtccttttggcatctcagagctgatgaaggcaggtgtagatgcctggttacaag	205
CONSENSUS	GGTgCCCTGTCTTtTGGcATCTCAGAGCTGATGAAGGCAGGTGTAGATGCCTgGTACAAG	1020
kpn34-2r	gcaatcgcgaggagacagaatcc	21
kpn34-3r	ggaagaggggtgaataactacaatgtgccagcaatcgcgaggagacagaatcc	49
cc	CTCCTGGGCCAGGAAGAGGGTGAATACTACAATGTGCCAGCAATCGGGGAGACAGAATCC	112
d	CTCCTGGGcCAGGAAGAGGGTGAATACTACAATGTGcCAGCAATCGGGGAGACAGAATCC	136
c	CTCCTGGGCCAGGAAGAGGGTGAATACTACAATGTGCCAGCAATCGCGGAGACAGAATCC	136
ava9-1	ctcctggggccaggaagaggggtgaataactacaatgtgccagcaatcgcgaggagacagaatcc	147
ava6-1	ctcctggggc.aggaagaggggtgaataactacaatgtgccagcaatcgcgaggagacagaatcc	147
ss15-2	ctcctggggccaggaagaggggtgaataactacaatgtgccagcaatcgcgaggagacagaatcc	155
pkc1	ctcctggggccaggaagaggggtgaataactacaatgtgccagcaatcgcgaggagacagaatcc	162
ss15	ctcctggggccaggaagaggggtgaataactacaatgtgccagcaatcgcgaggaga	257
CONSENSUS	CTCCTGGGcCAGGAAGAGGGTGAATACTACAATGTGCCAGCAATCGcGGAGACAGAATCC	1080

Figure A1: (continued)

ava6-2r		agagcatgtcaag	13
smr1-2lor		agagcatgtcaag	13
smr1-2r		agagcatgtcaag	13
ava6-3r		tgccctacccaagagcatgtcaag	23
kpn34-2r	atcgatgaactcacttctaatataaagaaactaccaatgcctacccaagagcatgtcaag		81
kpn34-3r	atcgatgaactcacttctaatataaagaaactaccaatgcctacccaagagcatgtcaag		109
cc	ATCGATGATCTCACTTCTAATATAAAGAACTCCCAATGTCTCCCCAAGAGTATGTCAAG		172
d	ATCGATGAACCTCACTTCTAATATAAAGAACTACCAATGCCTACCCAAGAGTATGTCAAG		196
c	ATCGATGAACCTCACTTCTAATATAAAGAACTACCAATGTCTCCCCAAGAGCATGTCAAG		196
ava9-1	atcgatgaactcacttctaatataaagaaactacca		184
ava6-1	atcgatgaactcacttctaatataaagaaactacca.tgccctacc		192
ss15-2	atcgatgaactcacttctaatataaagaaactaccaatgcctacccaagagcatgtcaag		215
pkc1	atcgatgaactcacttctaatataaagaaactaccaatgcctacccaagagcatgtcaag		222
CONSENSUS	ATCGATGAaCTCACTTCTAATATAAAGAACTaCCAaTGcCTaCCCAAGAGcATGTCAAG		1140
ava6-2r	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		73
smr1-2lor	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		73
smr1-2r	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		73
ava6-3r	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		83
kpn34-2r	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		141
kpn34-3r	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		169
cc	TCTCAGAACTCTATCTCAATGACAGGTATGGGTGTTGTTAGAGCAA		218
d	cCTCAGATCTCTAaCTCAATGTcAGGTATGGGTGTTGTTAGAGCAAGCGATCaCATCTtC		256
c	CCTCAGATCTCTATCTCAATGTcAGGTATGGGTGTTGTTAGAGCAAGCGATTACATCTTC		256
ss15-2	cctcaga.ctctaactcaatgtcaggtatgg		246
pkc1	cctcagaactctaactcaatgtcaggtatgggtgttgttagagcaagcgatttcaacttc		282
CONSENSUS	cCTCAGAACTCTAaCTCAATGTcAGGTATGGGTGTTGTTAGAGCAAGCGATtCaaCTTC		1200
kpn32-2		cc	2
kpn32-3		cc	2
2bsp-2r		cc	2
ava6-2r	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagggtacc		133
smr1-2lor	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagggtacc		133
smr1-2r	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagggtacc		133
ava6-3r	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagggtacc		143
kpn34-2r	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagg		196
kpn34-3r	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagg		224
d	cTaTCAGTGCTTGGAAGG		276
c	CTATCAGTTCTTGGAAGG		276
pkc1	ctatcagtgccttggaagaggcagcttcggcaagggttatggttagcagagaagaagggtacc		342
CONSENSUS	CTATCAGTgCTTGGAAGGcAGGCTTCGGCAAGGTTATGTTAGCAGAGAAGAGGGTACC		1260
pkc2		gacgatgatgtg	12
kpn32-2	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		62
kpn32-3	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		62
2bsp-2r	gatgaccctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		62
ava6-2r	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		193
smr1-2lor	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		193
smr1-2r	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		193
ava6-3r	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		203
pkc1	gatgaactctacgccatcaagatccttaagaaggatgtcatcatccaggacgacgatgtg		402
CONSENSUS	GATGAaCtCTACGCCAtCAAGatCCTTAAGAAGGaTGTcATCATCCAGGACGAcGATGTG		1320

Figure A1: (continued)

441-4	tttcttgacg	10
441-3	tttcttgacg	10
f	CAGCAAACCCGCTTTCTTGACG	22
e	CAGCAAACCCGCTTTCTTGACG	22
dd	CcTGCCCAGCAAACCCGCTTTCTTGACG	28
pkc2	gagtgcagcatgacggaaaagaggggtcctgggcctgccagcaaaccgctttcttgacg	72
kpn32-2	gagtgcacccatgacggaaaagaggggtcctgggcctgccagcaaaccgctttcttgacg	122
kpn32-3	gagtgcacccatgacggAAAAGaggggtcctgggcctgccagcaaaccgctTTTcTTgacg	122
2bsp-2r	gagtccacccatgacggacaagaggggtcctgggcctgccagcaaaccgctttcttgacg	122
ava6-2r	gagtgcacccatgacggaaaagaggggtc	220
smr1-2lor	gagtgcacccatgacggaaaagaggggtcctgggcctgccagcaaaccgctttcttgacg	253
smr1-2r	gagtgcacccatgacggaaaagaggggtcctgggcctgccagcaaaccgctttcttgacg	253
ava6-3r	gagtgcacccatgacggaaaagaggggtc	230
pkc1	gagtgcacccatgacggaaaagaggggtc	429
CONSENSUS	GAGTgCacCATGACGGAAAGAGGGTCCTGGgCCTGCCCAGCAAACCCGCTTTCTTGaCG	1380
ss17	aat	3
441-2	ttctttgtcatggagttcgtaaat	24
441-4	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggagttcgtaaat	70
441-3	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggagttcgtaaat	70
f	GcGC'TTCACTCATGCTTCCAGACAATGGACAGACTCTTCTGTGTCATGGAGTTCGACAAT	82
e	GCGCTTCACTCATGCTTCCAGACAATGGACAGACTCTTCTGTGTCATGGAGTTCGACAAT	82
dd	GCGCTTCACTCATGCTTCCAGACAATGGACAGACTCTTCTGTGTCATGGAGTTCGACAAT	88
pkc2	gcgcttcactcatgcttccagacaatgaacagactcttctttgtcatggagttcgtaaat	132
kpn32-2	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggagttcgtaaat	182
kpn32-3	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggagttcgtaaat	182
2bsp-2r	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggagttcgtaaat	182
smr1-2lor	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggag	304
smr1-2r	gcgcttcactcatgcttccagacaatggacagactcttctttgtcatggagttcgtaaat	313
CONSENSUS	GCGCTTCACTCATGCTTCCAGACAATGgACAGACTCTTCTtTGTCATGGAGTTCGtCAAT	1440
ss17-2	ggcaagttcagggaaacctcatgctgta	27
441-5	ttccagatccagaaagtcggcaagttcagggaaacctcatgcagta	45
ss17	gggggagatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	63
441-2	gggggagatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	84
441-4	gggggagatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	130
441-3	gggggagatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	130
f	GGGGGAGATCTCATGTTCCAGATCCAGAAAGTCGGCAAGTtCAGGGAACCTCATGCTGTA	142
e	GGGGGAGATCTCATGTTCCAGATCCAGAAAGTCGGCAAGTtCAGGGAACCTCATGCTGTA	142
dd	GGGGGAGATCTCATGTTCCAGATCCAGAAAGTCGGCAAGTACAGGGAACCTCATGCTGTA	148
pkc2	gggggagatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	192
kpn32-2	ggggg.agatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	242
kpn32-3	gg.g.agatctcatggttccagatccagaaagtcg	216
2bsp-2r	gggggagatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	242
smr1-2r	ggggg.agatctcatggttccagatccagaaagtcggcaagttcagggaaacctcatgctgta	373
CONSENSUS	GGgGgAGATCTCATGTTCCAGATCCAGAAAGTCGGCAAGTtCAGGGAACCTCATGctGTA	1500

Figure A1: (continued)

441-1r	ggcttattctacctccattcacaagggtgtgatatac	36
ss17-2	ttctatgcagctgagatagcagtaggcttattctacctccattcacaagggtgtgatatac	87
441-5	ttctatgcagctgagatagcagtaggcttattctacctccattcacaagggtgtgatatac	105
ss17	ttctatgcagctgagatagcagtaggcttattctacctccattcacaagggtgtgatatac	123
441-2	ttctatgcagctgagatagcagtaggcttattctacctccattcacaagggtgtgatatac	144
441-4	ttctatgcagctgagatagcagtaggcttattctacctccattcacaagggtgtgatatac	190
441-3	ttctatgcagctgagatagcagtaggcttattctacctccattcacaagggtgtgatatac	190
f	TTCTATGGAGcTGAGATAGGAGGAGGCTTATTCTCCCTCCACACACAGGGTGTGATATAC	202
e	TTCTATGcAGcTGAGATAGGAGGAGGCTTATTCTCCCTCCATACACAAGGTGTGATATAC	202
dd	TTCTATGGAGCTGAGATAGGAGGAGGCTTATTCTCCCTCCAtACACAAGGTGTGATATAC	208
pkc2	ttctatgcagctgagataggaggaggcttattctacctccattcacaagggtgtgatatac	252
kpn32-2	ttctatgcagctg	255
2bsp-2r	ttctatgcag	252
smr1-2r	ttctatgcag	383
CONSENSUS	TTCTATGcAGCTGAGATAGcAGtAGGCTTATTCTaCCTCCAttCACAAgGTGTGATATAC	1560
441-1r	agagatctcaaactagataaatgtactagtagatgcagaagggtcacatcaagattgctgac	96
ss17-2	agagatctcaaactagataaatgtactagtagatgcagaagggtcacatcaagattgctgac	147
441-5	agagatctcaaactagataaatgtactagtagatgcagaagggtcacatcaagattgctgac	165
ss17	agagatctcaaactagataaatgtactagtagatgcagaagggtcacatcaagattgctgac	183
441-2	agagatctcaaactagataaatgtactagtagatgcaga	182
441-4	agagatctcaaactagataaatgtactagtagatgcagaagggtcacatcaagattgctgac	250
441-3	agagatctcaaactagataaatgtactagtagatgcaga	228
f	AGAGATCTCAATCTAGATAATGTTCTAGGAGATAGAGaGGGACACATCAAGATTGcTGTC	262
e	AGAGATCTCAATCTAGATAATGTTCTAGGAGATAGAGAGGGACACATCAAGATTGGTGTc	262
dd	AGAGATCTCAATCTAGATAATGTTCTAGGAGATAGAGaGGGACACATCAAGATTGCTGTc	268
pkc2	agagatctcaa.ctagataaatgtactagtagatgcagaagggtcacatcaagattgctgac	312
CONSENSUS	AGAGATCTCAaACTAGATAATGTaCTAGtAGATgcAGAgGGtCACATCAAGATTGcTGaC	1620
441-1r	tttggtatgtgcaaggaacacatgaacgagggcgacacaacgagaacggttctgcggaaca	156
ss17-2	tttggtatgtgcaaggaacacatgaacgagggcgacacaacgagaacggttctgcggaaca	207
441-5	tttggtatgtgcaaggaacacatgaacgagggcgacacaacgagaacggttct	217
ss17	tttggtatgtgcaaggaacacatgaacgagggcg	217
441-4	tttggtatgtgcaaggaacacatgaacgagggcgacacaacgagaacggttct	302
f	TGTGGTGTGTACAAGGaACACATGAGCGaGGGgGACACAACGAGAAC	309
e	TGTGGTATGTACAAGGAACACATGAACGAGGGCGACACAACGAGAACG	310
dd	TGTGGTATGTACAaGGAACACATGAACGAGGGGGACACAACGAGA	313
pkc2	tttggtatgtgcaaggaacacatgaacgagggcgacacaacgagaacggttctgcgggaca	372
CONSENSUS	TtTGGTaTGTgCAAGGAACACATGAaCGAGGGcGACACAACGAGAACGtTCTGCGGaACA	1680
ava9-2r	tgact.g	7
2br1rev	ctggttgactgg	11
nr1-3r	gagattgtagcatatcagccatattggcaaggctggttgactgg	42
ava9-6r	tcgctcccagattgtagcatatccaccatattggcaaggctggttgactgg	50
441-1r	ccagactacatcgctcccagattgtagcatatcagccatattggcaaggctggttgactgg	216
ss17-2	ccagactacatcgctcccagattgtagcatatcagccatattggc	252
pkc2	acagactacatcgctcccagattg	397
CONSENSUS	cCAGACTACATCGCTCCCGAGATTGTAGCATATCagCCATATGGCAAGGCTGTTGACTgG	1740

Figure A1: (continued)

nr1-2r	cctccatttgatggtgaa	18
ava9-5r	tgtttagcaggacagcctccatttgatggtgaa	32
nr	TGTATGAAATGTTAGCAGGACAACCTCCATTGATGGTGAA	41
ava9-3r	ttggtgtttt.ctgtatgaaatgtttagcaggacagcctccattg.atggtgaa	53
ava9-2r	tgggccttttggtgttttactgtatgaaatgatagcaggacagcctccatttgatggtgta	67
2br1rev	tgggccttttggtgttttactgtatgaaatgtttagcaggacacccctccatttgatggtgaa	71
nr1-3r	tgggccttttggtgttttactgtatgaaatgtttagcaggacagcctccatttgatggtgaa	102
ava9-6r	tgggccttttggtgttttactgtatgaaatgtttagcaggacagcctccatttgatggtgaa	110
441-1r	tggg	220
CONSENSUS	TGGGCCTTTGGTGTTTTaCTGTATGAAATGtTAGCAGGACAgCCTCCATTtgATGgTGaA	1800

nr1-2r	gatgaagatgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	78
ava9-5r	gatgaagatgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	92
nr	GATGAAGATGAATTGTTTCAATCTATCATGGAACATGTACCCTCATATCCCCAAATCAATG	101
ava9-3r	gatgaagatgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	113
ava9-2r	gatgaa..tgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	127
2br1rev	gatgaagatgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	131
nr1-3r	gatgaagatgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	162
ava9-6r	gatgaagatgaattgtttcaatctatcatggaacatgtaccctcatatcccaaatacaatg	170
CONSENSUS	GATGAAGaTGAATTGTTTCAATCTATCATGGAACATGTACCCTCATATCCCCAAATCAATG	1860

2bsr1-2r	gggtttttgacaaagcatcctggtaaccgggta	33
nr1-2r	tcaagagaatctgtgactatgtgcaaaggggtttttgacaaagcatcctggtaaccgggta	138
ava9-5r	tcaagagaatctgtgactatgtgcaaaggggtttttgacaaagcatcctggtaaccgggta	152
nr	TCAAGAGAATCTGTGACTATGTGCAAAGGGTTTTTGACAAAGCATCCTGGTAAGCGGTTA	161
ava9-3r	tcaagagaatctgtgactatgtgcaaaggggtttttgacaaagcatcctggtaaccgggta	173
ava9-2r	tcaagagaatctgtgactatgtgcaaaggggtttttgacaaagcatcctggtaaccgggta	187
2br1rev	tcaagagaatctgtgactatgtgcaaaggggtttttgacaaagcatcctggtaaccgggta	191
nr1-3r	tcaagagaatctgtgactatgtgcaaaggggtttttgacaaagcatcctggtaaccgggta	222
ava9-6r	tcaagagaa	179
CONSENSUS	TCAAGAGAAtCTGTGACTATGTGcAAaGGgTTTTTGACAAAGCATCCTGGTAAGCGGTTA	1920

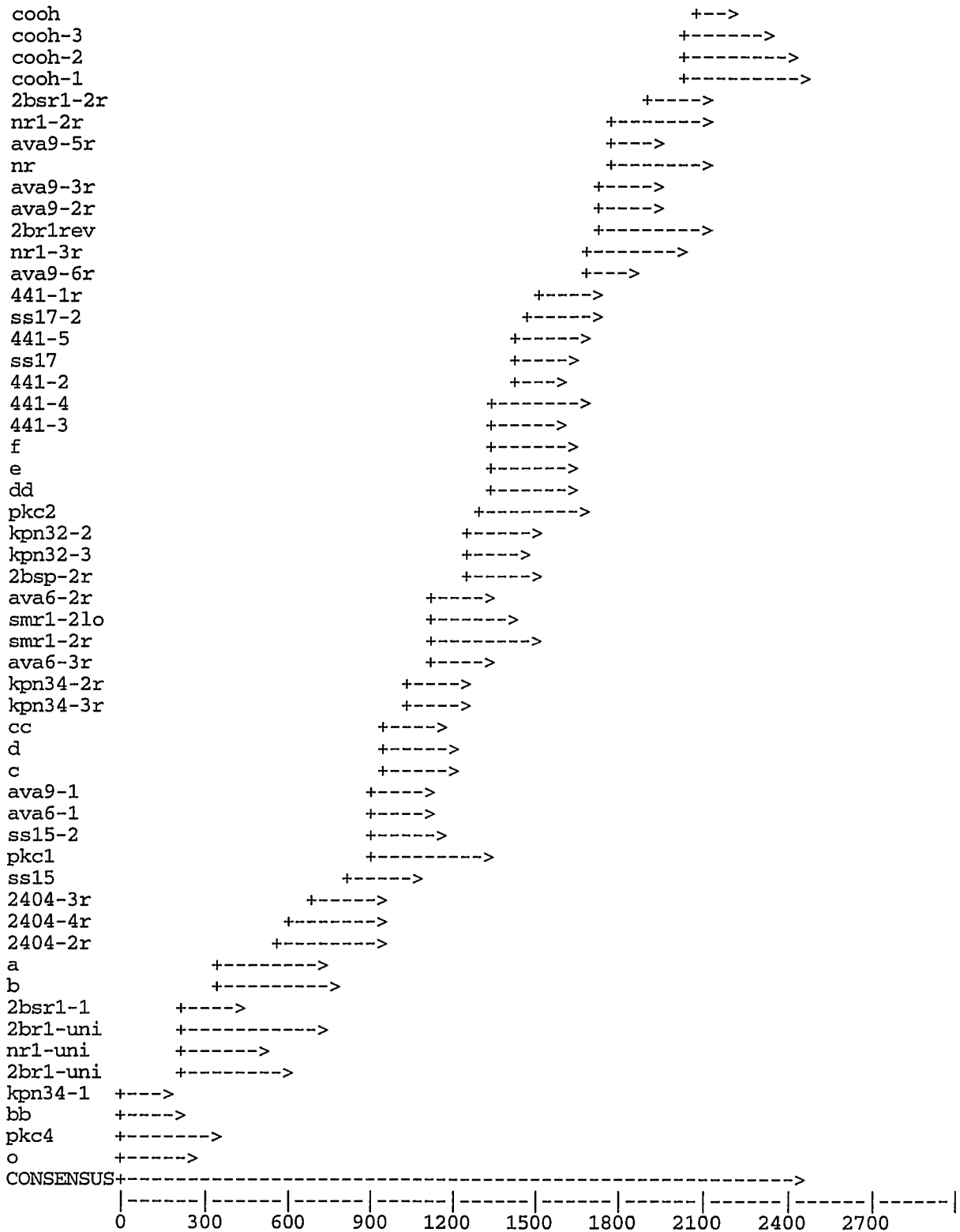
2bsr1-2r	ggaagtggacctacaggggacccggacatcagagagcatcaagtcttccggcgattcgct	93
nr1-2r	ggaagtggacctacaggggaacaggacatcagggagcatcaattcttccgctcgatcgac	198
ava9-5r	ggaagtggac	162
nr	GGAAGTGGACCTACAGGGGAACAGGACATCAGGGAGCATCAATTCTTCCGTCGAATCGAC	221
ava9-3r	ggaagtggac	183
ava9-2r	ggaagtggac	197
2br1rev	ggaagtggacctacaggggaacaggacatcagggagcatcaattcttccgctcgatcgac	251
nr1-3r	ggaagtggacctacaggggaacaggacatcagggagcatcaattcttccgctcgatcgac	282
CONSENSUS	GGAAGTGGACCTACAGGGGAaCaGGACATCAGgGAGCATCAATCTTCCGTCGAaTCGac	1980

2bsr1-2r	tgggagaaactagcttgagagaaaatacagcctccgtttgtgccgtcattgagaaacccc	153
nr1-2r	tgggagaaactagctaacagagaaaatacagcctccgtttgtgccgtcagtgagaaacccc	258
nr	TGGGAGAAACTAGCTAACAGAGAAATACAGCCTCCGTTTGTGCCGTCAGTGAGAAACCCC	281
2br1rev	tgggagaaactagctaacagagaaaatacagcctccgtttgtgccgtcagtgagaaacccc	311
nr1-3r	tgggagaaactagctaacagagaaaatacagcctccgtttgtgccgtcagtgagaaacccc	342
CONSENSUS	TGGGAGAAACTAGCTaacAGAGAAATACAGCCTCCGTTTGTGCCGTCAGTGAGAAACCCC	2040

**Figure A1: (continued)**

cooh		gacc	4
cooh-3	ctgctgaaaactttgaccctatttcacaaaaatcccgtgtgcgcttaccgccgacc		56
cooh-2	ctgctgaaaactttgaccctatttcacaaaaatcccgt. .gcgcttaccgccgacc		56
cooh-1	gctgctgaaaactttgaccctatttcacaaaaatcccgtgtgcgcttaccgccgacc		57
2bsr1-2r	agaactgctgaagactttgaccctatttcacaaaaatcccgtgtgcgcttaccgccgacc		213
nr1-2r	agagctgctgaaaactttgaccctatttcacaaaaatcccgtgtgcgcttaccgccgacc		318
nr	AGAGCTGCTGAAAACTTTGACCCCTATTTCACAAAAATCCCGTGTGCGCTTACCCCGACC		341
2br1rev	agagctgctgaaaagtttgaccctatttcacaaaaatcccgtgtgcgcttaccgccgacc		371
nr1-3r	agagctgctgaaa		355
CONSENSUS	AGAGCTGCTGAAaAcTTTGACCCCTATTTCaCaaAAATcCCGTgtgCgCTTACCcCGaCC		2100
cooh	gacaaactcattatcatgaatattcaggacgaattccaaggctttaccttcgtgaacgag		64
cooh-3	gacaaactcattatcatgaatattcaggacgaattccaaggctttaccttcgtgaacgag		116
cooh-2	gacaaactcattatcatgaatattcaggacgaattccaaggctttaccttcgtgaacgag		116
cooh-1	gacaaactcattatcatgaatattcaggacgaattccaaggctttaccttcgtgaacgag		117
2bsr1-2r	gacaaattcagtatcatgaa		233
nr1-2r	gacaaactcattatcatgaatattcaggacgaattccaa		357
nr	GACAAACTCATTATCATGAATATTTCAGGACGAATTC		377
2br1rev	gacaaactcattatcatgaatattcaggacgaattc		407
CONSENSUS	GACAAAcTCAcTATCATGAATATTTCAGGACGAATTCcAAGGCTTTACCTTCGTGAACGAG		2160
cooh	gtcttcgatggctatgtgcgtagtttgcccagctagt. ccaa		106
cooh-3	gtcttcgatggctatgtgcgtagtttgcccagctagtgcgaagtttgtagctcccaccgt		176
cooh-2	gtcttcgatggctatgtgcgtagtttgcccagctagtgcgaagtttgtagctcccaccgt		176
cooh-1	gtcttcgatggctatgtgcgtagtttgcccagctagtgcgaagtttgtagctcccaccgt		177
CONSENSUS	GTCTTCGATGGCTATGTGCgTAGTTTGCCcAGCTAgTgCCAAGTTTGTAGCTCCcACCGT		2220
cooh-3	cgctgttcctacgcctacttgggtgttcccaacgtatccaaaatgtcaccagtttatacat		236
cooh-2	cgctgttcctacgcctacttgggtgttcccaacgtatccaaaatgtcaccagtttatacat		236
cooh-1	cgctgttcctacgcctacttgggtgttcccaacgtatccaaaatgtcaccagtttatacat		237
CONSENSUS	CGCTGTTCCTACGCCTACTTGGTGTTCcAACGTATCCAAAATGTCACCAGTTTATACAT		2280
cooh-3	gtgctgtcctttttctttctttttgttacttgatgtgtacatagtagtagtagtag		296
cooh-2	gtgctgtcctttttctttctttttgttacttgatgtgtacatagtagtagtagtagtag		296
cooh-1	gtgctgtcctttttctttctttttgttacttgatgtgtacatagtagtagtagtagtag		297
CONSENSUS	GTGCTGTCTTTTCTTTCTTTTGTACTTGATGTGTACATAGTACATGTAGGTAGTAG		2340
cooh-3	aagtt		301
cooh-2	aagttggatgtagtcatgagtgccgaagacagtgaatcggtacattaatttgtttgaag		356
cooh-1	aagttggatgtagtcatgagtgccgaagacagtgaatcggtacattaatttgtttgaag		357
CONSENSUS	AAGTTGGATGTAGTCATGAGTGCCTGAAGACAGTGAATCGGTACATTAAATTGTTTGAAG		2400
cooh-2	ttttagcattacagaggaatagtg		380
cooh-1	ttttagcattacagaggaatagtgaaatgaatagctagcatataatggatctgtgattagtg		417
CONSENSUS	TTTTrGCATTACAGAGGAATAGTGAATGAATAGCTAGCATATAATGGATCTGTGATTAGT		2460
cooh-1	caagaatgtcgtac		431
CONSENSUS	CAAGAATGTCGTAC		2474

Figure A1: (continued)



**Figure A2: Arrow diagram of sequence data BigPic output**

```

ACGAACATTTAATCGAAGGCGGATATTTCTCGCAGTCCTATTGTGCAAGAACGAAGCACAT
1  -----+-----+-----+-----+-----+-----+-----+ 60
TGCTTGTAATTAGCTTCCGCCATATAAGAGCGTCAGGATAACACGTTCTTGCTTCGTGTA

a      T N I * S K A D I L A V L L C K N E A H -

TAACATTGGTTAAATGTGCCCCGTCGTAATTACTGATTACTGTATCGCTATCATTGTTGGGT
61 -----+-----+-----+-----+-----+-----+ 120
ATTGTAACCAATTTACACGGGCAGCATTAATGACTAATGACATAGCGATAGTAAACCCCA

a      * H W L N V P V V I T D Y C I A I I W G -

                                A
                                v
                                a
                                I
                                I

AGTACATGTATACGGTAGCGGAGGCCGAGGTCCAACGTTGTACTTCCATTCCAAAAGTGG
121 -----+-----+-----+-----+-----+-----+ 180
TCATGTACATATGCCATCGCTCCGGCTCCAGGTTGCAACATGAAGGTAAGGTTTTCACC

a      S T C I R * R R P R S N V V L P F Q K W -

                                E
                                c
                                o
                                R
                                I

ATATTTACATTTGGTGTGCGCCGAGACAGTGGATATAAAATGTGCAATTCGACACTTGAG
181 -----+-----+-----+-----+-----+-----+ 240
TATAAATGTAAACCACACGCGCTCTGTCACTTATATTTTACAGCTTAAGCTGTGAAGTC

a      I F T F G V R R D S G Y K M S N S T L E -

ATGAAGGGATTGTGCGAGGAGGGGAGCTTTGCGTCAGAAGAACGTGCATGAGATAAAAAAT
241 -----+-----+-----+-----+-----+-----+ 300
TACTTCCCTAAACGCTCCTCCCTCGAAACGCAGTCTTCTTGCACGTACTCTATTTTTTA

a      M K G F A R R G A L R Q K N V H E I K N -

CACAAATTCATCCCGAGGTTTTTCAAGCAGCCGACATTTTGTAGCCATTGCAAGGATTC
301 -----+-----+-----+-----+-----+-----+ 360
GTGTTTAAGTAGGGCTCCAAAAGTTCGTCGGCTGTAAAACATCGGTAACGTTCTTAAAG

a      H K F I P R F F K Q P T F C S H C K D F -

ATTTGGGGATTGTGCAAGCAAGGGTTTCAGTGCAAAGTGTGCAGTTTGTAGTTCATAAG
361 -----+-----+-----+-----+-----+-----+ 420
TAAACCCCTAAACGTTCTGTTCCCAAAGTCACGTTTCACACGTCAAACATCAAGTATTC

a      I W G F G K Q G F Q C K V C S F V V H K -

```

**Figure A3: Restriction map of the sea urchin PKC clone**

B  
S  
t  
X  
I

```

CGCTGCCATGAATTTGTCACCTTTCCAGTGCCCTGGACTCGACCCAGGAGTCGATTCTGAC
421 -----+-----+-----+-----+-----+-----+ 480
GCGACGGTACTTTAAACAGTGAAAGGTCACGGGACCTGAGCTGGGTCTCAGCTAAGACTG

a      R C H E F V T F Q C P G L D P G V D S D -

GATCCTAGGAACAAGCATAAGTTCAAAGTTCACAGTTATAACAGTCCCACATTCTGTGAC
481 -----+-----+-----+-----+-----+-----+ 540
CTAGGATCCTTGTTCGTATTCAAGTTTCAAGTGTCAATATTGTCAGGGTGTAAGACACTG

a      D P R N K H K F K V H S Y N S P T F C D -

CACTGTGGGTCTCTCCTATATGGGCTCTATCACCAGGGCATGAAATGTGGAGCATGTGAC
541 -----+-----+-----+-----+-----+-----+ 600
GTGACACCCAGAGAGGATATACCCGAGATAGTGGTCCCGTACTTTACACCTCGTACACTG

a      H C G S L L Y G L Y H Q G M K C G A C D -

ATGAATGTACACAAACGATGTCAGAAGTCAGTACCAAATTTATGTGGAGCAGACCATACA
601 -----+-----+-----+-----+-----+-----+ 660
TACTTACATGTGTTTGCTACAGTCTTCAGTCATGGTTTAAATACACCTCGTCTGGTATGT

a      M N V H K R C Q K S V P N L C G A D H T -

GAGAGACGAGGTCTGATCAAGGTCAAAGCTGAGGTCAATTGGAAACAAACTCCAGGTCACA
661 -----+-----+-----+-----+-----+-----+ 720
CTCTCTGCTCCAGCATAGTTCCAGTTTCGACTCCAGTAACCTTTGTTTGAGGTCCAGTGT

a      E R R G R I K V K A E V I G N K L Q V T -

A
v
a
I
I
GTTGCTGAAGCCAAGAACCTAATTCGATGGACCCTAATGGATTGTCAGATCCTTTTGTG
721 -----+-----+-----+-----+-----+-----+ 780
CAACGACTTCGGTTCTTGATTAAAGGCTACCTGGGATTACCTAACAGTCTAGGAAAACAC

a      V A E A K N L I P M D P N G L S D P F V -

AAACTCAAACCTCATTCCAGATCAGAAACGAGAGACTAAGAAGAAGACTAGGACTATTAAA
781 -----+-----+-----+-----+-----+-----+ 840
TTTGAGTTTGAGTAAGGTCTAGTCTTTGCTCTCTGATTCTTCTCTGATCCTGATAATTT

a      K L K L I P D Q K R E T K K K T R T I K -

```

**Figure A3:(continued)**

```

      GGAAGCCTAAATCCAACCTGGGGAGAATCATTTGATTTTAACTTAGAAGACACGGACAGG
841  -----+-----+-----+-----+-----+-----+ 900
      CCTTCGGATTTAGGTTGGACCCCTCTTAGTAAACTAAAATTGAATCTTCTGTGCCTGTCC

a      G S L N P T W G E S F D F N L E D T D R -

                                     A
                                     v
                                     a
                                     I
                                     I
      AACCGGAGGCTTCTGGTTGAGGTGTGGGACTGGGACCGTGCCACCCGCAATGACTTCATG
901  -----+-----+-----+-----+-----+-----+ 960
      TTGGCCTCCGAAGACCAACTCCACACCCCTGACCCTGGCACGGTGGGCGTTACTGAAGTAC

a      N R R L L V E V W D W D R A T R N D F M -

      GGTGCCCTGTCTTTGGCATCTCAGAGCTGATGAAGGCAGGTGTAGATGCCTGGTACAAG
961  -----+-----+-----+-----+-----+-----+ 1020
      CCACGGGACAGGAAACCGTAGAGTCTCGACTACTTCCGTCCACATCTACGGACCATGTTT

a      G A L S F G I S E L M K A G V D A W Y K -

      CTCTGGGCCAGGAAGAGGGTGAATACTACAATGTGCCAGCAATCGCGGAGACAGAATCC
1021 -----+-----+-----+-----+-----+-----+ 1080
      GAGGACCCGGTCCTTCTCCCACTTATGATGTTACACGGTCGTTAGCGCCTCTGTCTTAGG

a      L L G Q E E G E Y Y N V P A I A E T E S -

      ATCGATGAACTCACTTCTAATATAAAGAACTACCAATGCCTACCCAAGAGCATGTCAAG
1081 -----+-----+-----+-----+-----+-----+ 1140
      TAGCTACTTGAGTGAAGATTATTTCTTTGATGGTTACGGATGGGTTCTCGTACAGTTC

a      I D E L T S N I K K L P M P T Q E H V K -

      CCTCAGAACTCTAACTCAATGTCAGGTATGGGTGTTGTTAGAGCAAGCGATTTCAACTTC
1141 -----+-----+-----+-----+-----+-----+ 1200
      GGAGTCTTGAGATTGAGTTACAGTCCATACCCACAACAATCTCGTTCGCTAAAGTTGAAG

a      P Q N S N S M S G M G V V R A S D F N F -

                                               K
                                               p
                                               n
                                               I
      CTATCAGTGCTTGGAAGGAGCAGCTTCGGCAAGGTTATGTTAGCAGAGAAGAAGGGTACC
1201 -----+-----+-----+-----+-----+-----+ 1260
      GATAGTCACGAACCTTTCCGTCGAAGCCGTTCCAATACAATCGTCTCTTCTTCCCATGG

a      L S V L G K G S F G K V M L A E K K G T -

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Figure A3: (continued)

**Figure A3: (continued)**

**Figure A3: (continued)**

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1681 CCAGACTACATCGCTCCCGAGATTGTAGCATATCAGCCATATGGCAAGGC'TGTTGACTGG
-----+-----+-----+-----+-----+-----+-----+
GGTCTGATGTAGCGAGGGCTCTAACATCGTATAGTCGGTATACCGTTCCGACAAC'TGACC
a      P D Y I A P E I V A Y Q P Y G K A V D W -

1741 TGGGCCTTTGGTGT'TTTACTGTATGAAATGTTAGCAGGACAGCCTCCATTTGATGGTGAA
-----+-----+-----+-----+-----+-----+-----+
ACCCGGAAACCACAAAATGACATACTTTACAATCGTCCTGTCGGAGGTAAACTACCACTT
a      W A F G V L L Y E M L A G Q P P F D G E -

1801 GATGAAGATGAATTGTTTCAATCTATCATGGAACATGTACCCTCATATCCCAAATCAATG
-----+-----+-----+-----+-----+-----+-----+
CTACTTCTACTTAACAAAGTTAGATAGTACCTTGTACATGGGAGTATAGGGTTTAGTTAC
1860

a      D E D E L F Q S I M E H V P S Y P K S M -

1861 TCAAGAGAATCTGTGACTATGTGCAAAGGGT'TTTTGACAAAGCATCCTGGTAAGCGGTTA
-----+-----+-----+-----+-----+-----+-----+
AGTTCTCTTAGACACTGATACACGTTTCCCAAAAAC'TGTTTCGTAGGACCATTCGCCAAT
a      S R E S V T M C K G F L T K H P G K R L -

      A
      v
      a
      I
      I
1921 GGAAGTGGACCTACAGGGGAACAGGACATCAGGGAGCATCAATTCTTCCGTCGAATCGAC
-----+-----+-----+-----+-----+-----+-----+
CCTTCACCTGGATGTCCCCCTTGTCTGTAGTCCCTCGTAGTTAAGAAGGCAGCTTAGCTG
1980

a      G S G P T G E Q D I R E H Q F F R R I D -

1981 TGGGAGAAACTAGCTAACAGAGAAATACAGCCTCCGTTTGTGCCGTCAGTGAGAAACCCC
-----+-----+-----+-----+-----+-----+-----+
ACCCTCTTTGATCGATTGTCTCTTTATGTGCGAGGCAAACACGGCAGTCACTCTTTGGGG
2040

a      W E K L A N R E I Q P P F V P S V R N P -

2041 AGAGCTGCTGAAAAC'TTTGACCCCTATTTTCAAAAAATCCCGTGTGCGCTTACCCCGACC
-----+-----+-----+-----+-----+-----+-----+
TCTCGACGACTTTTGAAACTGGGGATAAAGTGTTTTTAGGGCACACGCGAATGGGGCTGG
2100

a      R A A E N F D P Y F T K I P C A L T P T -

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Figure A3: (continued)

E  
C  
O  
R  
I

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2101  GACAAACTCATTATCATGAATATTCAGGACGAATTCCAAGGCTTTACCTTCGTGAACGAG 2160
      -----+-----+-----+-----+-----+-----+
      CTGTTTGAGTAATAGTACTTATAAGTCCTGCTTAAAGGTTCCGAAATGGAAGCACTTGCTC

a      D K L I I M N I Q D E F Q G F T F V N E -

      GTCTTCGATGGCTATGTGCGTAGTTTGCCAGCTAGTGCCAAGTTGTAGCTCCCACCGT
2161  -----+-----+-----+-----+-----+ 2220
      CAGAAGCTACCGATACACGCATCAAACGGGTCGATCACGGTTCAAACATCGAGGGTGGCA

a      V F D G Y V R S L P S * C Q V C S S H R -

      CGCTGTTTCCTACGCCTACTTGGTGTTCCTCAACGTATCCAAAATGTCACCAGTTTATACAT
2221  -----+-----+-----+-----+-----+ 2280
      GCGACAAGGATGCGGATGAACCACAAGGGTTGCATAGGTTTACAGTGGTCAAATATGTA

a      R C S Y A Y L V F P T Y P K C H Q F I H -

      GTGCTGTCTTTTTTCTTTCTTTTGTACTTGTATGTGTACATAGTACATGTAGGTAGTAG
2281  -----+-----+-----+-----+-----+ 2340
      CACGACAGGAAAAAGAAAGAAAAACAATGAACATACACATGTATCATGTACATCCATCATC

a      V L S F F F L F V T * C V H S T C R * * -

      AAGTTGGATGTAGTCATGAGTGCCTGAAGACAGTGAATCGGTACATTAATTTGTTTGAAG
2341  -----+-----+-----+-----+-----+ 2400
      TTCAACCTACATCAGTACTCACGGACTTCTGTCTACTTAGCCATGTAATTAAACAACTTC

a      K L D V V M S A * R Q * I G T L I C L K -

      TTTTAGCATTACAGAGGAATAGTGAATGAATAGCTAGCATATAATGGATCTGTGATTAGT
2401  -----+-----+-----+-----+-----+ 2460
      AAAACGTAATGTCTCCTTATCACTTACTTATCGATCGTATATTACCTAGACACTAATCA

a      F * H Y R G I V N E * L A Y N G S V I S -

      CAAGAATGTCGTAC
2461  -----+----- 2474
      GTTCTTACAGCATG

a      Q E C R -

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Enzymes that do cut:

AvaII    BstXI    EcoRI    KpnI    PvuII

Enzymes that do not cut:

BamHI    HindIII    PstI    SacI    SalI    SmaI    XhoI

**Figure A3:(continued)**