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Genetic and Molecular Analyses of *Pkc53E* in *Drosophila melanogaster*

by

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ABSTRACT

Protein kinase C (PKC) is a family of ubiquitous serine-threonine kinases that play pivotal roles in many cellular processes, including growth, division, and differentiation. There are three known members of the *Drosophila melanogaster* PKC gene family, each with distinct tissue- and developmental stage-specific expression. One of these genes, *Pkc53E*, encodes at least four transcripts. The powerful genetic and molecular tools available for the study of *D. melanogaster* gene function have enabled the systematic description of several signal transduction molecules, many of which are highly conserved from *Drosophila* to humans. This dissertation describes genetic and molecular characterizations of the *D. melanogaster Pkc53E* gene.

A PCR-based *P* element screen of more than 118,000 flies did not detect any mutations at the *Pkc53E* locus. Several explanations for the inability to isolate a *Pkc53E* mutant, including non-permissive gene structure and dominant mutant lethality, are discussed.

RNAse protection analysis (RPA), reverse transcription-polymerase chain reaction (RT-PCR), and rapid amplification of cDNA ends (RACE) were used to elucidate *Pkc53E* transcript structures. RPA indicated that transcript structure was more complex in adult heads than adult ovaries or testes. However, the more sensitive RT-PCR and RACE techniques suggested that the level of transcript complexity was maintained, at least in ovaries. Further, the differences in transcript structure occur in the 5′ untranslated regions of the transcripts. Evidence is presented supporting differential polyadenylation of *Pkc53E* transcripts and the inclusion of both novel and previously designated intronic sequences within transcripts. Possible roles for these transcripts are discussed. The molecular results are summarized and predictions about the structure of the *Pkc53E* transcripts are made.

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LIST OF ABBREVIATIONS

A_{600}	absorbance at 600 nm
AAP	abriged adapter primer
AKAP	A kinase anchoring protein
AP	adapter primer
aPKC	atypical PKC
Arf	ADP-ribosylation factor
ATP	adenosine 5′-triphosphate
β-PDGFR	β-platelet derived growth factor receptor
°C	degrees Celcius
C1, C2, C3, C4	conserved PKC domains
Ca^{2+}	calcium
CAPP	ceramide-activated protein phosphatase
cDNA	complementary DNA
cGMP	cyclic guanosine 5′-monophosphate
CI	24:1 chloroform:isoamyl alcohol
CIAP	calf intestinal alkaline phosphatase
cPKC	classical PKC
cpm	counts per minute
CRD	cysteine-rich domain
CTP	cytidine 5′-triphosphate
dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytidine 5′-triphosphate
DEPC	diethyl pyrocarbonate
DG	diacylglycerol
dGTP	2′-deoxyguanosine 5′-triphosphate
DIG	digoxigenin
DNAse	deoxyribonuclease
dNTP	2′-deoxynucleotide 5′-triphosphate
Dr	*Drop*
dTAP	oligo$(dT)_{17}$-adapter primer
DTT	dithiothreitol
dTTP	2′-deoxythymidine 5′-triphosphate
EGF	epidermal growth factor
ERK	extracellular-signal regulated kinases
FcεRI	IgE receptor found on mast cells and basophils
FFAs	free *cis*-unsaturated fatty acids
g	gram(s)
GCG	Genetics Computer Group
GM-CSF	granulocyte-macrophage colony-stimulating factor
GS	1,3-β-glucan synthase
GSK-3	glycogen synthase kinase-3
GSP	gene-specific primer

GTP	guanosine 5′-triphosphate
I-κB	inhibitor-κB
IL	interleukin
IP_3	inositol-3,4,5-triphosphate
kb	kilobase(s)
kbp	kilobase pair(s)
kDa	kilodalton(s)
LTP	long-term potentiation
LysoPC	lysophosphatidylcholine
M	molar
MAPK	mitogen-activated protein kinases
MARCKS	myristoylated alanine-rich C-kinase substrate
μCi	microCurrie(s)
mCi	milliCurrie(s)
MCS	multiple cloning site
MDBK	Mandin Darby bovine kidney
MEK	MAP or ERK kinase
MEKK	MAP or ERK kinase kinase
μF	microFarad(s)
μg	microgram(s)
mg	milligram(s)
μl	microlitre(s)
ml	millilitre(s)
mm	millimetre(s)
μM	micromolar
mM	millimolar
mmol	millimole(s)
N	normal
NF-κB	nuclear factor-κB
NF-AT	nuclear factor of activated T cells
norpA	*no receptor potential A*
nPKC	novel PKC
nt	nucleotide(s)
Ω	Ohm(s)
ORF	open reading frame
ΨPKCζ	PKCζ pseudogene
p110α	PI3-kinase catalytic domain
p85α	PI3-kinase regulatory domain
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidyl choline
PCI	25:24:1 buffer-saturated phenol:chloroform:isoamyl alcohol
PCR	polymerase chain reaction

PDGF-B	platelet derived growth factor B
PEG	polyethylene glycol
PI	phosphatidyl inositol
PI3-kinase	phosphatidyl inositol 3-kinase
PICK	proteins that interact with C-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidyl inositol-3,4,5-trisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKC_i	PKC inhibitor pseudo-substrate peptide
PKD	protein kinase D
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
$polyA^+$	polyadenine
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PS	phosphatidylserine
PSD	phosphorylation site domain
RACE	rapid amplification of cDNA ends
RACK	receptor of activated C-kinase
rdgB	*retinal degeneration B*
RNAse	ribonuclease
rp49	ribosomal protein 49
RPA	RNAse protection analysis
RT-PCR	reverse transcription-PCR
ry	*rosy*
Sb	*Stubble*
T_A	annealing temperature
TGF-α	transforming growth factor-α
T_m	melting temperature (*ie.* temperature at which ½ of primers anneal)
TMS	Third Multiple Singson
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
TRE	TPA-responsive element
trp	*transient receptor potential*
tur	*turnip*
U	unit(s)
UAS	upstream activating sequence
UTP	uridine 5′-triphosphate
UTR	untranslated region
v/v	volume/volume
V	volt(s)

V1, V2, V3, V4, V5	variable PKC domains
w	*white*
w/v	weight/volume
Wg	Wingless
x *g*	times gravity
y	*yellow*

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

I. INTRODUCTION

Signal transduction, the process by which a cell transmits information about its external environment to the nucleus, has been a subject of intense study for several years. The importance of this process is evidenced by the realization that most, if not all, known proto-oncogenes represent proteins involved in signal transduction pathways at all levels: ligands, receptors, adaptors, transducers, and effectors (Hug and Sarre, 1993). In general, signal transduction involves ligand binding to its receptor, thereby triggering a cascade of events, often involving the production of second messengers and the phosphorylation and/or dephosphorylation of one or more transducer proteins. However, receptors with inherent kinase activity have been identified and these receptors do not usually produce second messengers, but instead utilize various adaptor proteins to mediate protein-protein interactions to initiate the signal transduction cascade (Erpel and Courtneidge, 1995; Malarkey *et al.*, 1995). The consequence of most signal transduction events is a change in cellular behaviour mediated by changes in gene expression. Of course, signals may also be transduced from inside cells to the outside, as with hormone or neurotransmitter release (Fashena and Zinn, 1995; Südhof, 1995).

The ability of a cell to coordinate its response to multiple external cues involves the cross-talk between different signal transduction pathways (Bygrave and Roberts, 1995; Nishizuka, 1995; Szamel and Resch, 1995). Examples of this cross-talk include, but not are restricted to, (1) sharing limiting components between pathways; (2) activation of multiple effectors by a single ligand-receptor interaction; (3) differential responses to varying levels of second messengers; and (4) differential responses of transcription factors to phosphorylation and/or dephosphorylation by multiple kinases and/or phosphatases. The ligands, receptors, and nuclear effectors (transcription factors) involved in signal transduction are relatively well-characterized compared to the molecules involved in the transduction of the signal from the cell surface to the nucleus. Among these transducers are the mitogen-activated protein kinases (MAPKs, also known as extracellular-signal regulated kinases, or ERKs) and their cognate protein kinase activators (Cobb and Goldsmith, 1995;

Winston and Hunter, 1996), other serine-threonine kinases (Inagaki *et al.*, 1994; Newton, 1996b), receptor and non-receptor tyrosine kinases (Erpel and Courtneidge, 1995; Malarkey *et al.*, 1995; Taniguchi, 1995), phospholipid-metabolizing enzymes (Majerus, 1996; Nakamura *et al.*, 1993; Nishizuka, 1995), receptor and non-receptor phosphatases (Fashena and Zinn, 1995; Inagaki *et al.*, 1994), and GTP-binding proteins and exchange factors (Burgering and Bos, 1995; Coleman and Sprang, 1996; Daum *et al.*, 1994; Symons, 1996). It has become clear that isoenzyme families are common with signal transduction molecules (Burgering and Bos, 1995; Daum *et al.*, 1994; Dekker and Parker, 1994; Erpel and Courtneidge, 1995; Symons, 1996; Winston and Hunter, 1996). Among these are the family of protein kinases C (PKCs), ubiquitous serine-threonine kinases involved in several cellular events, including growth, division, and differentiation (Buchner, 1995; Newton, 1996b; Szamel and Resch, 1995). The following is a brief overview of the family of mammalian PKCs and the lessons we have learned from the study of PKCs in yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster*. Next, I give a précis of some of the tools used for the research outlined in this thesis. Together this sets the stage for the continued study of *Pkc53E* of *Drosophila melanogaster*.

II. Protein Kinase C

A. Mammalian PKC: An Enzyme Family

Attention initially focussed on PKCs after they were identified as major cellular receptors for tumour promoting phorbol esters such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and phorbol 12-myristate 13-acetate (PMA). The mammalian PKCs are a family of at least 11 different isoenzymes encoded by 10 different genes. These isoenzymes can be classified into four groups, based on their structures and activator requirements. The classical PKCs (cPKCs) include the phosphatidylserine (PS)-, diacylglycerol (DG)-, and Ca^{2+}-dependent PKCs α, β_I, β_{II}, and γ. The novel PKCs (nPKCs) are also PS- and DG-dependent, but Ca^{2+}-independent, and include PKCs δ, ϵ, η, and θ. The atypical PKCs (aPKCs), PKCs ζ and λ/ι, are both DG- and Ca^{2+}-independent, but can be activated by some membrane phospholipids (Nishizuka, 1995). PKCμ is the prototype of a fourth group

of PKC isoenzymes with unusual subcellular distribution and inhibitor sensitivities (Johannes *et al.*, 1994; 1995). Some of the structural characteristics, regulation, and functions of PKCs are discussed below.

1. PKC Structure

The diversity within the mammalian PKC family arises from the transcription of different PKC genes, as well as alternative splicing of PKCβ pre-mRNA (Coussens *et al.*, 1986; 1987; Knopf *et al.*, 1986; Ohno *et al.*, 1987). This alternative splicing generates messages that differ in the 3′ coding and untranslated regions (UTRs) of β_I and β_{II} mRNA. These differences result in amino acid sequences that diverge in the final 50 (β_I) or 52 (β_{II}) amino acids. The 3′ UTR of β_I mRNA is approximately 333 nucleotides (nt) longer than that of β_{II} mRNA (Coussens *et al.*, 1987). No consensus polyadenylation signal precedes the site of polyadenine (polyA$^+$) addition in β_I mRNA. This may cause a reduction in polyadenylation efficiency, resulting in reduced processing of the β_I pre-mRNA and/or reduced transcript stability (Coussens *et al.*, 1986; 1987). The expression of the PKCβ transcripts is regulated at the level of alternative splicing in response to developmental cues in haematopoesis (Desdouits *et al.*, 1996) and in response to insulin in BC3H-1 myocytes (Lehel *et al.*, 1995a).

The carboxyl terminal 50/52 amino acids of PKCs β_I and β_{II} share approximately 45% sequence similarity along their length (Coussens *et al.*, 1987). However, these domains appear to define domains for isoenzyme-specific protein-protein interactions (Blobe *et al.*, 1996). The carboxyl terminus of PKCβ_{II} contains an actin binding site not found in PKCβ_I. Interaction *in vitro* between PKCβ_{II} and actin significantly increased PKCβ_{II} autophosphorylation and altered its substrate specificity. Further, upon PMA-treatment of several cell lines (Molt-4 T-lymphoblastoid cells, K562 erythroleukemia cells, and BJA-B, B-lymphoblastoid cells) PKCβ_{II} translocated to the microfilament-based cytoskeleton where it was protected from down-regulation.

Multiple PKCζ-hybridizing transcripts have also been identified. Andrea and Walsh (1995) identified a transcriptionally active PKCζ pseudogene (ΨPKCζ) that encodes two brain-specific transcripts of 2.5 and 4.7 kb. However, these transcripts lack an initiating

methionine codon so are translationally inactive. The authentic PKCζ transcript in rat brain is 3.1 kb with 2.7 and 4.7 kb transcripts in other tissues. The latter two transcripts are thought to arise from alternative polyadenylation. Recently, a novel 1.75 kb PKCζ-related transcript has been identified in rat hearts and brains. The authors speculated that the ΨPKCζ transcripts may have an as yet unidentified regulatory role (Andrea and Walsh, 1995).

Figure 1.1 depicts a schematic comparison of PKCs and their known domain structures. All PKCs have an amino terminal regulatory domain and a carboxyl terminal catalytic domain separated by variable domain 3 (V3). The prototypical cPKCs have four conserved domains (C1-C4) interspersed among five variable domains (V1-V5). The C1 domain usually contains a pseudo-substrate domain and one or two cysteine-rich domains (CRDs; Newton, 1996b). Each CRD binds two atoms of zinc and, in DG-dependent PKCs, these are the sites of phorbol ester and DG binding (Hommel *et al.*, 1994; Hubbard *et al.*, 1991; Quest *et al.*, 1994). Studies with PKCα demonstrated the presence of both high and low affinity activator binding sites. The first CRD binds PKC activators with affinities in the order DG > TPA > bryostatin I, with the affinities reversed for the second CRD. This helps explain why coactivation of PKC with DG and TPA is greater than either activator alone and why bryostatin I partially inhibits activation induced by phorbol ester (Slater *et al.*, 1996). PKCs γ and δ have a similar non-equivalence between their CRDs (Quest and Bell, 1994; Szallasi *et al.*, 1996). Furthermore, it appears that phorbol esters increase the affinity of PKC for membranes to a much greater extent than DG (Newton, 1996b, data in press). Binding of phorbol esters or DG to the CRD of PKCζ has not been detected. Site-directed mutagenesis of this CRD to match the putative DG-binding motif of cPKCs does not result in DG binding, showing that residues outside the recognized motif play role(s) in activator binding (Kazanietz *et al.*, 1994).

CRDs may also play a role in the subcellular localization of PKCε. Fragments of PKCε containing only the CRDs localize to the Golgi apparatus in NIH3T3 mouse fibroblasts, whereas the CRDs from PKCα or PKCβ do not (Lehel *et al.*, 1995a). When these PKCε fragments also contain the hinge region or the N-terminal 33 amino acids

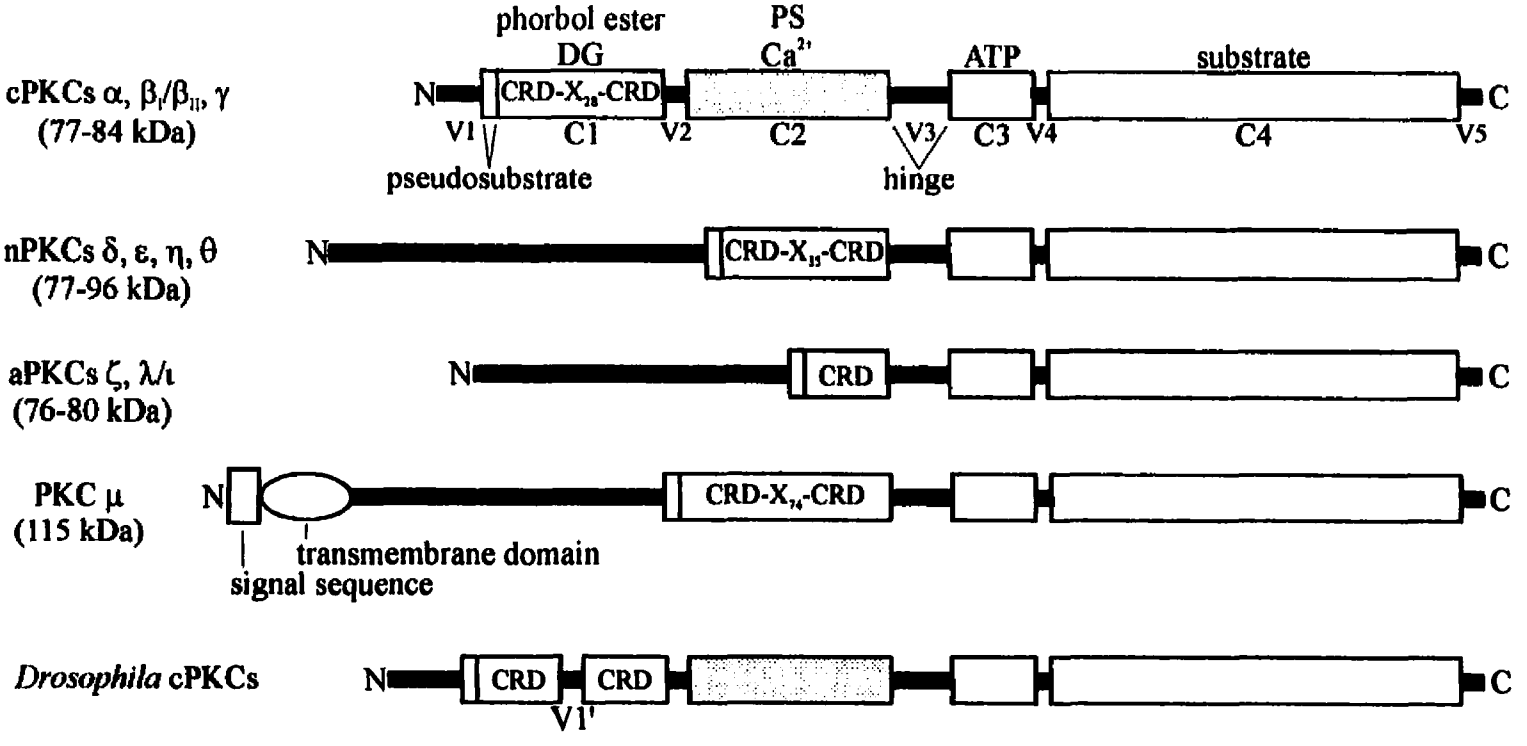

FIGURE 1.1. Schematic representation of mammalian PKC structures and the *Drosophila* cPKCs, *Pkc53E* and *inaC*. The amino terminal regulatory region contains the pseudo-substrate domain, the C1 domain and the C2 domain. C1 is comprised of one or two CRDs, each of which binds two atoms of zinc, and together bind DG and phorbol esters. The C2 domain confers Ca^{2+}-specificity for divalent cation binding. This also appears to be the site of PS binding. The hinge region is the site of proteolytic degradation during down-regulation. The catalytic domain contains the ATP-binding lobe (C3) and the substrate-binding lobe (C4). Variable regions are shown in black (▬). The variable regions are thought to confer different catalytic potential and intracellular localizations to the different isoenzymes.

The predicted amino acid sequences of *Pkc53E* and *inaC* classifies them as cPKCs. *Drosophila* PKCs contain a V1' domain where there is increased variability between the two CRDs as compared to mammalian PKCs.

Not to scale. See text for details. (Figure modified from Newton, 1996b.)

including the pseudo-substrate domain, the fragments localize to the plasma membrane (Lehel *et al.*, 1995a; b).

In vitro studies of Golgi secretory function has implicated PKC in the regulation of Golgi vesicle formation (Simon *et al.*, 1996b). However, this appears to be independent of kinase activity (Simon *et al.*, 1996a). Overexpression of either holo-PKCϵ or PKCϵ CRD fragments inhibit Golgi glycosaminoglycan secretion and protein sulfation (Lehel *et al.*, 1995a). PKCμ is localized to the Golgi apparatus in HepG2 cells and when overexpressed in COS and HeLa cell transfectants (Prestle *et al.*, 1996). Exogenous expression of PKCμ enhances glycosaminoglycan secretion and protein sulfation (Prestle *et al.*, 1996), implicating PKCs ϵ and μ as antagonistic regulators of secretory processes.

The C2 domain, which is present in cPKCs, but absent from other PKCs, is the site of Ca^{2+} binding. The amino terminus of the C2 domain contains residues that confer specificity for Ca^{2+}. When these residues are missing, Mn^{2+} and Mg^{2+} can substitute for Ca^{2+} (Luo and Weinstein, 1993). C2 also appears to be the site of PS binding (Newton, 1996b). A pseudo-receptor of activated C-kinase (pseudo-RACK1) site, important in PKC regulation and subcellular localization, is present in the middle of the C2 region in cPKCs and within the regulatory region of other PKCs (Ron *et al.*, 1994; see below).

The nPKCs contain a C2-like (or E2) domain (Sossin and Schwartz, 1993). This domain has the conserved residues that maintain the fold of the domain, but most of the coordinating oxygens in the Ca^{2+}-binding site are absent (Newton, 1996b).

All PKCs have both the C3 domain, which contains the ATP-binding motif, and the C4 domain, which contains the rest of the conserved catalytic motifs. However, the catalytic domain of PKCμ more closely resembles the catalytic domains of cAMP-dependent protein kinases (PKAs) with respect to inhibitor sensitivity (Johannes *et al.*, 1995). PKCμ is also unique in that it contains an amino terminal signal peptide that is cleaved from the mature kinase leaving an amino terminal transmembrane domain, suggesting that PKCμ is constitutively associated with membranous structures. Furthermore, PKCμ also lacks the pseudo-substrate domain characteristic of the other PKC isoenzymes (Johannes *et al.*, 1994).

V3 comprises the hinge region of PKC. Proteolytic cleavage of PKC at this site by calpain I or trypsin results in the release of a cofactor/coactivator independent catalytic fragment. It has been suggested that cleavage at this site is the first step in PKC down-regulation (Parker *et al.*, 1995; and see below). Definitive roles for the other variable domains have not yet been reported. It has been suggested that these sequences confer substrate-specificity and/or isoenzyme-specific functions to PKCs. However, chimeric $\alpha^{Reg}/\beta_{II}^{Cat}$ or $\beta_{II}^{Reg}/\alpha^{Cat}$ PKCs possess the isoenzyme-specific functions of their respective catalytic domains, at least in K562 cells (Walker *et al.*, 1995). This suggests that V1 and V2 are dispensable for some isoenzyme-specific functions.

2. PKC Regulation

Biochemical studies of PKC both *in vitro* and *in situ*, using mammalian cell culture, have demonstrated that PKC activity is controlled in at least two ways. PKC is rendered catalytically competent by both *trans*- and autophosphorylation events (Keranen *et al.*, 1995; Lee, J.Y. *et al.*, 1996). The availability of Ca^{2+} and/or lipid activators, which have been the subject of several recent reviews (Buchner, 1995; Nakamura *et al.*, 1993; Newton, 1996b; Nishizuka, 1995; Szamel and Resch, 1995), regulates the enzymatic activity of catalytically competent PKCs.

Regulation by phosphorylation: Newton and her colleagues (Dutil *et al.*, 1994; Keranen *et al.*, 1995) have demonstrated that three distinct phosphorylations of $PKC\beta_{II}$ are necessary before the enzyme can respond to second messengers. Newly synthesized $PKC\beta_{II}$ is associated with a detergent insoluble cell fraction. *Trans*-phosphorylation by an as yet unidentified PKC kinase on Thr^{500} in the activation loop renders $PKC\beta_{II}$ catalytically competent and promotes autophosphorylation on Thr^{641} at the top of the catalytic core. A second autophosphorylation on Ser^{660} releases mature $PKC\beta_{II}$ into the cytosol ready to respond to lipid activators. The electrophoretic mobility of $PKC\beta_{II}$ is not affected by Thr^{500} phosphorylation, but sequentially decreases with phosphorylation at Thr^{641} and Ser^{660} (Keranen *et al.*, 1995).

Dephosphorylation of $PKC\beta_{II}$ with protein phosphatase 1 (PP1) removes all three phosphates rendering the enzyme inactivatable. However, protein phosphatase 2A (PP2A)

action leaves the phosphate on Thr^{641} and this enzyme is fully activatable (Dutil *et al.*, 1994; Keranen *et al.*, 1995). In mature $PKC\beta_{II}$, Thr^{500} is only partially phosphorylated. These data imply that phosphorylation on Thr^{641} relieves the requirement for Thr^{500} phosphorylation. Thr^{641} autophosphorylation may play a critical role in anchoring the carboxyl terminus away from the substrate-binding site (Keranen *et al.*, 1995, unpublished observations).

The regulation of PKC by dephosphorylation *in vivo* is suggested by studies of ceramide inactivation of PKCα. Lee *et al.* (1996) demonstrated that in Molt-4 cells ceramide inhibits PKCα by dephosphorylation, possibly through the activation of the PP2A-like ceramide-activated protein phosphatase (CAPP). This ceramide-induced inactivation of PKCα is maintained through PKCα immunoprecipitation, suggesting that it cannot be restored by autophosphorylation. Given the results of Newton and her colleagues with respect to PP1 and PP2A effects on PKC phosphorylation, this suggests that CAPP, unlike the PP2As tested by Newton's group, is able to dephosphorylate PKCα at the residue equivalent to Thr^{641}; that PP2As are able to dephosphorylate the residue equivalent to Thr^{641} in PKCα, but not $PKC\beta_{II}$; or that another phosphatase, possibly PP1 or PP1-like, is involved in ceramide-induced inhibition of PKCα. Further studies will be necessary to differentiate between these possibilities.

PKC isoenzyme specificity may be regulated by cross-talk with tyrosine kinase pathways. Tyrosine phosphorylation of PKCδ is rapidly induced in response to the activation of the IgE receptor (FcεRI) found on mast cells and basophils. This phosphorylation only occurs after PKCδ is translocated to the membrane and tyrosine phosphorylation of PKCδ prevents its association with FcεRI. Tyrosine phosphorylated PKCδ showed reduced activity toward FcεRI, but increased activity toward myelin basic protein peptide and histone HIII (Halleem-Smith *et al.*, 1995). In 32D cells (a monocytic cell line) tyrosine phosphorylation of PKCδ occurred in response to TPA and prolonged TPA treatment induced the expression of cell surface molecules indicative of monocytic differentiation (Li *et al.*, 1995). This suggests that tyrosine phosphorylation and activation of PKCδ may play a role in monocyte differentiation.

Regulation by calcium ions: A single Ca^{2+} binding site increases the affinity of cPKCs for negatively charged lipids (reviewed in Newton, 1996b). It has been proposed that Ca^{2+} binding at C2 might clamp together the upper and lower lobes, thereby orienting bulky aromatic and basic amino acids appropriately for membrane lipid interactions. In nPKCs, the C2-like domain may already have this closed conformation, thereby conferring Ca^{2+}-independence.

Regulation by lipids: The regulation of PKCs through the receptor-mediated breakdown of membrane lipids has been intensively studied and is the subject of several recent reviews (Buchner, 1995; Nakamura *et al.*, 1993; Nishizuka, 1995; Szamel and Resch, 1995; Figure 1.2). One mechanism of PKC activation is the activation of phospholipase C (PLC) by the binding of ligand to a receptor, either directly or through associated trimeric G proteins. PLC mediates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into DG and inositol-3,4,5-triphosphate (IP_3). IP_3 diffuses through the cytoplasm where it mediates the release of Ca^{2+} from internal stores. The increase in Ca^{2+} concentrations increases cPKCs affinity for PS located at the inner surface of the plasma membrane. DG remains in the membrane, where it can activate PKC that translocates to the membrane and/or is localized to the membrane through protein-protein interactions (see below) or by transmembrane domains (PKCμ).

The DG produced through the activation of PLC rapidly disappears due to the activity of DG kinase in phosphatidyl inositol (PI) turnover and therefore can support only transient PKC activation. The level of DG, however, often increases again with relatively slow onset. Analysis of the fatty acid composition of this DG indicates that it is derived from phosphatidyl choline (PC). Phospholipase D (PLD) hydrolyzes PC into phosphatidic acid (PA) and choline. PA is rapidly converted to DG by a phosphomonoesterase. Because of the difference in fatty acid composition, the DG derived from PC is not a good substrate for DG kinase and is more slowly degraded by DG lipase. The second DG wave may take part in the sustained activation of PKC that is essential for long-term cellular responses such as growth and differentiation (Nishizuka, 1995).

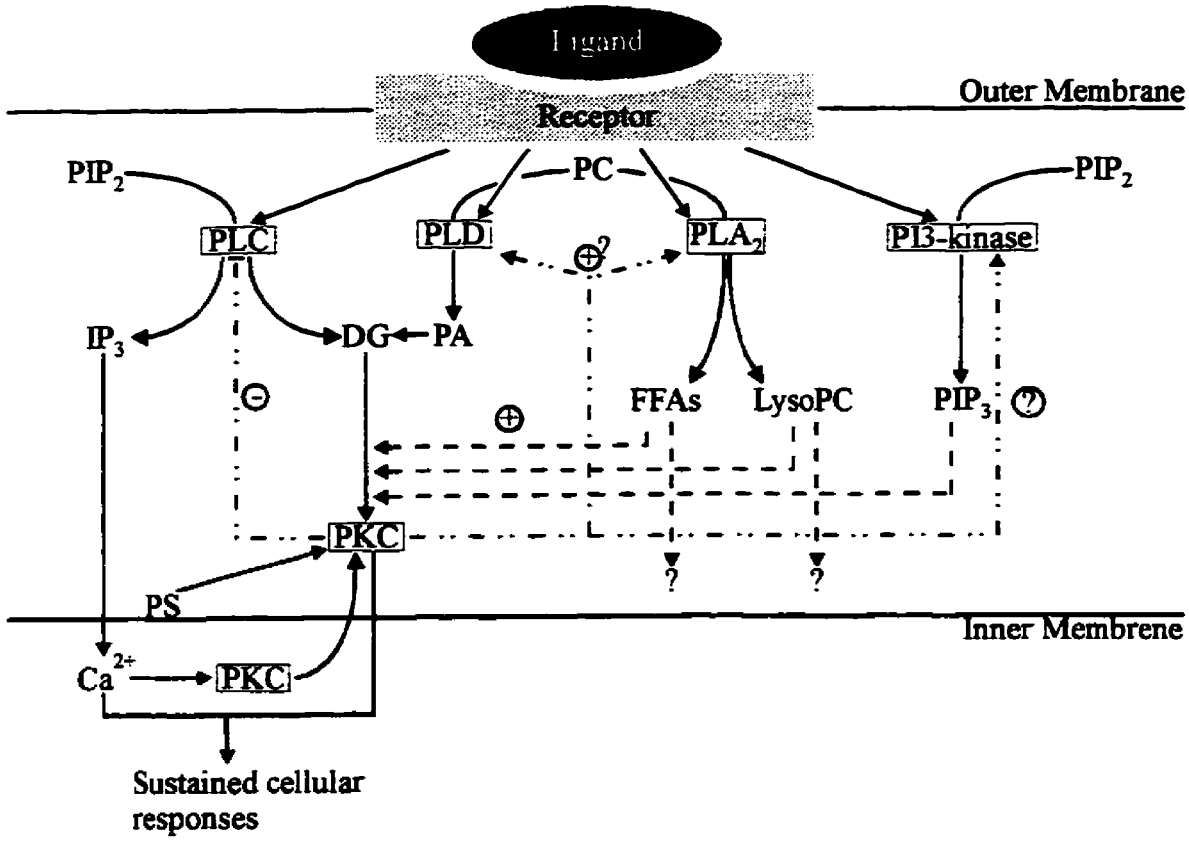

FIGURE 1.2. Schematic representation of PKC activation. Receptor activation by bound ligand activates PLCs and/or PI3-kinase. The archetypical PKC activation involves PLC-mediated hydrolysis of PIP_2 into IP_3 and DG. IP_3 diffuses into the cytosol where it effects the release of Ca^{2+} from internal stores. DG remains in the membrane where it activates membrane translocated DG-dependent PKCs. A second, more sustained DG wave is elicited by the PLD-mediated hydrolysis of PC. PLA_2 and PI3-kinase produce other lipids that can potentiate the DG-dependent PKC responses, or activate the aPKCs. See text for further details. (Figure modified from Nishizuka, 1995.)

Work from several laboratories has suggested that cPKCs have a positive regulatory role in the activation of PLD (Lopez *et al.*, 1995; Ohguchi *et al.*, 1996; Singer *et al.*, 1996). This activation of PLD appears to be independent of PKC kinase activity (Singer *et al.*, 1996), and evidence from one group suggests that the presence of ATP is necessary to maintain the level of PIP_2, a necessary cofactor for PLD activity (Ohguchi *et al.*, 1996). It was also shown that RhoA-GTP (a small GTP-binding protein) (Ohguchi *et al.*, 1996; Singer *et al.*, 1996) and ADP-ribosylation factor (Arf; Singer *et al.*, 1996) acted synergistically with PKC for PLD activation. These data suggest a mechanism for a positive regulatory feedback loop during PKC activation.

Activation of phospholipase A_2 (PLA_2) results in the hydrolysis of PC into free *cis*-unsaturated fatty acids (FFAs) and lysophosphatidylcholine (LysoPC). Both these molecules have been shown to potentiate cellular responses elicited by DG and TPA, most likely through PKC. While the mechanisms of PLA_2 activation have not been clearly determined, it has been shown that FFA release is affected by PKC activators and inhibitors and the presence of GTP-γ-S, suggesting the involvement of both PKC and G proteins (Nishizuka, 1995).

The production of DG in response to cellular stimuli can account for the activation of DG-responsive PKCs, but the aPKCs have been shown to be DG-unresponsive. Studies in rat 3Y1 fibroblasts, HepG2 cells, and COS1 cells have provided insight into the mechanism of aPKC activation (Akimoto *et al.*, 1996). Whereas PKCs α, δ, or ∈ enhance TPA-stimulated activation of TPA-responsive element (TRE)-reporter constructs, PKCs ζ and λ mediate TRE-reporter expression in response to serum, epidermal growth factor (EGF), and transforming growth factor-α (TGF-α). EGF causes a transient rise in phosphatidyl inositol-3,4,5-trisphosphate (PIP_3) levels. PIP_3 can be produced by the phosphorylation of PIP_2 by phosphatidyl inositol 3-kinase (PI3-kinase). Studies using the overexpression of PI3-kinase catalytic (p110α) or regulatory (p85α) domains, PI3-kinase inhibitors, and β-platelet derived growth factor receptors (βPDGRs) deficient in PI3-kinase, but not PLCγ, activation, demonstrated that both PKCλ- and non-PKCλ-mediated TRE activation depended on PI3-kinase activity. EGF-dependent phosphorylation of PKCλ and

translocation of PKCλ from and within the nucleus also requires PI3-kinase activation. This suggested that PI3-kinase, perhaps through PIP_3 production or through the activation of an intermediary protein, can activate PKCλ. PI3-kinase activation and PIP_3 production are also implicated in PKCζ activation (Nakanishi *et al.*, 1993).

Another regulatory feedback loop of PKC may involve the regulation of PI3-kinase. PKCs δ and ϵ co-immunoprecipitated with PI3-kinase when expressed in TF-1 erythroleukemia cells, but PKCs α, β, μ, ζ, and θ did not. Granulocyte-macrophage colony-stimulating factor (GM-CSF), and to a lesser extent interleukin (IL)-5, but not IL-3, increased PKCδ-PI3-kinase association, but not that of PKCϵ and PI3-kinase. This increased association correlated well with increased PI3-kinase activity (Ettinger *et al.*, 1996). FcϵRI cross-linking induced rapid tyrosine phosphorylation of PKCδ (Halleem-Smith *et al.*, 1995; discussed above). However, no PI3-kinase was found in immunoprecipitates of PKCδ from FcϵRI activated RBL-2H3 cells, suggesting that PKCδ-PI3-kinase association is independent of the SH2 domains in PI3-kinase p85α and of PKCδ tyrosine phosphorylation. The role of PKCδ-PI3-kinase association is not clear, but may be distinct from mitogenic functions, and related to differentiation mediated by GM-CSF and IL-5, effects distinct from those of IL-3 (Ettinger *et al.*, 1996).

Regulation through subcellular localization: PKCs are differentially expressed within and among a variety of tissues and cell types (*eg.* Goodnight *et al.*, 1995; also see Buchner, 1995; Inagaki *et al.*, 1994; Mochly-Rosen, 1995; Nishizuka, 1995, for reviews). The biological functions of PKCs likely depend upon not only receptor activation, but also on the appropriate co-expression and co-localization of both PKC isoenzymes and PKC substrates. Further, the localized production of lipid activators may also affect PKC function within cells. For example, a separate lipid cycle within the nucleus has been described and there are reports of differential Ca^{2+} level regulation between the cytosol and nucleus (Buchner, 1995; Divecha *et al.*, 1993, for reviews). Substrate and some other binding proteins are thought to position inactive PKC at subcellular locations, with PKC activation releasing the kinase. Other binding proteins, such as RACKs, may localize the activated PKC (Newton, 1996a, see below).

Mochly-Rosen and her colleagues (Ron and Mochly-Rosen, 1995) identified three specific PKC binding proteins termed RACKs. Isolation and characterization of RACK1 revealed a 36 kilodalton (kDa) protein that is a homologue of the β subunits of G proteins (Mochly-Rosen *et al.*, 1991; Ron *et al.*, 1994). Full-length RACK1 is neither a substrate nor an inhibitor of PKC. However, a RACK1-derived peptide, rVI, blocks PKC-RACK1 binding and activates PKCβ autophosphorylation and histone phosphorylation in the absence of PKC activators *in vitro*, while inhibiting PKCβ function *in vivo* (Ron and Mochly-Rosen, 1994; Ron *et al.*, 1995). A region similar to rVI is found in PKCs. The effects of this peptide suggest that in the PKC enzyme this sequence functions as a pseudo-RACK1 site, conferring another level of regulation on PKC enzymes. In inactive PKC, the pseudo-RACK1 site in the regulatory domain binds to the PKC RACK1-binding site, contributing to the inactive conformation of PKC previously attributed to pseudo-substrate-substrate binding site interaction. Upon activation of PKC, the enzyme unfolds making the RACK1-binding site available for RACK1 binding. The ability of rVI peptide to activate PKCβ *in vitro* was ascribed to disruption of the folded conformation of PKC by interfering with pseudo-RACK1-RACK1-binding site interactions (Ron and Mochly-Rosen, 1995). Inhibition of PKCβ functions *in vivo* was attributed to the mislocalization of PKCβ (Ron *et al.*, 1995). PKCs δ and ∈ also bound to the pseudo-RACK1 peptide, though to a lesser extent than PKCβ, suggesting that RACK1 may not be entirely isoenzyme specific (Ron *et al.*, 1994).

Staudinger *et al.* (1995) identified five proteins that interact with C-kinase (PICKs) using the yeast two-hybrid system with the bovine PKCα catalytic domain as bait and a T cell complementary DNA (cDNA) library. Characterization of one of these, PICK1, identified a 55 kDa perinuclear protein that acted as a PKC substrate and is bound by PKC regardless of phosphorylation state in COS cells. Sequence analysis of PICK1 revealed an ATP/GTP binding motif and an acidic carboxyl terminus, however the functionality of these domains was not evaluated.

Liao *et al.* (1994) used a blot overlay assay that identified several proteins that bound to the pseudo-substrate and other C domains of PKCα. Many of these proteins also

bound PS, suggesting that many PKC binding proteins also bind PS. Mochly-Rosen and her colleagues used the PS-binding annexin proteins to demonstrate that not all proteins that bind PS also bind PKC and that the presence of PS may alter the conformation of PKC thereby revealing sites for protein-protein interaction (Mochly-Rosen *et al.*, 1991).

In yeast, Ste5p acts as a scaffold protein, co-localizing the kinases of the pheromone response MAPK signal transduction cascade, enabling their efficient sequential phosphorylation and activation (Herskowitz, 1995). The PKA anchoring proteins (AKAPs or A kinase anchoring proteins) seem to function as scaffold proteins in mammalian cells (Newton, 1996a). Klauck *et al.* (1996) demonstrated that AKAP79 binds to PKC, PKA, and calcineurin, localizing these proteins at post-synaptic densities. The authors suggested that this localization positions these kinases and phosphatase for rapid substrate reactions upon nerve stimulation and possibly for regulatory interactions. Alexandra Newton (1996a) reports unpublished observations that calcineurin dephosphorylates PKC at one of the carboxyl terminal sites that regulate its function, suggesting that calcineurin might provide a rapid "off-switch" for PKC.

Down-regulation of PKC: One consequence of phorbol ester or sustained-DG long-term activation of PKCs is their proteolytic degradation (reviewed by Parker *et al.*, 1995). The initial step in this degradation, at least for some isoenzymes, is the exposure of the hinge (V3) region upon activation. The mechanisms responsible for this down-regulation have not been fully characterized. Three protease-sensitive sites have been identified in PKCε by expressing different portions of the PKCε protein in NIH3T3 cells (Lehel *et al.*, 1995b). One was the previously identified hinge region. Proteolysis at this site releases a catalytic domain free from activator dependence. Another site was identified between the ATP-binding site and the catalytic fold. It was suggested that proteolysis at this site could inactivate the catalytic domain, preventing uncontrolled PKC activity. A final site was identified between the pseudo-substrate domain and the CRDs. Proteolysis at this site was proposed to disrupt the ability of PKCε to resume its folded conformation, thereby exposing the other protease-sensitive sites.

Increased vesicle trafficking appears to correlate with the ability of cells to mediate the down-regulation of PKC. For example, the expression of a kinase-negative PKCδ in *Schizosaccharomyces pombe* did not induce increased vesicular traffic nor induce PKC down-regulation. However, co-expression of the kinase-negative PKCδ with wild-type PKCδ both increased vesicular traffic and restored down-regulation of both kinases (Goode *et al.*, 1995).

When overexpressed in an R6 rat embryo fibroblast cell line, PKCβ is resistant to TPA-induced down-regulation. Furthermore, in this context, PKCβ protected PKCϵ from normal down-regulation, whereas PKCα overexpression did not (Borner *et al.*, 1995). The mechanism by which PKCβ down-regulation is prevented is not known, but may be due to increased PKCβ synthesis, possibly since the PKCβ promoter is TPA responsive (Obeid *et al.*, 1992). The work of Blobe *et al.* (1996) shows that PKCβ_{II} translocated to the cytoskeleton is protected from down-regulation, whereas PKCs β_I and β_{II} translocated to the membrane are not. These authors suggest that sequestration of PKCβ_{II} protects it from proteolysis.

Recently, Lee *et al.* (1996) demonstrated that PKCα is ubiquinated and degraded by the proteasome following bryostatin I stimulation of LLC-MK_2 epithelial cells. Ubiquination followed bryostatin I-induced dephosphorylation of PKCα. Both dephosphorylation and degradation were prevented by lactacystin, a selective inhibitor of the proteosome.

Cross-talk between signal transduction cascades: Figure 1.3 illustrates some examples of cross-talk between signal transduction cascades. Cross-talk is necessary in order for the cell to coordinate responses to a variety of extracellular signals. The tyrosine phosphorylation of PKCδ and the synergy between RhoA, Arf, and PKC in PI3-kinase activation is discussed above. One pathway to enhance TRE-reporter expression in NIH3T3 mouse fibroblasts is mediated by PKCδ. This PKCδ-enhanced expression is inhibited by dominant negative Ras protein, suggesting that Ras is a downstream mediator of PKCδ-mediated TPA-induced TRE expression (Hirai *et al.*, 1994). PKC involvement in Ras pathways is also indicated by the prevention of $p21^{Ras}$-mediated activation of nuclear

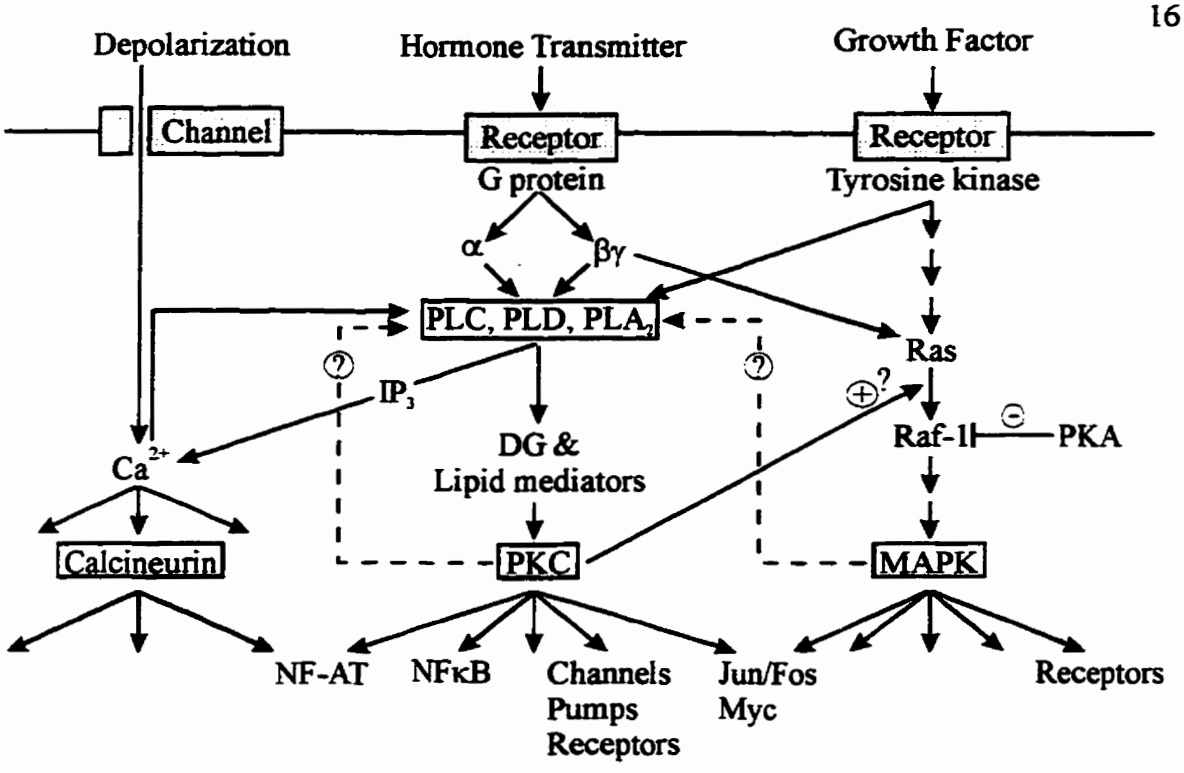

FIGURE 1.3. Schematic representation of cross-talk between different signal transduction pathways. The coordination of cellular responses to a variety of signals is accomplished by interaction, or cross-talk, between signal transduction cascades. This complex network allows a cell to fine-tune gene expression and other cellular behaviours. (See text for further details. Figure modified from Nishizuka, 1995.)

factor-κB (NF-κB) in *Xenopus laevis* oocytes and NIH3T3 fibroblasts by inhibitors of PKCζ (Diaz-Meco *et al.*, 1993; Dominguez *et al.*, 1993).

Cooperation between Ca^{2+}-activated calcineurin and PKC is demonstrated by the activation of nuclear factor of activated T cells (NF-AT). Functional NF-AT consists of a cytoplasmic phosphoprotein $NF\text{-}AT_p$ and a nuclear component, $NF\text{-}AT_n$, which contains the Jun/Fos transcription factors. Dephosphorylation of $NF\text{-}AT_p$ by calcineurin allows translocation of $NF\text{-}AT_p$ to the nucleus where it combines with the newly synthesized $NF\text{-}AT_n$ to produce a functionally active transcription factor. New synthesis of $NF\text{-}AT_n$ is dependent on PKC activation stimulated via T-cell antigen receptor or by phorbol esters (reviewed by Lehel *et al.*, 1995a; Szamel and Resch, 1995).

3. PKC Function

Most work attempting to elucidate the function of mammalian PKCs has used phorbol esters to elicit changes in cellular behaviour and then associates these changes with PKC activity. Unfortunately, results using phorbol esters have many caveats. (1) Phorbol esters are much longer lasting than some of the DG and other lipid second messengers produced by receptor-mediated or channel-mediated activation. Additionally, as mentioned above, phorbol esters do not bind to the same PKC sites, or with the same affinity, as DG. This suggests that PKC activation by phorbol esters may not accurately reflect PKC activation by endogenous second messengers. (2) Several non-PKC proteins have CRDs that bind phorbol esters, *eg. unc-13* of *C. elegans*, n-chimaerin, DG kinase, and protein kinase D (PKD; Ahmed *et al.*, 1991; Kazanietz *et al.*, 1995; Van Lint *et al.*, 1995). This suggests that activation of proteins other than PKC may be responsible for some phorbol ester-induced responses. This is complicated by the recent report that PKD is activated by PKCs ϵ and η, but not PKCζ, in COS-7 cells (Zugaza *et al.*, 1996). (3) TPA treatment does not affect all PKC isoenzymes equally. Studies of Mandin Darby bovine kidney (MDBK) cells showed that TPA treatment caused the activation and translocation of PKCα to the plasma membrane. The same treatment caused the translocation of PKCβ to the nuclear membrane, but did not activate it (Simboli-Campbell *et al.*, 1994). The above illustrates the difficulties with interpretation of phorbol ester-induced changes in cell

behaviour. Nevertheless, the use of phorbol esters has provided much insight into the function of PKCs and in conjunction with other tools for studying PKC action (see examples below) yields convincing evidence for PKC function in cell growth and development.

PKCs-specific inhibitors, anti-PKCs-specific antibodies, and ATP-binding deficient dominant-negative PKC mutants are additional powerful tools for the elucidation of PKC function. In addition, genetic studies with yeast, *Caenorhabditis elegans*, and *D. melanogaster* are rapidly revealing the roles of PKCs in these organisms, and as with several other signal transduction pathways, there is accumulating evidence that these pathways may be conserved in mammals. These biochemical and genetic studies have revealed important roles for PKCs in several cellular processes.

PKC and transcription control: The activation of PKC has be implicated in the transcriptional regulation of several genes. One of PKCs earliest characterized transcriptional effects involved the activation of c-Jun, a component of the AP-1 transcriptional activator. AP-1 binding to the TRE is essential for TPA inducibility. The activation of PKC stimulates the dephosphorylation of residues in the DNA binding domain of c-Jun (Boyle, W.J. *et al.*, 1991). These residues are phosphorylated by glycogen synthase kinase-3 (GSK-3). It appears that the *Drosophila* Wingless (Wg; Wnt oncogene homologue) gene product acts through a PKC to inactivate GSK-3 (Cook *et al.*, 1996). The simplest model would involve PKC phosphorylation and inactivation of GSK-3, however this has not yet been demonstrated. Other intermediates may also be involved.

NF-κB is a transcription factor that is activated in response to several stimuli, including TPA treatment (reviewed in Hill and Treisman, 1995). NF-κB is a heterodimer localized to the cytosol, when inactive, through binding to inhibitor-κB (IκB). Upon activation, IκB dissociates from NF-κB, allowing its translocation to the nucleus, where it serves as a transcriptional activator. Dissociation of IκB from NF-κB has been correlated with phosphorylation of IκB. Hirano *et al.* (1995) showed that the overexpression of PKCε, but not of PKCs α, δ, or θ, activates transcription from a κB-responsive reporter construct. Further, they demonstrated that in response to TPA, neither the PKCε regulatory domain nor a kinase-negative PKCε could activate reporter expression, and in

fact exerted dominant-negative effects on wild-type PKCε with respect to reporter expression. These results suggested that PKCε may be involved in NF-κB activation in response to TPA.

Others have shown that NF-κB is activated in response to TNF-α, insulin, and constitutively active *Bacillus cereus* PC-PLC and that this activation is correlated with *X. laevis* oocyte maturation (Diaz-Meco *et al.*, 1993; Dominguez *et al.*, 1993). Overexpression of PKCζ or the PKCζ catalytic domain increases NF-κB activation in NIH3T3 cells. Further, activation of NF-κB by TNF-α or *B. cereus* PC-PLC is inhibited by the expression of a kinase-negative PKCζ (Diaz-Meco *et al.*, 1993). Using *X. laevis* oocytes, Dominguez *et al.* (1993) demonstrated that PKCζ-specific pseudo-substrate inhibitor peptides, but not cPKCs-specific pseudo-substrate inhibitor peptides, block *B. cereus* PC-PLC-, insulin-, and $p21^{Ras}$-induced NF-κB activation. Taken together these data indicate that PKCs have important roles in TPA-, insulin-, phospholipid-induced, and $p21^{Ras}$-mediated NF-κB activation. Studies with 3T3-L1 adipocytes suggested that PKC may affect Ras signalling through an inhibitory effect on GTPase-activating protein (Schuber *et al.*, 1996).

PKCs have also been implicated in the transcriptional regulation of other TPA-responsive genes. Li *et al.* (1996) have shown that dominant-negative PKCδ can inhibit transformation of NIH3T3 cells by the *sis* proto-oncogene (homologue to PDGF-B). This inhibition of transformation appeared to be the result of inhibition of TPA-responsive gene expression as indicated by decreased TRE-reporter construct, c-*myc*, and c-*fos* expression.

Isoenzyme-specific functions in TPA responses are also indicated by the work of Reifel-Miller *et al.* (1996). These authors demonstrated that two different TPA-responsive promoters, collagenase and urokinase plasminogen activator, exhibit differential responses to PKC isoenzymes. These studies demonstrate that PKCs play important roles in the regulation of gene transcription. However, the precise mechanisms of PKCs influence await clarification.

PKC and cell cycle control: A role for PKCs in the regulation of the cell cycle has become increasingly clear. Early studies of the vitamin D_3 receptor in MDBK cells

demonstrated that receptor activation induced the nuclear translocation and activation of PKCβ, which correlated with increased Ca^{2+}- and phospholipid-dependent phosphorylation of nuclear proteins (Simboli-Campbell *et al.*, 1994). Work with other cell lines has shown that the relative levels of PKCα and PKCβ determine whether a cell undergoes differentiation or proliferation. When expression of PKCα is increased, K562 cells or R6 rat embryo fibroblasts stop dividing and the former begin megakaryocytic differentiation. However, when PKCβ is overexpressed, cells continue to divide and become resistant to signals that normally induce differentiation (Borner *et al.*, 1995; Murray *et al.*, 1993). In at least one case, the proliferative phenotype was associated with the secretion of a growth factor, capable of inducing the division of resting cells (Borner *et al.*, 1995). These effects appear to be mediated by the catalytic domain as chimeric α/β enzymes retain the phenotype of their associated catalytic domain (Walker *et al.*, 1995; see above).

There is increasing evidence that $PKC\beta_{II}$ influences cell cycle progression through the phosphorylation of lamin B. *In vitro* $PKC\beta_{II}$ phosphorylates lamin B at Ser^{405}, a prominent mitotic phosphorylation site. This phosphorylation correlates with $PKC\beta_{II}$ translocation from the cytosol to the nuclear envelope at G2/M transition *in situ*. Furthermore, phosphorylation of lamin B at this site by $PKC\beta_{II}$ leads to lamin B solubilization indicative of nuclear envelope breakdown *in vitro* (Goss *et al.*, 1994). In human promyelocytic leukemia (HL60) cells, chelerythrine, a $PKC\beta_{II}$ inhibitor, blocks Ser^{405} phosphorylation of lamin and arrests cells in G2, without affecting the activity of several other kinases, including $p34^{cdc2}$/cyclin B kinase, PKA, Ca^{2+}/calmodulin-dependent kinase, or tyrosine kinases. In addition to Ser^{405} phosphorylation, phosphorylation at other sites on lamin B, not directly dependent on $PKC\beta_{II}$ phosphorylation, is also blocked. (Hompson and Fields, 1996). This suggests that $PKC\beta_{II}$ may regulate other lamin kinases.

Recent reports by Sauma and Friedman (1996) showed that PKCβ-induced dedifferentiation and proliferation of a differentiated colon carcinoma cell line (HD3) correlated with constitutive activation of several members of the MAPK cascade, including ERK1, $p57^{MAPK}$, and ERK3 (Sauma and Friedman, 1996, and in press data cited therein). It remains to be determined whether the MAPK cascade is responsible for the other lamin B

phosphorylations. These studies indicate that phosphorylation of lamin B is an important part of $PKC\beta_{II}$-mediated proliferation.

Further support for a role of PKCβ in cell proliferation comes from the recent evaluation of PKCβ knock-out mice. Mice deficient for PKCβ show reduced numbers of B1 lymphocytes and reduced antigen-receptor mediated B cell proliferation. Further, some aspects of their humoral immune response were also affected. The unaffected *in vitro* proliferation of splenic T cells from $PKC\beta^{-}/\beta^{-}$ mice suggested that distinct PKC isoforms mediate B cell receptor and T cell receptor signal transduction (Leitges *et al.*, 1996).

Murray *et al.* (1994) demonstrated the presence of a selective $PKC\beta_{II}$ activator in the nuclear envelope. Preliminary work indicated that it was likely a lipid cofactor. Further characterization of this activator should help define the mode(s) of $PKC\beta_{II}$ regulation at the nucleus.

PKC and synaptic transmission: Several physiological substrates of PKCs are located at the synapses in mammalian brain. These include myristoylated alanine-rich C-kinase substrate (MARCKS) protein, neurogranin, and neuromodulin. These proteins each have a phosphorylation site domain (PSD) for PKC phosphorylation. The PSD is also the site of interaction with calmodulin and in the case of MARCKS protein, with actin. Binding of calmodulin occurs in the presence of Ca^{2+} for MARCKS and in the absence of Ca^{2+} for neurogranin and neuromodulin. The binding of calmodulin to the PSD inhibits PKC phosphorylation (Sheu *et al.*, 1995). In BALB/MK mouse keratinocytes Ca^{2+} inhibits PKC-mediated phosphorylation of MARCKS by enhancing calmodulin binding to the PSD (Chakravarthy *et al.*, 1995). Phosphorylation of any of these proteins at the PSD inhibits their interaction with calmodulin (or actin).

It has been suggested that a function of neurogranin is to sequester calmodulin to the cell membrane. Ca^{2+} influx upon depolarization would releases calmodulin near its site of action. Phosphorylation of neurogranin by PKC could then regulate the reassociation of neuromodulin and calmodulin (Gamby *et al.*, 1996). The inhibition of PKC activation abolishes long-term potentiation (LTP) in rat hippocampal slices (Ramakers *et al.*, 1995). It was further shown that the phosphorylation of presynaptic neuromodulin preceded that of

post-synaptic neurogranin with the data suggesting that both these processes were part of the normal physiological response termed LTP (Ramakers *et al.*, 1995).

Synaptic transmission involves a cascade of protein-protein interactions (reviewed by Südhof, 1995). PKC has been shown to phosphorylate at least two proteins involved in either the fusion of synaptic vesicles (SNAP-25) or the regulation of synaptic vesicle fusion (Munc-18), at least *in vitro* (Fujita *et al.*, 1996; Shimazaki *et al.*, 1996). These findings suggest that PKC plays a role in the regulation of synaptic transmission.

B. *Saccharomyces cerevisiae*: *PKC1* and a MAP Kinase Cascade

S. cerevisiae has one identified PKC gene, *PKC1*, that was first isolated as a cell cycle defect and later determined to be necessary for cell wall integrity (Herskowitz, 1995; Levin *et al.*, 1990). The predicted amino acid sequence of the *PKC1* gene product, Pkc1p, is most similar to mammalian cPKCs, however Pkc1p has an amino terminal extension characteristic of some nPKCs and aPKCs. Pkc1p also exhibits an expansion of the V2 domain relative to known cPKCs (Levin *et al.*, 1990). Early biochemical studies indicated that Pkc1p activity was independent of phospholipids, DG, and Ca^{2+} (Antonsson *et al.*, 1994; Watanabe *et al.*, 1994), however this is likely due to improper *in vitro* assay conditions (see below). Furthermore, Antonsson *et al.* (1994) demonstrated that in subcellular fractionations, the Pkc1p is associated with a large protein complex that is resistant to dissociation with high salt, alkali buffer, and nonionic detergents.

Yeast strains harbouring temperature-sensitive alleles of *PKC1* grow slowly at permissive temperatures and undergo rapid cell lysis at non-permissive temperatures. Several genes in this pathway have been isolated by their ability to suppress the lethality of *pkc1Δ* disruption mutants or the temperature-sensitive lethality of other *PKC1* alleles (Irie *et al.*, 1993; Lee, K.S. and Levin, 1992; Lee, K.S. *et al.*, 1993b). Epistasis experiments with these genes demonstrated that *PKC1* regulates a MEKK-MEK-MAPK module (Figure 1.4; Herskowitz, 1995). This module comprises a MAP or ERK kinase kinase (MEKK) homologue encoded by *BCK1* (Lee, K.S. and Levin, 1992), that can be phosphorylated *in vitro* by Pkc1p (Levin *et al.*, 1994), two functionally redundant MAP or

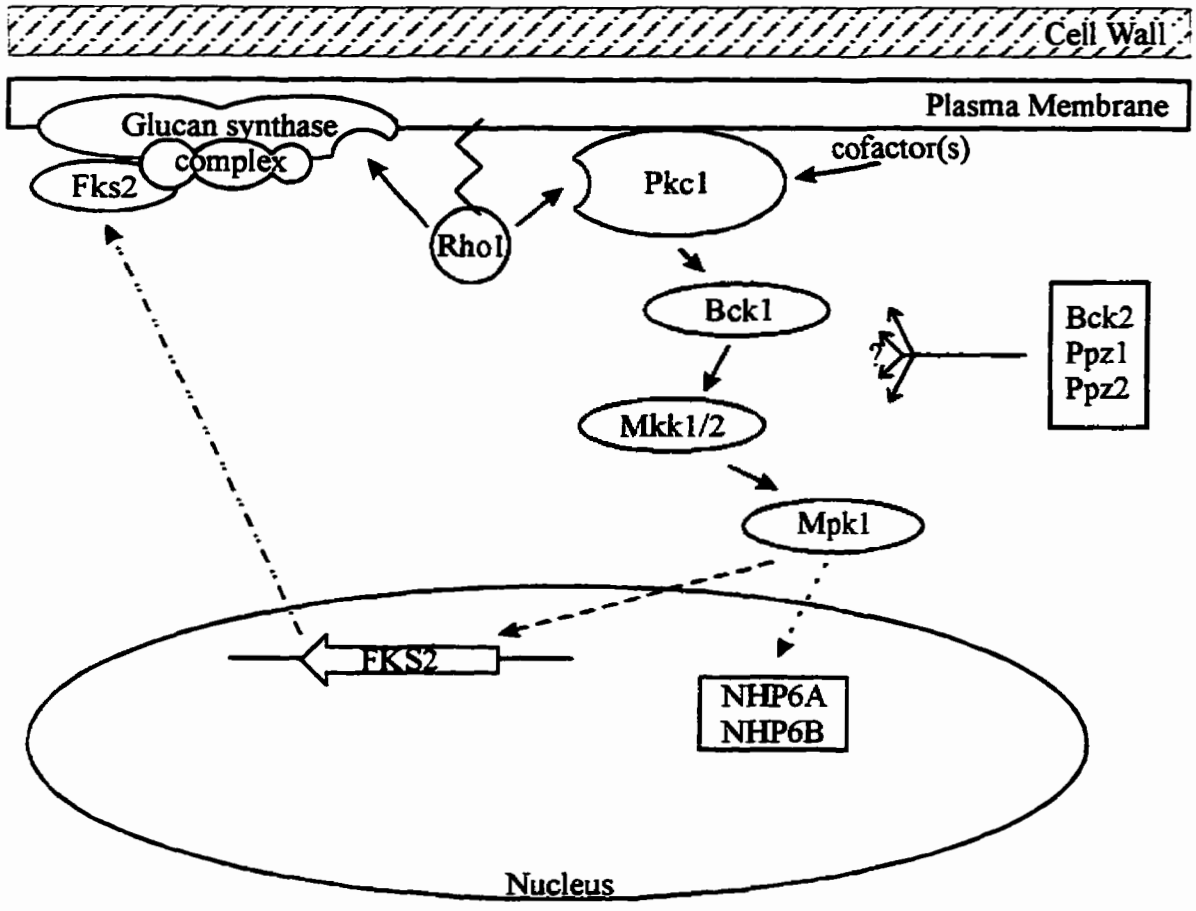

FIGURE 1.4. Schematic representation of the *PKC1* signalling cascade in *S. cerevisiae*. Cell wall integrity is maintained at elevated temperatures by the *PKC1*-dependent thermal induction of *FSK2*, an essential component of the cell wall synthesis machinery. The cell lysis and budding defects of *Pkc1* mutants are likely the result of a defect in cell wall synthesis. (See text for details; Figure adapted from Qadota *et al.*, 1996).

ERK kinase (MEK) homologues encoded by *MKK1* and *MKK2* (Irie *et al.*, 1993), and a MAPK homologue encoded by *MKP1* (Lee, K.S. *et al.*, 1993b).

Yeast contain a number of MEKK-MEK-MAPK modules, one of which mediates pheromone responses. The pheromone response module contains two functionally redundant MAPK homologues, *FUS3* and *KSS1* (Herskowitz, 1995). It is interesting that the *X. laevis* M phase MAPK can partially complement *MPK1* defects, but neither the pheromone response nor the cell cycle defects of either *FUS3* or *KSS1*, suggesting partial functional conservation between vertebrate and yeast MAPKs (Lee, K.S. *et al.*, 1993b).

As mentioned above, early biochemical studies with Pkc1p suggested that its activity was cofactor independent. However, one of the same reports demonstrated that mutation of the pseudo-substrate domain rendered Pkc1p constitutively active both *in vivo* and *in vitro*, suggesting that Pkc1p is subject to pseudo-substrate regulation similar to that seen with most mammalian PKCs. Additionally there appears to be a Pkc1p kinase in yeast, again correlating with the regulation of mammalian PKCs (Watanabe *et al.*, 1994). Recent genetic and biochemical studies have demonstrated that the small GTPase, Rho1p, activates Pkc1p (Kamada *et al.*, 1996; Nonaka *et al.*, 1995). The interaction of Rho1p-GTP with Pkc1p makes Pkc1p responsive to PS (Kamada *et al.*, 1996). The site of interaction of Rho1p-GTP has been mapped to a region containing the pseudo-substrate domain and the C1 (DG-binding) domain of Pkc1p (Nonaka *et al.*, 1995), suggesting that Rho1p-GTP may substitute for DG in Pkc1p activation (Kamada *et al.*, 1996). Alternatively, Rho1p-GTP may induce a conformational change that exposes PS binding sites.

Further work from David Levin's group (Qadota *et al.*, 1996) has demonstrated an essential role for Rho1p in the 1,3-β-glucan synthase (GS) complex, the enzyme responsible for constructing polymers of 1,3-β-glucan in the cell wall. They have also reported that thermal induction of the *FSK2* gene, encoding another GS subunit, is regulated by *PKC1* and *MPK1* (unpublished results cited in Kamada *et al.*, 1996). These results clarify the mechanism by which defects in the *PKC1*-mediated kinase cascade affect cell wall integrity (Figure 1.4). Several lines of evidence indicate that Rho protein function(s) in cellular morphogenesis may be evolutionarily conserved between bud formation in yeast and stress

fibre and focal adhesion formation in mammalian cells (Bussey, 1996). Whether other components of the yeast pathway are also conserved remains to be determined.

The observation that loss of *PKC1* function results in a cell lysis defect at all temperatures, while deletion of any of *BCK1*, *MKK1* and *MKK2*, or *MPK1* results in cell lysis only at high temperatures, suggests that *PKC1* regulates a bifurcated pathway (Irie *et al.*, 1993). Several genes have been identified that impinge on *PKC1* function (for examples see Costigan *et al.*, 1994; Irie *et al.*, 1993; Lee, K.S. *et al.*, 1993b; Levin *et al.*, 1990). These include two functionally redundant PP1-related protein phosphatases, *PPZ1* and *PPZ2*, and a novel protein, *BCK2* (Lee, K.S. *et al.*, 1993a). Epistasis experiments indicate that *PPZ1*, *PPZ2*, and *BCK2* mutations are additive with respect to Pkc1-MAPK pathway kinase mutations. This suggests that these genes act in an adjacent pathway regulating cellular integrity. However, these same experiments were unable to determine the relative order of action within this adjacent pathway.

Yoshida *et al.* (1994) screened for mutants that exhibited temperature-sensitive cell lysis and sensitivity to staurosporine, a potent PKC inhibitor, to characterize *SST4*. *SST4* cell lysis defects, but not staurosporine sensitivity, could be suppressed by overexpression of *PKC1*. *SST4* was identified as a phosphatidyl inositol 4-kinase, suggesting that phosphoinositol lipid metabolism might help regulate the *PKC1* pathway (Herskowitz, 1995).

Two functionally redundant high mobility group 1-like proteins have been identified that function downstream of *MPK1*, *NHP6A* and *NHP6B*, as their overexpression rescues *pkc1Δ* and *mpk1Δ* defects. Nhp6Ap and Nhp6Bp are not *in vitro* substrates for MPK1 (Costigan *et al.*, 1994). Analyses of *nhp6Δ* mutants suggest a bifurcating pathway regulated by *MPK1* with both Nhp6p-dependent and Nhp6p-independent branches.

Yeast presents an easily accessible experimental model with which to study regulatory pathways. Much has already been learned about the function of *PKC1* and the further elucidation of this pathway should be quick to follow. However, unlike more complex metazoans, yeast contains a single known PKC gene rather than a multigene family, indicating the need for studies on more complex, but still genetically manipulable organisms.

C. *Caenorhabditis elegans* PKC: A Multigene Family

The PKC homologue *tpa-1* was identified in *C. elegans* with screens for resistance to TPA (Tabuse *et al.*, 1989). Molecular analysis of this gene has revealed two transcripts differing in their 5′ sequences, *tpa-1A* and *tpa-1B*, with cognate proteins TPA-1A and TPA-1B, as recognized by Northern and Western blotting, respectively (Sano *et al.*, 1995). *tpa-1B* lacks 0.4 kb of 5′ sequence, encompassing the first four exons of the *tpa-1* gene. Both transcripts contain the splice leader, SL1, and are polyadenylated. It is not known whether alternative splicing of a common pre-mRNA or use of an alternative promoter is responsible for the truncated 5′ structure of *tpa-1B*. The authors speculated that a promoter in intron IV is responsible for *tpa-1B* transcription, since both CAAT and TATA box structures are present (Sano *et al.*, 1995).

TPA-1A and TPA-1B putative protein sequences each contain a pseudo-substrate domain and CRDs, as well as the PKC kinase domain. However TPA-1A has an amino terminal extension similar to that found in nPKCs, most similar to mammalian PKCδ (Sano *et al.*, 1995).

The work of C. S. Rubin and colleagues (Islas-Trejo *et al.*, 1997; Land *et al.*, 1994) have identified two more PKC structural genes. One of these, *kin-13*, encodes a single polypeptide, PKC1B, most closely related to PKCϵ among mammalian PKCs, including ≥50% similarity in the amino-terminal extension characteristic of nPKCs. PKC1B mRNA and protein are coordinately regulated during post-embryonic development, however embryos express a high level of PKC1B mRNA with only minimal amounts of PKC1B protein. These results suggest that this isoenzyme is subjected to both transcriptional and post-transcriptional and/or post-translational regulation. *In situ* immunohistochemistry demonstrates that PKC1B is exclusively expressed in approximately 75 chemo- and mechanosensory neurons and related interneurons. Protein levels and promoter activity persist in adults, albeit at a reduced level, and PKC1B protein is found along the complete length of several neurons. These data implicate PKC1B as a target of phospholipid second messengers at multiple sites within neurons that comprise part of the sensory circuitry of *C. elegans*.

The identification of TPA-1A, TPA-1B, and PKC1B as separate nPKC isoenzymes, the purification of a Ca^{2+}-dependent PKC activity (Sassa and Miwa, 1992), and the recent cloning of a gene encoding a Ca^{2+}-dependent PKC isoenzymes, PKC2 (Islas-Trejo *et al.*, 1997), indicate that *C. elegans* expresses a multiplicity of PKC isoforms. Studies with this organism should help illuminate some of the multiple roles of PKC in growth and development.

D. *Drosophila melanogaster* PKC: A Multigene Family

The *D. melanogaster* PKC gene family is comprised of three genes, *Pkc53E*, *inaC*, and *Pkc98E* (FlyBase, 1996; Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989), suggesting a lower level of complexity than that seen in mammals. However, as seen in Table 1.1, two of these genes encode several developmentally and spatially regulated transcripts. It remains to be determined whether these transcripts each encode unique isoenzymes, or if the transcript differences reflect the presence of different post-transcriptional regulatory elements in the RNAs and/or the use of alternative transcription start sites from different promoters.

TABLE 1.1. Developmental Expression of *Drosophila* PKC RNAs.

	Pkc53E[1]	*inaC*[2]	*Pkc98E*[2]
Embryo	n.d.[3]	n.d.	4.3, 4.5, 5.5 kb
Larva	2.4, 3.3 kb	n.d.	5.5 kb
Pupa	2.4, 4.0, 4.3 kb	2.4 kb	5.5 kb
Adult	2.4, 4.0, 4.3 kb	2.4 kb	5.5 kb

[1] Natesan, 1990; [2] Schaeffer *et al.*, 1989; [3]not detected

1. *Protein kinase C 53E, Pkc53E*

The genomic and cDNA sequences of *Pkc53E* were identified using a bovine PKCα probe (Rosenthal *et al.*, 1987). The nucleotide sequence of the identified open reading frame (ORF), compiled from a pair of overlapping cDNAs, predicts a protein that is most similar to the cPKCs with a molecular weight of 75 kDa. Rosenthal *et al.* (1987) reported

the presence of three, equally abundant transcripts in adult heads of about 4.3, 4.0 and 2.4 kilobases (kb) each. No transcripts were detected in embryos. Work in our laboratory (Natesan, 1990; and data presented here) suggests that the 2.4 kb transcript may be more abundant than either the 4.3 or 4.0 kb transcript. Transcription of this gene has been shown to occur in most neurons of the adult head, including the photoreceptor cells (Hughes, 1993; Schaeffer *et al.*, 1989).

Studies using TPA have demonstrated that *Pkc53E* is transcriptionally induced to high levels in embryos, peaking after about 6 to 9 hours of treatment, followed by a rapid down-regulation of transcription to undetectable levels (Hughes, unpublished results; Natesan, 1990). These transcripts were localized to the developing brain and tracheal system (Hughes, 1993).

2. *inactivation no afterpotential C, inaC*

A second PKC gene was cloned from a cDNA library using bovine PKCs α and β probes (Schaeffer *et al.*, 1989). This gene maps about 50 kilobase pairs (kbp) away from *Pkc53E*, produces a single mRNA of 2.4 kb, and again is predicted to be most similar to cPKCs (Schaeffer *et al.*, 1989). *inaC* was identified as the structural gene for eye-PKC by Smith *et al.* (1991), who demonstrated that a *P* element containing the complete coding region for eye-PKC could completely rescue the *inaC* mutation. To date this is the best characterized *Drosophila* PKC due to the availability of several *inaC* mutant alleles.

inaC mutants are defective in light adaptation, likely due to the exhaustion of the excitatory response (Hardie *et al.*, 1993). Genetic and biochemical analyses have identified several genes that appear to play a role in the eye-PKC-mediated visual transduction cascade. These include *no receptor potential A* (*norpA*), the structural gene for a PLC (Ranganathan *et al.*, 1995); *transient receptor potential* (*trp*), encoding a novel light-dependent, IP_3-responsive Ca^{2+} channel (Hardie *et al.*, 1993); and *retinal degeneration B* (*rdgB*) that encodes a novel integral membrane protein with an ATP-binding site, Ca^{2+}-binding activity, and PI transfer activity (Smith *et al.*, 1991; reviewed in Ranganathan *et al.*, 1995).

Recently, analysis using the fly *Calliphora*, has demonstrated that InaD is likely a substrate of the *Calliphora* eye-PKC homologue (Huber *et al.*, 1996). These authors point out that many of the visual transduction studies in *Drosophila* have been successfully complemented by biochemical studies in other fly species (*eg. Calliphora, Musca*). Four lines of evidence indicate that InaD is a direct substrate for eye-PKC. (1) Anti-InaD antibodies coimmunoprecipitate a complex of InaD, eye-PKC, the *norpA* PLC, and the *trp* Ca^{2+} channel from *Calliphora* rhabdomeral membranes. (2) Treatment of rhabdomeric membrane preparations with phorbol esters increased InaD phosphorylation; (3) Treatment with the PKC inhibitor, bisindolylmaleimide, reduced InaD phosphorylation. (4) Five consensus PKC phosphorylation sites are conserved between *Calliphora* and *Drosophila* InaD, but the consensus cAMP- and cGMP-dependent phosphorylation sites in *Drosophila* InaD are absent from *Calliphora* InaD.

These data, coupled with earlier work (see above), suggest a model for eye-PKC activation in visual transduction in *Drosophila* (Figure 1.5). Light activation of rhodopsin results in the G protein-mediated activation of PLC. PLC activates eye-PKC through the production of DG and IP_3-mediated Ca^{2+} release. Activated eye-PKC then phosphorylates InaD. The colocalization of PLC, eye-PKC, and InaD at the rhabdomeral membrane facilitates rapid signal transduction.

While RdgB function is not yet understood, it is clear that RdgB functions in a pathway with *norpA* PLC since several *norpA* alleles suppress retinal degeneration in $rdgB^9$ mutants (FlyBase, 1996). Further, the data suggest that light activation of eye-PKC is responsible for the light-dependent retinal degeneration of *rdgB* mutants. It is attractive to hypothesize that *rdgB* functions as an antagonist of eye-PKC activation (Ranganathan *et al.*, 1995).

3. *Protein kinase C 98E, Pkc98E*

In addition to *inaC*, Schaeffer *et al.* (1989) identified a third, more highly divergent PKC gene, *Pkc98E* (FlyBase, 1996). This gene encodes a major 5.5 kb transcript in adult heads and bodies. *Pkc98E* is the only *Drosophila* PKC gene with detectable mRNA expression in embryos, where the expression of the 5.5 kb transcript is reduced, but 4.3 and

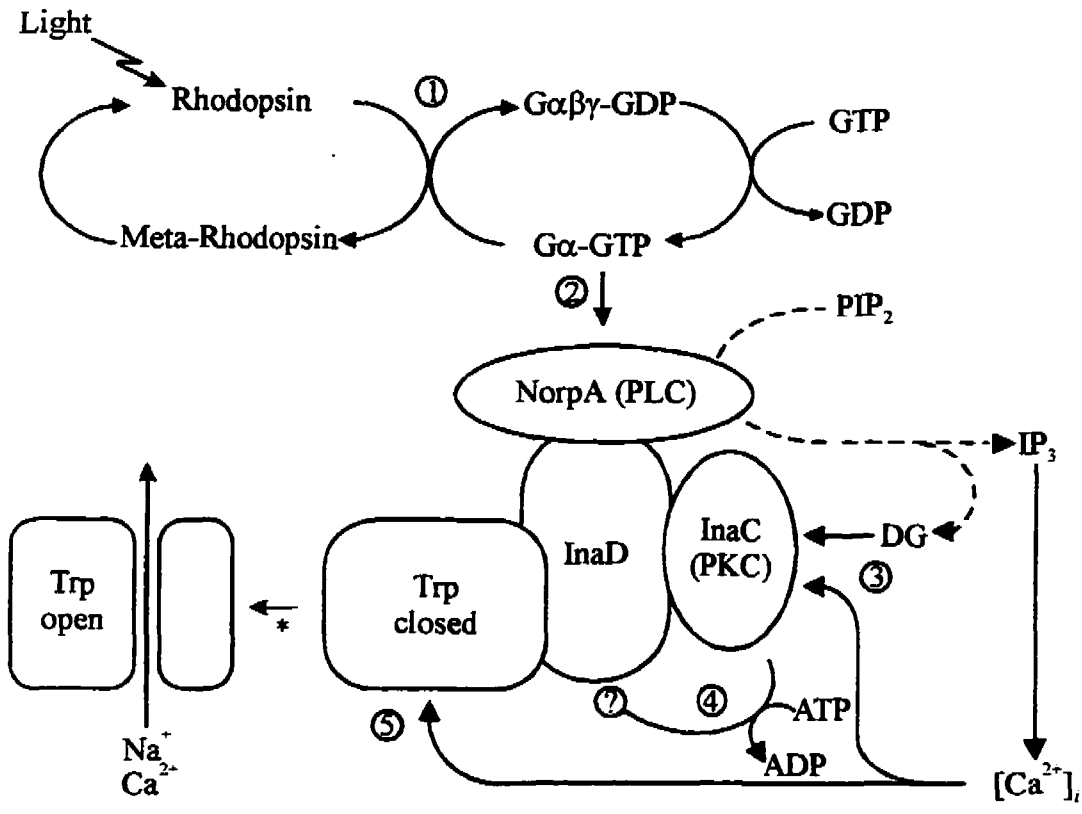

Figure 1.5. Schematic diagram of *Drosophila* InaC in phototransduction. (1) Light activation of rhodopsin activates GDP-GTP exchange on a trimeric G protein. Gα and Gβγ dissociate. (2) Gα-GTP stimulate PLC (*norpA*) which generates lipid second messengers. DG remains at the membrane, while IP_3 diffuses into the cytosol and stimulates Ca^{2+} release from internal stores. (3) DG and Ca^{2+} activate PKC (*inaC*). (4) PKC phosphorylates InaD. InaD may act as a scaffolding protein. The function of this phosphorylation has not been reported. (5) Ca^{2+} further causes a conformational change in the Trp ion channel, opening the channel. *This arrow does not indicate dissociation, but rather a conformational change. (Figure modified from Ranganathan *et al.*, 1995).

4.5 kb transcript expression is increased (Schaeffer *et al.*, 1989). The predicted amino acid sequence for this gene suggests that it is likely an nPKC, most similar to PKCϵ (Kruger *et al.*, 1991).

4. Functions of *Drosophila* PKCs

Flies mutant at *turnip* (*tur*) exhibit poor learning performance (Lindsley and Zimm, 1992). While early studies were somewhat inconclusive, recent work implicates a role for PKC activity in associative learning in *Drosophila* (FlyBase, 1996). PKC activity appears to be influenced by *tur* gene function. In fact, recent work reporting the cloning of the *tur* gene has shown that tur^{+} encodes a Rho-like GTP-binding protein (FlyBase, 1996). Work with *S. cerevisiae* has shown that RHO1 is essential for the activation of PKC1 in the cell wall integrity pathway (see above). This suggests evolutionary conservation of PKC regulatory mechanisms.

A recent report using a transgene expressing a PKC inhibitor pseudo-substrate peptide (PKC*i*) suggests that a PKC activity is required for the generation of type I and type II processes in developing neuroblasts (Broughton *et al.*, 1996). However, it has not been determined whether PKC53E or PKC98E is responsible for this activity. Due to the high conservation of PKC pseudo-substrate domains it is likely that PKC*i* inhibits multiple PKC isoenzymes. Clearly, more studies need to be done on these genes in order to understand the multiple functions of PKC outside of visual transduction. The elucidation of signal transduction cascades in *Drosophila* has often provided major insights into signal transduction in mammals and other organisms (Artavanis-Tsakonas *et al.*, 1995; Kandel and Abel, 1995; Siegfried and Perrimon, 1994; Yamamoto, 1994). The study of PKC in *Drosophila* has the potential to be a very fruitful avenue of research.

III. *P*-ELEMENT MUTAGENESIS OF *DROSOPHILA*

P elements are transposable elements that require both a *P* element encoded transposase and host supplied factors. Autonomous *P* elements encode a functional transposase and can effect their own transposition, as well as the transposition of nonautonomous transposase-deficient *P* elements. *P* elements have provided *Drosophila*

geneticists with a powerful set of tools with which to approach genetic and developmental research (Engels, 1996, is a recent comprehensive review).

P elements are used for the identification and cloning of genes and for their reintroduction into the genome. Several characteristics of *P* elements make them particularly suitable for these applications. (1) The existence of M strains, lab stocks devoid of endogenous *P* elements, allows the creation of stocks containing only selected *P* elements. (2) Transposase can easily be added or removed using appropriate genetic crosses. The most commonly used transposase source is the P[ry^+; Δ2-3(99B)] (Robertson *et al.*, 1988). (3) *P* elements are highly mobile and retain this mobility even with extensive modifications to their internal sequences. (4) Insertion of *P* elements into genes usually generates null or hypomorphic alleles. Insertion has been detected in both coding (*eg.* f^{hd}, $w^{hd80KI7}$, $Pka\text{-}C1^{01272}$) and non-coding regions of genes (*eg. N*, vg^{21}, *y*) (Chia *et al.*, 1986; FlyBase, 1996; Kelley *et al.*, 1987). (5) Recently, the double-strand DNA breaks created by *P* element excision have been utilized to effect gene replacement and targeted gene insertion (Engels, 1996).

Several approaches for *P* element mutagenesis have been developed, the most efficient of which involves the use of an immobile genomic copy of the transposase gene, P[ry^+; Δ2-3(99B)], with one or more mobile, but nonautonomous, *P* elements as ammunition. A cross between one parent with the transposase source and the other with the ammunition elements yields progeny in whose germline *P* element mobilization and mutagenesis occurs. The mutations are stabilized in the next generation by segregation from the transposase source (see Materials and Methods).

There are two general strategies for selecting ammunition elements (Engels, 1996). One involves the use of a chromosome carrying as many highly mobile elements as possible, such as the Birm-2 chromosome which has 17 small nonautonomous *P* elements inserted along its length. This maximizes the likelihood of obtaining the desired mutation, but isolation of the mutation from the rest of the *P* elements is often a very time-consuming process. The second strategy involves the use of a smaller number of artificially constructed *P* elements. This usually entails a larger screen, but mutations are more easily isolated and

characterized, especially with ammunition elements carrying a visible *Drosophila* marker, a bacterial origin of replication, and a bacterial selectable marker to permit cloning of sequences adjacent to the insertion site by plasmid rescue (*eg.* P[lacW], Bier *et al.*, 1989). Once a *P* element insertion has been generated, additional genetic variability can be generated by reintroduction of the transposase alone, which induces internal or flanking deletions, or with exogenous templates to induce site directed mutagenesis via gene replacement (Engels, 1996).

PCR can be used to detect insertions in genes for which sequence, but not mutational phenotype, information is available. These types of screens rely on the absolute necessity of conserved 31-bp inverted repeats at the ends of *P*-elements for transposition. Transposition of a *P*-element into the gene of interest can translocate the conserved 31-bp sequence *cis* to gene-specific sequences. PCR using primer pairs consisting of gene-specific sequences and the 31-bp repeat sequence enable the detection of a PCR product. True amplification products can be differentiated from spurious products by Southern hybridization (see Methods). Table 1.2 shows data from some reported screens. In the screen done by Dalby *et al.* (1995) mutations at the cytogenetic location nearest 53E, 54D, occurred at a frequency of 3/16,100, giving an indication as to the size of the screen that must be undertaken.

TABLE 1.2. Comparison of mutation frequencies between different *P*-element mutagenesis screens.

Ammunition Chromosome	No. of Inserts	Location of Insert	No. of Flies Screened	Screening Method	Ref
Birm-2[a]	1	87AB	6316	Direct screen with GSP + P-element primers	1
Birm-2	0	72E	6316	Direct screen with GSP + P-element primers	1
Birm-2	3[b]	10D1-2	900	Direct screen with GSP + P-element primers	4

TABLE 1.2. Comparison of mutation frequencies between different *P*-element mutagenesis screens (continued).

Ammunition Chromosome	No. of Inserts	Location of Insert	No. of Flies Screened	Screening Method	Ref
C(1)RM 4[PlacW]	1[c]	45D	140	Plasmid rescue/Inverse PCR	3
C(1)RM 4[PlacW]	1[c]	90D	140	Plasmid rescue/Inverse PCR	3
C(1)RM 4[PlacW]	0[c]	NR	140	Plasmid rescue/Inverse PCR	3
C(1)RM 4[PlacW]	1[c]	71F	160	Plasmid rescue/Inverse PCR	3
C(1)RM 4[PlacW]	3[c]	NR	160	Plasmid rescue/Inverse PCR	3
C(1)RM 4[PlacW]	2[c]	NR	160	Plasmid rescue/Inverse PCR	3
C(1)RM 4[PlacW]	3	54D	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	4	64D	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	1	67A	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	2	98A	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	1	NR	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	0	NR	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	0	69D	16,100	Inverse PCR[d]	2
C(1)RM 4[PlacW]	0	NR	11,900[e]	Inverse PCR[d]	2
C(1)RM 4[PlacW]	0	NR[f]	11,900[e]	Inverse PCR[d]	2
C(1)RM 4[PlacW]	0	NR[f]	11,900[e]	Inverse PCR[d]	2
C(1)RM 4[PlacW]	1	68E	6,900[e]	Inverse PCR[d]	2
C(1)RM 4[PlacW]	0	32F	5,700[e]	Inverse PCR[d]	2
Birm-2	5	100EF	15,000	Direct screen with GSP + P-element primers	1,5

TABLE 1.2. Comparison of mutation frequencies between different *P*-element mutagenesis screens (continued).

Ammunition Chromosome	No. of Inserts	Location of Insert	No. of Flies Screened	Screening Method	Ref
Birm-2	0	NR	15,000	Direct screen with GSP + P-element primers	1,2
Birm-2	0	NR	15,000	Direct screen with GSP + P-element primers	1,2
Birm-2	0	NR	15,000	Direct screen with GSP + P-element primers	1,2

Notes: [a]Birm-2 is the second chromosome from the Birmingham P-strain and has 17 non-autonomous *P*-elements on it.

[b]This screen was used to detect inserts at sn^{w} in a known hot spot for *P*-element insertion. The insertion frequency shown here agrees well with other reports of *sn* mutagenesis.

[c]These inserts were identified by hybridization of inverse PCR products derived from rescued plasmids to an array of cDNAs. Two hybridized to the same DNA, but likely represent independent insertions.

[d]These inserts were identified by inverse PCR of digested and circularized genomic DNA hybridized against an array of genomic clones of the genes of interest, except as in Note f. They are not necessarily within the coding region of the gene.

[e]These represent screens of a subset of the 16,100 flies used in this paper.

[f]Cloned cDNAs were used as the targets for hybridization in these two subsets.

NR Not reported

References: 1. Ballinger and Benzer, 1989; 2. Dalby *et al.*, 1995; 3. Hamilton *et al.*, 1991; 4. Kaiser and Goodwin, 1990; 5. Pereira *et al.*, 1992

IV. PROJECT GOALS

A. Mutagenesis of *Pkc53E*

As see with yeast and *Drosophila* eye-PKC above, the availability of mutations enables the use of powerful genetic techniques to elucidate gene functions. *Drosophila* has

three known PKC genes and therefore provides an ideal complex animal model in which to study PKC function. One goal of this project was to use *P* element mutagenesis to produce an insertional mutant of *Pkc53E*. The generation of such a mutant would enable the generation of deletion mutants through remobilisation of the *P* element and perhaps site-directed mutagenesis using the recently elucidated technique of targeted gene replacement (Engels, 1996). While it is predicted that a null-*Pkc53E* allele would be recessive lethal, the use of such a mutation for mosaic analysis would provide information as to the tissue-specific function of *Pkc53E* in development. This type of analysis can be done using genetic crosses with the FLP/FRT (flip recombinase/flip recombinase target) technique (Simpson, 1993).

B. Molecular Analysis of *Pkc53E*

As described earlier, several transcripts that hybridize to *Pkc53E*-specific probes can be detected by Northern hybridization of *Drosophila* RNA. However, the transcript sequence differences have not been elucidated nor has it been demonstrated whether functional PKCs are encoded by each of these transcripts. The second goal of this thesis was to examine transcript structures of adult *Pkc53E* mRNA, using the common molecular techniques of ribonuclease (RNAse) protection analysis (RPA), rapid amplification of cDNA ends (RACE), reverse transcription polymerase chain reaction (RT-PCR), and nucleic acid sequencing.

RPA experiments were designed to identify regions of transcript variability due to alternative splicing. This information was then used to design PCR and reverse transcription primers within conserved transcript sequences to try to amplify variant sequences. Restriction mapping and nucleic acid sequencing was then done to identify novel *Pkc53E* transcript sequences.

CHAPTER 2: MATERIALS AND METHODS

I. MATERIALS:

A. Bacterial Strains

Plasmids were propagated in commercially available strains of *E. coli*. *E. coli* DH10B, DH5α, DH5αF'IQ and DM1 cells were obtained from GIBCO/BRL. *E. coli* XL-1 Blue cells were obtained from Stratagene. All strains except for DM1 are suitable for blue/white selection on X-gal containing medium. DM1 cells are mutated in both methylation pathways and are therefore suitable for propagation of plasmid DNA for digestion by methylation-sensitive restriction endonucleases.

B. *Drosophila melanogaster* Stocks[1]

1. Oregon-R (OR)

Individuals of this stock are believed to contain the wild type alleles of all genes. This stock was derived from wild flies collected at Roseburg, Oregon prior to 1925 and has been maintained in this laboratory for several years.

2. C(1)RM, *y w* 4P[lac*W*] (B-#3697)

Females of this stock have two X chromosomes attached proximally to the same centromere. Four copies of the P[lac*W*] *P*-element are inserted along the length of the ammunition chromosome of C(1)RM, *y w* 4P[lac*W*]; +/+; +/+. The genes contained within P[lac*W*] are described in Figure 2.1. Digestion of genomic DNA for plasmid rescue can be done using the enzyme sites shown on either side of the bacterial sequences, except for *Hind*III (Bier *et al.*, 1989).

Two visible recessive mutations are also present on this chromosome. *y* (*yellow*, 1-0.0) causes a loss of melanotic pigment giving a yellow cuticle. The *w* (*white*, 1-1.5)

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[1] Stock descriptions and gene locations were taken from Lindsley and Zimm (1992) or FlyBase (1996). Fly stocks not maintained in this laboratory were obtained from the Bloomington Stock Centre and have their associated stock numbers following the stock name.

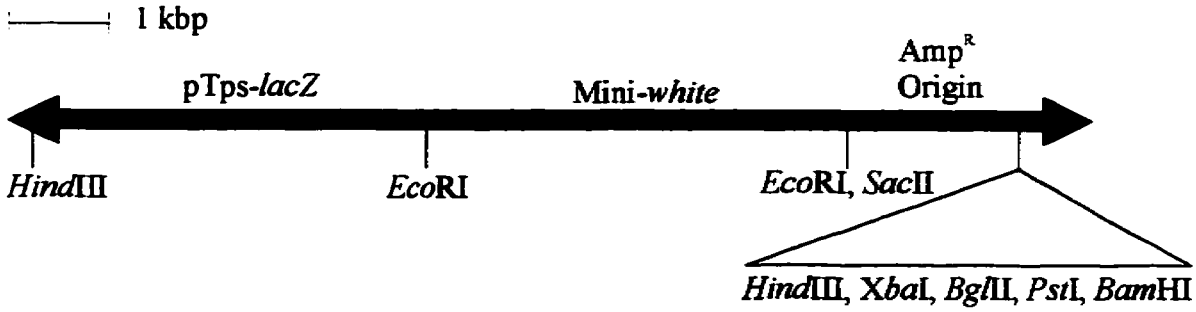


FIGURE 2.1. P[lac*W*] *P* element. Four copies of this *P*-element are found on the ammunition chromosome of C(1)RM, *y w* 4P[*lac*W]; +/+; +/+. The genes contained within this element are pTps-*lacZ* (an enhancer trap with the *E. coli* β-galactosidase gene under the control of the *P*-element transposase minimal promoter), mini-*white* (the w^+ marker), Amp^R (β-lactamase conferring ampicillin resistance to *E. coli* transformed by plasmid rescue), and origin (an origin of replication for plasmid propagation in *E. coli* transformed by plasmid rescue). Digestion of genomic DNA for plasmid rescue can be done using the enzyme sites shown on either side of the bacterial sequences, except for *Hind*III (From Bier *et al.*, 1989). I used *Eco*RI and *Xba*I for the plasmid rescue and sequencing that I did.

mutation normally causes a white eye phenotype, but this is masked by the presence of the P[lac*W*] elements, so the eyes are red. The autosomes are wild type.

Males of this strain are X/Y and phenotypically wild type. Females are C(1)RM/Y and have the yellow mutant phenotype. Male progeny receive their X chromosomes from their fathers and their Y chromosomes from their mothers. The C(1)RM chromosome is passed only through the females. Breakdown of the C(1)RM chromosome is detected by the appearance of *y* males.

3. *w; *Dr*^{1}/TMS, *kar ry*^{2} *Sb*^{1} P[*ry*$^{+t7.2}$ Δ2-3(99B)]** (B-#1610)

This stock has the visible recessive mutation *w* that causes white eyes. The third chromosome is heterozygous with the visible dominant, recessive lethal mutation *Dr* (*Drop*, 3-99.2), that causes small eyes, on one homologue and the other a balancer, TMS (Third Multiple Singson), with the visible dominant, recessive lethal mutation *Sb* (*Stubble*, 3-58.22), that causes short thick bristles. Also on TMS is the recessive mutation *ry* (*rosy*, 3-52.0) that normally causes a reddish-brown eye colour, but is masked by the presence of a functional *ry*$^{+}$ allele in the linked *P*-element. P[*ry*$^{+}$ Δ2-3(99B)] is a non-transposable *P* element with a wild type *ry* (*ry*$^{+}$) allele and a transposase gene that constitutively produces transposase in all cells. Linkage is maintained by the presence of multiple inversions along TMS that repress recombination. The recessive visible mutation *kar* (*karmoisin*, 3-51.7) gives a bright-red eye colour, but is not relevant in this project.

C. Description of Cross Progeny

1. C(1)RM; +/+; +/TMS

First generation females from crossing C(1)RM, *y w* 4P[lac*W*] virgin females with *w*; *Dr*/TMS, *Sb ry* P[*ry*$^{+}$ Δ2-3(99B)] males. Selected females had yellow bodies, red eyes, and Stubble bristles. The P[lac*W*] elements in these females can transpose to other sites in the genome due to the presence of the P[*ry*$^{+}$ Δ2-3(99B)] transposase source.

2. *w*/Y; P[lac*W*]/+; +/+

First generation males from crossing C(1)RM; +/+; +/TMS females with *w*/Y; +/+; +/+ males. Selected males had non-white (yellow, orange, or red) eyes and wild type bristles. These flies have one or more P[lac*W*] elements located on either the Y chromosome and/or one or more autosomes, as indicated by the non-white eye colour. The transposed element is stable as the TMS transposase supply is no longer present.

Subsequent crosses of *w*/Y; P[lac*W*]/+; +/+ males with *w*/*w*; +/+; +/+ females maintains the P[lac*W*] chromosome for subsequent screening procedures.

D. Plasmid and Transformation Vectors

1. pBluescript™ II

These standard plasmid cloning vectors were purchased from Stratagene Inc. and contain a multiple cloning site (MCS) within the *lacZ* gene for blue/white selection. Both bacterial and M13 origins of replication are present for plasmid propagation and single-strand phagemid production. Ampicillin resistance is conferred on transformants by the β-lactamase gene. The GenBank accession numbers of pBluescript II KS+ and pBluescript II SK- are X52327 and X52330, respectively.

2. p0161

This plasmid was generously obtained from A. Rosenthal (Genentech, Inc.) and contains part of the published *Pkc53E* cDNA sequence (Rosenthal *et al.*, 1987) cloned into the *Eco*RI site of pSP64 (Ausubel *et al.*, 1996). The plasmid map is shown in Figure 2.2.

3. pSB29

This plasmid was constructed by Natesan (1991) and contains a genomic fragment of *Pkc53E* from the *Eco*RI site at the published +1 and a 3' *Eco*RI site 2.9 kbp away.

4. pHR0.6

This plasmid contains a 0.6 kbp *Eco*RI-*Hind*III genomic fragment of the *D. melanogaster* ribosomal protein 49 (rp49) gene subcloned into the pBR322 vector by Wong *et al.* (1981). This genomic segment spans nucleotides 240 (*Eco*RI) through 879 (*Hind*III), inclusive, from Genbank accession number X00848. The sole intron is located between

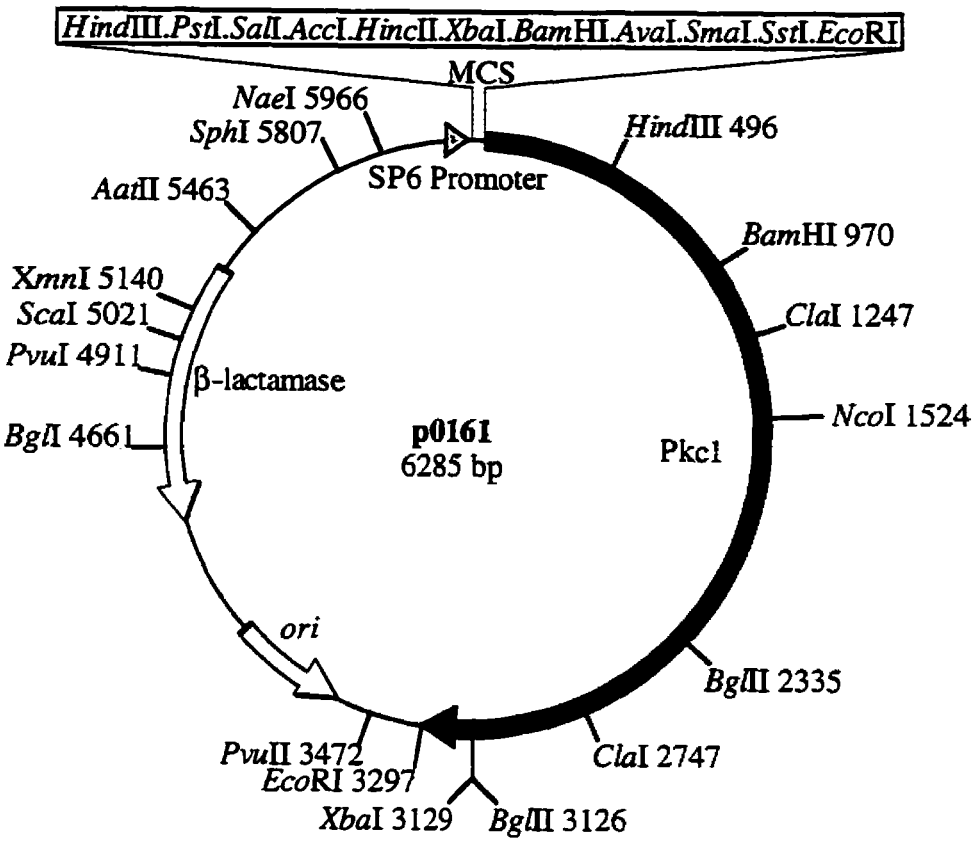


FIGURE 2.2. p0161. This plasmid contains the published 3.2 kbp *Pkc53E* cDNA sequence (in black; Rosenthal *et al.*, 1987) cloned in the sense orientation with respect to the SP6 RNA polymerase promoter of the cloning vector pSP64. This plasmid was generously provided by A. Rosenthal.

nucleotides 513 and 571, inclusive. Probes hybridizing to the rp49 message are often used as a control for loading on Northern blots, however expression of this message does vary between developmental stages and tissues (Al-Atia *et al.*, 1985).

E. cDNA Libraries

An adult head λgt10 cDNA library with a complexity of 2.75×10^5 was generously donated by G. Rubin (Department of Biochemistry, University of California, Berkley).

An ovary λgt22A cDNA library with a complexity of 5×10^5 was generously supplied by P. Tolias (Public Health Research Institute, New York).

F. Composition of Media and Solutions

The composition of media and solutions used throughout this project are specified in Table 2.1.

TABLE 2.1. Composition of media and solutions.

Name	Composition
6X DNA Loading Dye[a]	0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF 30% (v/v) glycerol
10 X RNA Loading Dye[a]	0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF 1 mM EDTA 50% glycerol
DIG Hybridization Detection Buffer Set:	
Hybridization Buffer	5X SSC 1.0% (w/v) blocking reagent (Boerhinger/Manheim) 0.1% (w/v) N-lauroylsarcosine 0.02% (w/v) SDS
Buffer 1	150 mM sodium chloride 100 mM maleic acid pH 7.5
Buffer 2	Buffer 1 with 1% (w/v) blocking reagent

TABLE 2.1. Composition of media and solutions (continued).

Name	Composition
Buffer 3	100 mM Tris-HCl 100 mM sodium chloride pH 9.5
Drosophila Ringers[d] (Tübingen and Düsseldorf)	10 mM Tris-HCl 182 mM potassium chloride 46 mM sodium chloride 3 mM calcium chloride pH 7.2
Exonuclease III Deletion Buffer Set:	
2X Exonuclease III Buffer	30 mM Tris-HCl, pH 8.0 1.32 mM magnesium chloride
5X S1 Nuclease Buffer	150 mM sodium acetate, pH4.6 1.5 M sodium chloride 5 mM zinc sulphate 25% (v/v) glycerol
S1 Nuclease Stop Buffer	0.8 M Tris-HCl, pH 8.0 20 mM EDTA, pH 8.0
S1 Nuclease Dilution Buffer	20 mM Tris-HCl, pH 7.5 0.1 mM zinc acetate 50 mM sodium chloride 5% (v/v) glycerol
GTE Buffer[a]	50 mM glucose 25 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0
LB Agar[c]	LB broth with 1.5% (w/v) agar
LB Broth[c]	1% (w/v) tryptone 0.5% (w/v) yeast extract 0.5% (w/v) sodium chloride pH 7.0
10X MOPS Buffer[a]	200 mM MOPS 100 mM sodium acetate 10 mM EDTA pH 7.0

TABLE 2.1. Composition of media and solutions (continued).

Name	Composition
SOC Medium[a]	2% (w/v) tryptone 0.5% (w/v) yeast extract 0.05% (w/v) sodium chloride 2.5 mM potassium chloride pH 7.0 10 mM magnesium chloride (added after autoclaving) 20 mM glucose (added after autoclaving)
20X SSC[a]	3 M sodium chloride 0.3 M sodium citrate pH 7.0
5X T4 DNA Ligase Buffer[d]	250 mM Tris-HCl, pH 7.6 50 mM magnesium chloride 5 mM ATP 5 mM DTT 25% (w/v) PEG-8000
50X TAE[a]	2 M Tris acetate 50 mM EDTA, pH 8.0
5X TBE[a]	0.446 M Tris borate 10 mM EDTA, pH 8.0
TE Buffer[a]	10 mM Tris-HCl 1 mM EDTA pH as indicated in procedure
Terrific Broth[d]	12 g tryptone 24 g yeast extract 4 ml glycerol Dissolved in 900 ml water. 2.3 g KH_2PO_4 12.5 g K_2HPO_4 Dissolved in 100 ml water. Solutions are autoclaved separately, cooled to room temperature, and mixed.

[a] Sambrook *et al.*, 1989; [b] Boehringer Mannheim Product Information; [c] Ashburner, 1989; [d] GIBCO/BRL Product Information

G. DNA Primers

Primers were selected by hand or based on recommendations using the computer program Oligo 4.0-s, version 4.06 (Wojciech Rychlik © 1992) and are listed in Table 2.2.

TABLE 2.2. Primers used for PCR or sequencing.

Name	Sequence (5′→3′)	T_m(°C)	Target
1	CGCGCTAACGCAAGTCGTTCGGA	74	*Pkc53E*
1b	AATTGTCCTTTTTGTGGTCTA	59	*Pkc53E*
1c	CGGAGGGCAGCGATAACAACG	68	*Pkc53E*
1Ra	GTCGGCGTGTTTTTATTAGGC	62	*Pkc53E*
2	GGGCTTCGGAAAGCAAGGATTTC	70	*Pkc53E*
2b	CAAGTTTGCTCCTATGTGGTG	62	*Pkc53E*
2Rb	CGAATCGATGCCCTTATCCTTGC	70	*Pkc53E*
2Rc	GCCCGGACAGATGAAGGTGAC	62	*Pkc53E*
2Rd	GCAAACTTGGCACTGAAATCC	62	*Pkc53E*
3	GCCCGCTGCAAGGAGAACGTGCC	78	*Pkc53E*
3b	GGAGATCAATGTCAAGGAGAA	60	*Pkc53E*
3Rb	CCAGACAGGATTTAGGCAAGC	64	*Pkc53E*
3Rc	GCGAGTCTTCTTCTTGGATTG	62	*Pkc53E*
3Rd	GTGATCGCATCCGCAAAGGCTGG	74	*Pkc53E*
4	CAGGACGACGACGTCGAGTGCAC	76	*Pkc53E*
4b	CCTGGCGCTGGGCGAAAAGCCAC	78	*Pkc53E*
4R	GAAGCCAACGAACTCCGACTG	69	*Pkc53E*
4Rb	GTGGGCGTCAAGTCTGTTTTC	64	*Pkc53E*
4Rc	AATCAGGTGTACCGCAGAAAG	62	*Pkc53E*
4Rd	AATGGCATACAACTCCTCGCT	62	*Pkc53E*
4Re	GATCCTTGTCTTCGGGCTTTA	62	*Pkc53E*

TABLE 2.2. Primers used for PCR or sequencing (continued).

Name	Sequence (5′→3′)	T_m(°C)	Target
P	CGACGGGACCACCTTATGTTATTTCATCATG	90	*P*-element end
AAP[1]	GGCCACGCGTCGACTAGTAC(GGGII)$_3$G	118	Oligo(dC) tail
AP[2]	GACTCGAGTCGACTAGTAC	58	Adapter sequence
dTAP[3]	GACTCGAGTCGACTAGTAC(T)$_{17}$	92	Poly(A) tail
T3	ATTAACCCTCACTAAAG	46	T3 promoter
T7	AATACGACTCACTATAG	46	T7 promoter

[1]Abriged adapter primer; [2]Adapter primer; [3]Oligo(dT)$_{17}$ adapter primer

Figure 2.3 shows the location of each *Pkc53E*-targeted primer relative to the published cDNA.

H. Suppliers of Enzymes and Reagents

Chemicals were supplied by GIBCO/BRL, Fisher Scientific, or VWR, unless otherwise indicated. Antibiotics were supplied by Sigma Chemical Company, unless otherwise indicated. Enzymes and enzyme reaction buffers were supplied by GIBCO/BRL, unless otherwise indicated.

II. METHODS:

A. *Drosophila*

1. *Drosophila* culture conditions

All stocks were maintained on standard cornmeal/yeast/sugar/agar medium (Lewis, 1960). Experimental and maintenance stocks were maintained at room temperature (18-22°C) on a 24 hour light cycle, unless otherwise indicated.

2. *P*-element mutagenesis and screening

For *P* element mutagenesis, I used a modification of the screening procedure of Ballinger and Benzer (1989) that uses PCR to screen for heterozygous insertional mutants.

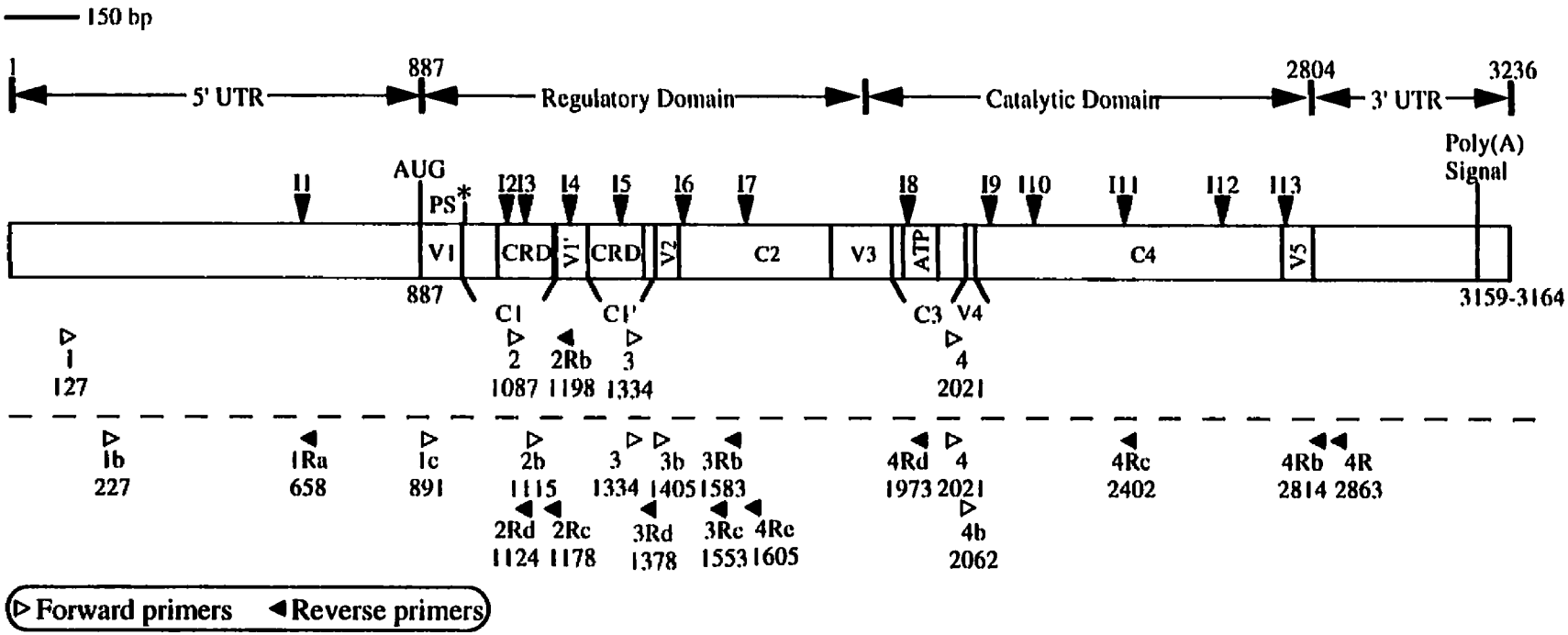


FIGURE 2.3. Location of *Pkc53E* primers relative to the published cDNA sequence. Primers are labelled with their name and the location of their 5′ end relative to the published +1 of transcription of *Pkc53E* as reported by Rosenthal *et al.* (1987). Each primer sequence was compared to the public sequence databases using the Blast search program (Altschul *et al.*, 1990). Primers 1, 2, 3, and 4 were used for *P* element mutagenesis PCR screening. Other primers were used for priming reverse transcription, 5′ RACE, RT-PCR, or 3′ RACE procedures. The triangles (◀, ▷) show primer locations with the position of the 5′ ends indicated immediately below each arrowhead. Primers used for *P* element mutagenesis screening are shown above the dashed line. Primers used for RACE and RT-PCR analyses are shown below the dashed line.

This screen relies on the absolute necessity of the conserved 31-bp inverted repeats at the ends of *P* elements for transposition. Transposition of a *P* element into the gene of interest can translocate the conserved 31-bp sequence *cis* to gene-specific sequences. PCR using primer pairs consisting of gene-specific sequences and the 31-bp repeat sequence enables the detection of a PCR product (Figure 2.4). True amplification products can be differentiated from spurious products by Southern hybridization.

I modified this screen so that flies with novel *P* elements are marked with the w^+ gene in a *w* background. Mobilization of *P* elements from an ammunition chromosome, using the genetic transposase source P[ry^+ Δ2-3(99B)], results in the transposition of the w^+ marker between the *P*-element ends. Male progeny with a *P* element were selected on the basis of a phenotypic change from white eyes to non-white eyes. These progeny were collected, crossed to *w* virgin females, and screened by PCR. True-breeding strains may then be established from positive primary screens through a series of subsequent crosses and screening steps (Figure 2.5).

3. Dissection of adult *Drosophila*

Adult *Drosophila*, 2-5 days old, were dissected in cold *Drosophila* Ringers solution under a dissecting microscope. Dissected tissues were immediately put into ice-cold *Drosophila* Ringers and kept on ice. Once enough tissues were collected, the Ringers solution was removed and the RNA was immediately extracted following the procedure below.

The isolation of 100 μg of total RNA required approximately 175 female heads, 235 male heads, 325 pairs of testes, 25 pairs of ovaries, or 10 whole female flies.

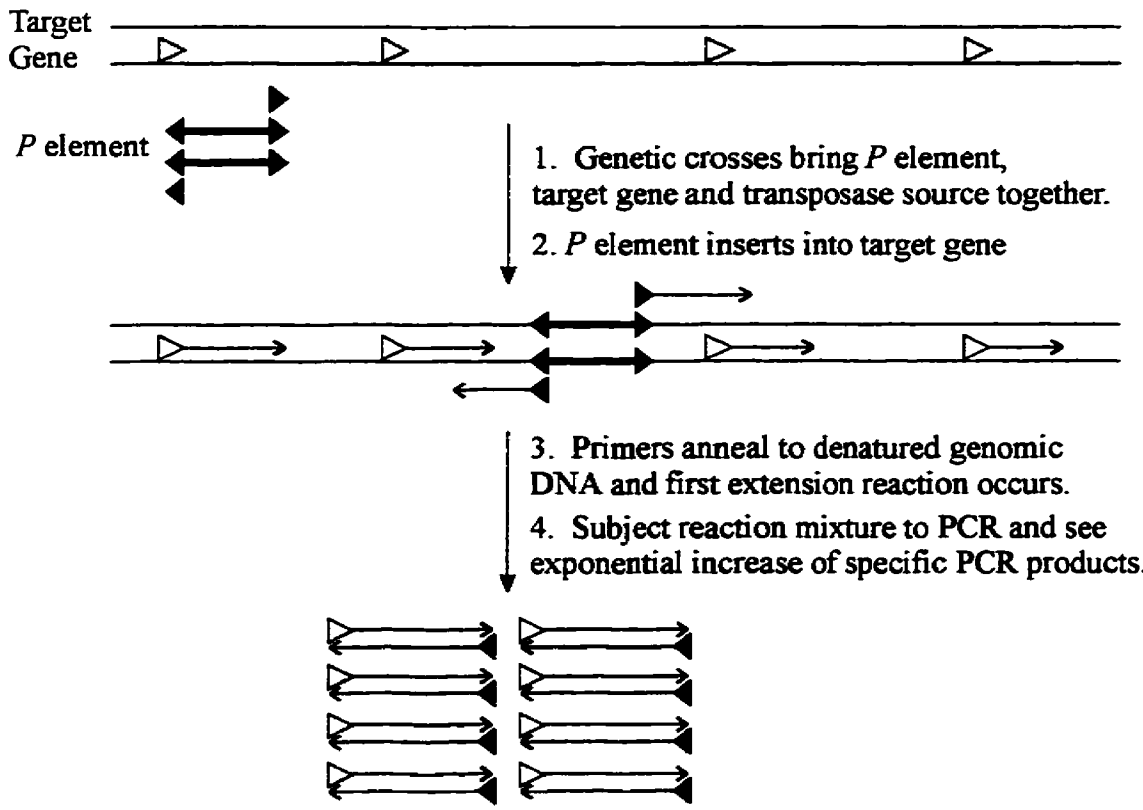


FIGURE 2.4. Schematic diagram of PCR screening for *P* element mutagenesis. Insertion of a *P* element into a gene of interest, brings the *P* element ends *cis* to a gene-specific primer (GSP). PCR using the *P* element primer and a GSP results in the appearance of a novel PCR product that will hybridize to gene-specific probes. Primers complementary to the *P* element 31-bp repeat are represented by the black triangles (◄ ►). GSPs are represented by the open triangles (▷).

FIGURE 2.5. Crosses for *P*-element mutagenesis. C(1)RM, *y w* 4P[*lac*W]; +/+; +/+ is the mutator strain with four P[*lac*W] elements (ammunition) on an attached-X chromosome. Only male progeny from Cross 2 with a *P*-element transposed onto an autosome or Y chromosome are w^+. *w*/Y; +/+; *Dr*/TMS, *Sb*, P[(ry^- Δ2-3)99B] supplies the chromosomal source of transposase. Cross 1 brings the chromosomal transposase (P[(ry^+ Δ2-3)99B]) and the ammunition chromosome into the same germline resulting in mobilization of the ammunition *P*-elements. Cross 2 allows the chromosomal transposase to be crossed out of the progeny, stabilizing the transposed *P* elements. Subsequent crosses serve to propagate the mutated chromosomes and allow screening.

Cross 1:

20♀♀ C(1)RM, *y w* 4P[*lac*W]; +/+; +/+ X 10♂♂ *w*/Y; +/+; *Dr*/TMS, *Sb*, P[*ry*$^{+}$Δ2-3)99B]

Cross 2:

5♀♀ C(1)RM, *y w* 4P[*lac*W]; +/+; +/TMS, *Sb*, P[*ry*$^{+}$Δ2-3)99B] X 3♂♂ *w*/Y; +/+; +/+

Cross 3:

10♂♂ *w*/Y; P[*lac*W]/+; +/+ (or) 10♂♂ *w*/Y; +/+; P[*lac*W]/+ X 20♀♀ *w*; +/+; +/+

Transfer at days 5 and 9.
Pool males from 5 bottles.
Analyze DNA *via* PCR and
Southern blot analysis.

positive — negative

Collect about 100 male and female *w*; P[*lac*W]/+; +/+ progeny from each of the 5 original bottles.
Maintain each set of males at 17°C.
Analyze each set of female DNA via PCR.

Discard

Cross 4:

5♂♂ *w*/Y; P[*lac*W]/+; +/+ X 20♀♀ *w*; +/+; +/+

Transfer at days 3, 6, and 9.
Pool males from 5 bottles.
Analyze their DNA *via* PCR and
Southern blot analysis.

positive — negative

Collect about 40 male and female *w*; P[*lac*W]/+; +/+ progeny from each of the 5 original bottles.
Maintain each set of males at 17°C.
Analyze each set of female DNA via PCR.

Discard

Cross 5:

1♂ *w*/*Y*; P[*lac*W]/+; +/+ X 15♀♀ *w*; +/+; +/+

Transfer at day 5.
Analyze DNA of first brood *via* PCR.

positive — negative

Discard

Make balanced stock by crossing to *w*; T(2;3)*ap*^{Xa}/SM5, *Cy*; TM3, *Sb*.
Determine site of insertion by direct sequencing of PCR product and/or ***in situ*** hybridization to polytene chromosomes.
Determine phenotype of homozygotes, if possible.

B. Nucleic Acid Isolation

1. Plasmid DNA

Bacterial cultures: Liquid cultures were grown in LB broth or Terrific Broth at 37°C on a rotary shaker at 200-300 rpm. When required, ampicillin was added to a final concentration of 100 μg/ml, streptomycin to a final concentration of 50 μg/ml, and/or tetracycline to a final concentration of 12 μg/ml. For long term storage, 1 ml aliquots of stationary liquid cultures were frozen at -80°C in 10% (v/v) glycerol.

For the growth of single colonies, bacteria were plated onto LB agar and grown at 37°C for 12-16 hours. When required, antibiotics were added to the appropriate final concentrations. For blue/white colony selection, X-gal was added to the medium to a final concentration of 50μg/ml or 40 μl of a 20 mg/ml solution was spread onto the surface of a 90 mm plate.

Crude small-scale plasmid isolation: For restriction enzyme screening, small-scale plasmid isolation was performed using the alkaline lysis plasmid mini-prep (Ausubel *et al.*, 1996), except that 100 μg/ml of deoxyribonuclease (DNAse)-free RNAse A (Pharmacia) was added to the GTE buffer.

Sequencing grade small-scale plasmid isolation: For sequencing of plasmids, small-scale plasmid isolation was performed from 4.5 ml of overnight culture by a modified alkaline-lysis/PEG precipitation procedure from Applied Biosystems, Inc., Terminator Cycle Sequencing Kit. Essentially, the bacterial pellet was resuspended in 200 μl of GTE buffer. The bacteria were lysed with 300 μl of 0.2 N NaOH/1% (w/v) SDS and inverted until clear. Cellular debris and chromosomal DNA was precipitated with 300 μl of 3.0 M potassium, 5.0 M acetate (Sambrook *et al.*, 1989), and incubated on ice for 5 minutes. The mixture was then centrifuged for 10 minutes at room temperature and the supernatant transferred to a clean tube. DNAse-free RNAse A (Pharmacia) was added to a final concentration of 20 μg/ml and incubated at 37°C for 1 hour.

To remove excess proteins and RNAse the supernatant was extracted twice with a 1:1 mixture of buffer-saturated phenol (GIBCO/BRL) and 24:1 (v/v) chloroform:isoamyl alcohol (PCI), followed by two extractions with 24:1 (v/v) chloroform:isoamyl alcohol (CI).

The aqueous phase was removed to a new tube and precipitated with an equal volume of 100% ethanol. The DNA pellet was washed with 500 μl of 70% ethanol and air-dried $\geq$10 minutes.

The DNA pellet was resuspended in 32 μl of water and reprecipitated by first adding 8 μl of 4 M NaCl and then 40 μl of autoclaved 13% polyethylene glycol $(PEG)_{8000}$, followed by thorough mixing and incubation on ice for 20 minutes. The plasmid DNA was pelleted by centrifugation at 12,000 x *g* for 15 minutes at 4°C in a fixed angle rotor. The pellet was washed with 500 μl of 70% ethanol and air dried. The DNA was then resuspended in 45 μl of water and stored at 4°C.

Large-scale plasmid isolation: For large-scale plasmid isolation, 100 ml to 500 ml of an overnight bacterial culture was pelleted and processed as per the sequencing plasmid isolation with the omission of the reprecipitation with PEG. Volumes were scaled up proportional to the amount of bacterial culture used.

2. Genomic DNA isolation

Isolation for PCR screening of mutagenized flies: DNA was isolated from adult flies for PCR with the method of Ballinger and Benzer (1989), with DNAse-free RNAse A added to the extraction buffer to a final concentration of 100 μg/ml. In addition, the DNA solution was extracted once with PCI and once with CI prior to the reprecipitation of the DNA.

Isolation for plasmid rescue, long PCR, and Southern blot analyses: DNA was isolated from 10 adult flies by homogenization in 250 μl of DNAzol™ (GIBCO/BRL). Extracellular debris and RNA was removed by centrifugation at 16,000 x *g* at room temperature for 5 minutes. The DNA was precipitated from the supernatant with 125 μl of 100% ethanol, mixed by gentle inversion and incubated at room temperature for 3 minutes. The DNA was pelleted by centrifugation at 16,000 x *g* for 5 minutes, washed twice with 1 ml of 95% ethanol and air dried briefly. The pellet was resuspended in 8 mM NaOH at 37°C. If required, the solution was neutralized with HEPES buffer as per the manufacturer's protocol.

3. Isolation of total RNA

RNA was isolated from 25 adult flies, 75 dissected heads, 75 pairs of dissected ovaries or 150 pairs of dissected testes with 500 μl of TRIzol™ reagent (GIBCO/BRL) according to manufacturer's protocols. To remove extracellular debris prior to phase separation, an additional centrifugation step (12,000 x *g* for 10 minutes) was included during the isolation process. The RNA pellet was stored at -20°C under 75% ethanol until resuspension in diethyl pyrocarbonate (DEPC)-treated water. After resuspension and quantization of the RNA, aliquots were made up to 50 μl volumes with DEPC-treated water and reprecipitated with 200 mM LiCl and 3 volumes of 100% ethanol and stored at -20°C until use. Immediately prior to use, the RNA was centrifuged at 12,000 to 16,000 x *g* for 15 minutes at 4°C or room temperature. The RNA pellet was washed with 75% ethanol, air dried, and resuspended in DEPC-treated water or hybridization buffer at the appropriate concentration.

4. Isolation of polyA$^+$ RNA

PolyA$^+$ RNA was isolated from 5 to 10 μg of total RNA using prepacked oligo(dT) cellulose columns from GIBCO/BRL (catalogue number 15939-010), according to the protocol provided. The yield of polyA$^+$ RNA from total whole female RNA was approximately 0.8%; the yield from total ovary RNA was approximately 1.0%.

5. Quantization of nucleic acids

Nucleic acids were quantified with UV light on a spectrophotometer according to Sambrook *et al.* (1989).

C. Manipulations of DNA

1. Restriction endonuclease digestion

Digestion of DNA was performed according to enzyme supplier's protocols.

2. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate, identify, and isolate DNA fragments. Agarose gel electrophoresis was carried out using 0.8 to 1.5% agarose gels with 0.1 μg/ml ethidium bromide in 1X TAE buffer according to Sambrook *et al.* (1989).

3. Cloning of DNA fragments

Gel purification of DNA fragments: DNA fragments were excised from a 0.8% to 1.0% agarose gels, solubilized in 6 M sodium iodide, and purified with silica suspension in 3 M guanidine isothiocyanate, according to the protocol of Boyle and Lew (1995).

Vector preparation:

(1) Removal of restriction enzymes: When heat inactivation was not possible, restriction enzymes were removed from vector preparations by one extraction with PCI, followed by one extraction with CI. Vector DNA was precipitated from the aqueous phase with 160 mM LiCl and 2½ volumes of 95% ethanol.

(2) Dephosphorylation: To prevent self-ligation of vector in non-directional cloning reactions (single-enzyme vector digestion), the vector was dephosphorylated prior to ligation (5′ phosphates removed). Following vector digestion and gel purification, 0.5 μg to 2 μg of DNA was incubated in 20 μl volumes with 5-10 units (U) of calf intestinal alkaline phosphatase (CIAP) and 1X dephosphorylation buffer (GIBCO/BRL) at 37°C for ≥2 hours. The CIAP was inactivated by heating at 65°C for 10 minutes. Aliquots of dephosphorylated vector were added directly to ligation reactions.

Blunt-ending of DNA fragments: When ligation of incompatible restriction digest ends was done, blunt ends were produced using T4 DNA polymerase according to the enzyme supplier's protocols (GIBCO/BRL).

Ligation: Ligations were carried out in 10 to 20 μl volumes with 0.1 to 2 μg DNA (a molar ratio of vector:insert of 1:3 to 1:6), 1X ligation buffer with or without 5% (w/v) PEG_{8000} and 1 U T4 DNA ligase. The ligation mix was incubated at 15°C ≥12 hours. 1 to 10 μl of the ligation mixture was used to transform $CaCl_2$-competent *E. coli*. For electrotransformation, ligation mixtures were brought up to 50 μl volume with water and extracted once with PCI and once with CI. The DNA was precipitated from the supernatant with 160 mM LiCl and 2 volumes of 95% ethanol at -20°C for ≥30 minutes. The DNA was pelleted by centrifugation at 16,000 x *g* for 15 minutes, washed once with 70% ethanol, air dried, and resuspended in sterile ddH_2O.

Transformation of E. coli:

(1) Calcium chloride: Competent *E. coli* were prepared using calcium chloride and transformed as per Sambrook *et al.* (1989). For storage, sterile glycerol was added to 10% (v/v) and 200 µL aliquots of competent cells frozen at -70°C.

(2) Electrotransformation: LB broth with 0.2% glucose was inoculated with $^1/_{100}$ volume of a saturated culture of *E. coli* and grown at 37°C with aeration to an A_{600} of 0.75. The culture was chilled on ice for 15 minutes then collected by centrifugation (4000 x *g*) at 4°C for 10 minutes. The cells were washed by sequential resuspension and centrifugation in 1 volume of ice-cold sterile water, ½ volume of ice-cold sterile water, $^1/_{50}$ volume of ice-cold sterile 10% glycerol and finally in $^1/_{500}$ volume of ice-cold sterile 10% glycerol. This 500-fold concentrated suspension was used immediately or distributed in 40 µl aliquots, frozen on dry ice and stored at -70°C. Frozen cells were thawed on ice immediately before use.

For transformation, 1 to 4 µl of DNA in water was added to a 40 µl aliquot of cells. The mixture was transferred to the bottom of an electroporation cuvette (10-mm gap) on ice. Immediately before electroporation all moisture was removed from the outside of the cuvette with a tissue and the cuvette was placed in the safety chamber of the electroporator and pulsed. A field strength of 12.5 kV/cm and a pulse length of 5.0 ms with 200 Ω resistance and 25 µF capacitance was used. Immediately following the pulse, 1 ml of SOC broth was added and the mixture transferred to a 17 x 100 mm polypropylene tube and incubated at 37°C with shaking for 1 hour. 100 to 200 µl of this suspension was plated onto LB agar and incubated overnight. If more than 200 µl of this suspension needed to be plated, the cells were pelleted by brief centrifugation (12,000 to 16,000 x *g*) in a microcentrifuge and resuspended in 100 to 200 µl of LB broth.

4. Exonuclease III deletion of plasmid DNA

When convenient restriction endonuclease sites were not available for cloning, exonuclease III was used to remove unwanted DNA. Exonuclease III deletions were performed according to the procedures outlined in the *double-stranded* Nested Deletion Kit

(Pharmacia/P-L Biochemicals) with the exonuclease III buffer set listed in Table 2.1 and the changes outlined below.

Preparation of plasmid DNA: Briefly, 10 μg of plasmid DNA was linearized with *Kpn*I to give a 3′-protected overhang followed by digestion with an enzyme leaving a 5′ overhang or blunt end adjacent to the insert DNA (deletion target). The plasmid DNA was then extracted once with PCI and once with CI followed by precipitation with 165 mM NaCl, 5 μg glycogen and 2½ volumes of 95% ethanol at -20°C for ≥30 minutes. Digested DNA was collected by centrifugation at 16,000 x *g* for 10 minutes. The pellet was washed once with 70% ethanol and air dried briefly prior to resuspension at a concentration of 0.1 μg/μl in 10 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$.

Exonuclease III deletion: Prior to exonuclease III deletion reactions, 100 μl of 1X S1 nuclease buffer was made up in distilled water with 50 U S1 nuclease (Pharmacia). 3 μl aliquots were pipetted into each of 21 microfuge tubes labelled 0 to 20 and placed on ice.

For exonuclease III deletions 20 μl of 2X exonuclease III buffer and 20 μl of digested plasmid DNA were mixed together. The mixture was equilibriated at 30°C for 1 minute and a 2 μl aliquot (time=0 control) was removed, mixed in the appropriate tube of S1 nuclease/buffer, and kept on ice. Then 1 μl of 65 U/μl exonuclease III (GIBCO/BRL) was added to the DNA/exonuclease III buffer reaction mixture, gently mixed, and incubation at 30°C continued. Every 1 minute, 2 μl aliquots were removed, mixed in the appropriate tube of S1 nuclease/buffer, and kept on ice. After all of the timed aliquots had been removed and mixed with the S1 nuclease/buffer, the samples were incubated at room temperature for 30 minutes. 1 μl of S1 stop buffer was added to each sample and all were incubated at 65°C for 10 minutes to stop the reaction.

Agarose gel electrophoresis of 3 μl of each reaction was used to determine the correct sized deletions for further analysis, the selected deletions were ligated in a total volume of 10 μl in 1X DNA ligation buffer with 1.25 U of T4 DNA ligase overnight at 15°C. After ligation, the entire ligation mixture was used to transform 200 μl $CaCl_2$-competent *E. coli* DH10B. After colony growth, 10 to 15 colonies were picked at

random, grown in 5 ml overnight cultures, and analyzed by restriction digestion and agarose gel electrophoresis to choose the appropriate sized plasmid for sequencing.

5. DNA sequencing

Sequencing reactions: Plasmid DNA was sequenced using the T7 DNA Sequencing Kit (Pharmacia/LKB) with 2 μg plasmid DNA. The plasmid template was denatured with NaOH, according to Procedure C of the supplier's protocol. Sequencing reactions were analyzed by urea-polyacrylamide gel electrophoresis (PAGE).

PCR fragments were directly sequenced by primer walking using dideoxy-fluorescent nucleotide protocols on an ABI373 sequencer at the University of Calgary Core DNA Services Laboratory.

Urea-PAGE: Urea-PAGE was performed using 7M urea and 5% or 6% polyacrylamide in 1X TBE buffer (Ausubel *et al.*, 1996). Sequencing and RNAse protection gels were electrophoresed using a GIBCO/BRL SA32, SA66 or S2 apparatus according to manufacturer's instructions. Mini-gels were electrophoresed in a Biorad Protean-II apparatus at 175 V until the xylene cyanol FF dye was ½ to ¾ distance into the gel.

For drying, gels were lifted off of the gel plate using Whatman 3MM chromatography paper and dried on a Biorad 583 gel drier at 80°C under vacuum for 2 to 3 hours. Dried gels were exposed 48 to 72 hours at room temperature with Kodak X-OMAT AR film in an X-ray cassette.

Sequence analysis: Exposed films from sequencing gels were read manually and sequence was entered into the DNAsis program for contig assembly. For comparison to known sequences, either the Blast program (Altschul *et al.*, 1990) was used to compare determined sequences with the non-redundant database or the FASTA program of the Genetics Computer Group (GCG) was used to align sequences along their length.

6. Construction of plasmid vectors

The steps for the construction of the probe vectors used for RNA probe synthesis are described in Table 2.3.

For sequencing of intron 1, pSB29 was digested with *Stu*I and *Xba*I, followed by gel purification of the 4.3 kbp vector piece, blunt ending of the DNA with T4 DNA polymerase, and recircularization with T4 DNA ligase to produce pSB29ESt. PSB29ESt was digested with *Eco*RI and *Apa*I, followed by gel purification of the 3.7 kbp vector piece, blunt ending of the DNA with T4 DNA polymerase, and recircularization with T4 DNA ligase to produce pSB29ASt. This plasmid was sequenced by the University of Calgary Core Sequencing Facility using standard sequencing primers for pBluescribe M13+.

7. Plasmid rescue

Plasmid rescue was carried out according to the protocol outlined by Hamilton *et al.* (1991). Briefly, ≈5 μg of *Drosophila* genomic DNA was digested to completion with either *Eco*RI or *Xba*I. The restriction enzymes were then heat inactivated and the DNA circularized in a 250 μl volume with 2 to 5 U of T4 DNA ligase ≥12 hours at 15°C. Following PCI extraction and ethanol precipitation, the dried DNA pellet was resuspended in 5 μl of sterile ddH_2O and used to electrotransform *E. coli* DH10B cells. A schematic representation of plasmid rescue is given in Figure 2.6.

8. cDNA library screening

λ phage cDNA libraries were screened by plaque filter hybridization (Ausubel *et al.*, 1996). An antisense Pkc1 ^{32}P-labelled RNA probe, spanning nucleotides 1467 to 3072 (*Nco*I to *Xba*I) was used. The number of plaques screened was approximately two times the complexity of the library.

TABLE 2.3. Construction of probe vectors for RNAse protection assays.

Vector Name	Steps for Construction
p*Pkc1*(KS)	1. Digested p0161 with *Eco*RI and *Pvu*I; gel purified the 3.2 kbp Pkc1 cDNA⇒Pkc1 2. Cloned Pkc1 into pBluescript II KS+ linearized with *Eco*RI and dephosphorylated with CIAP; checked orientation with *Bam*HI digestion (released a 0.9 kbp fragment)⇒p*Pkc1*(KS)
p*Pkc1*(SK)	1. Digested p0161 with *Eco*RI and *Pvu*I; gel purified the 3.2 kbp Pkc1 cDNA⇒Pkc1 2. Cloned Pkc1 into pBluescript II SK- linearized with *Eco*RI and dephosphorylated with CIAP; checked orientation with *Bam*HI digestion (released a 0.9 kbp fragment)⇒p*Pkc1*(SK)
p*Pkc1*H696	1. Subjected p*Pkc1*(KS) to exonuclease III deletion. Isolated plasmid from three independent transformants from deletions of the approximate appropriate size and sequenced 3′ end of the Pkc1 insert⇒p*Pkc1*E696 2. Digested selected plasmid with *Hind*III and *Eco*RI; purified 3.2 kbp vector piece; blunted DNA ends with T4 DNA polymerase; recircularized with T4 DNA ligase⇒p*Pkc1*H696
p*Pkc1*BC	1. Cut p*Pkc1*(KS) with *Bam*HI; purified 5.3 kbp vector piece; circularized with T4 DNA ligase⇒p*Pkc1*BE 2. Cut p*Pkc1*BE with *Cla*I; purified 3.2 kbp vector piece; circularized with T4 DNA ligase⇒p*Pkc1*BC
p*Pkc1*CN	1. Cut p*Pkc1*(SK) with *Nco*I and *Pst*I; purified 4.4 kbp vector piece; blunted DNA ends with T4 DNA polymerase; circularized with T4 DNA ligase⇒p*Pkc1*EN 2. Cut p*Pkc1*EN with *Cla*I; purified 3.2 kbp vector piece; circularized with T4 DNA ligase⇒p*Pkc1*CN
p*Pkc1*NS	1. Cut p*Pkc1*(SK) with *Nco*I and *Eco*RV; purified 4.7 kbp vector piece; blunted DNA ends with T4 DNA polymerase; circularized with T4 DNA ligase⇒p*Pkc1*NE 2. Cut p*Pkc1*NE with *Xba*I and *Xho*I; purified 1.6 kbp piece⇒Xh-NE-X fragment 3. Cut Xh-NE-X fragment with *Sca*I; purified 298 bp piece⇒(Xh)NS fragment 4. Cloned (Xh)NS fragment into pBluescript II KS+ linearized with *Xho*I and *Eco*RV using T4 DNA ligase⇒p*Pkc1*NS

TABLE 2.3. Construction of probe vectors for RNAse protection assays (continued).

Vector Name	Steps for Construction
p*Pkc1*SBc	1. Cut p*Pkc1*BE with *Sca*I; purified 704 bp fragment→SS fragment 2. Cloned SS fragment into pBluescript II KS+ linearized with *Eco*RV and dephosphorylated with CIAP; checked orientation of fragment by digestion with *Bam*HI and *Bgl*II (correct orientation indicated by the release of a 191 bp fragment, not a 513 bp fragment)→p*Pkc1*SS(T7α) 3. Cut p*Pkc1*SS(T7α) with *Bam*HI and *Bcl*I; purified 3.2 kbp vector piece; circularized with T4 DNA ligase→p*Pkc1*SBc
p*Pkc1*BcBg	1. Cut p*Pkc1*NE with *Bgl*II and *Bam*HI; purified 3.8 kbp vector piece; circularized with T4 DNA ligase→p*Pkc1*NBg 2. Cut p*Pkc1*NBg with *Bcl*I and *Eco*RV; purified 3.2 kbp vector piece; blunted DNA ends with T4 DNA polymerase; circularized with T4 DNA ligase→p*Pkc1*BcBg
p*Pkc1*BgSt	1. Cut p*Pkc1*BE with *Stu*I and *Eco*RV; purified 4.6 kbp vector piece; circularized with T4 DNA ligase→p*Pkc1*BSt 2. Cut p*Pkc1*BSt with *Bam*HI and *Bgl*II; purified 3.2 kbp vector piece; circularized with T4 DNA ligase→p*Pkc1*BgSt
p*Pkc1*SC	1. Cut p*Pkc1*(KS) with *Sca*I; purified 2.6 kbp piece; cut this piece with *Eco*RV; purified 771 bp piece→SE fragment 2. Cloned SE fragment into pBluescript II KS+ linearized with *Eco*RV and dephosphorylated with CIAP→p*Pkc1*SE 3. Cut p*Pkc1*SE with *Cla*I; purified 3.2 kbp vector piece; circularized with T4 DNA ligase→p*Pkc1*SC
p*Pkc1*C3023	1. Subjected p*Pkc1*(KS) to exonuclease III deletion. Isolated plasmid from three independent transformants from deletions of the approximate appropriate size and sequenced 3′ end of *Pkc1* insert→p*Pkc1*E3023 2. Digested selected plasmid with *Cla*I and *Eco*RI; purified 3.3 kbp vector piece; blunted DNA ends with T4 DNA polymerase; recircularized with T4 DNA ligase (this reconstitutes the *Eco*RI site)→p*Pkc1*C3023
p*rp49*	1. Cut pHR0.6 with *Eco*RI and *Hind*III; purified 640 bp piece→ *rp49* fragment 2. Cloned *rp49* fragment into pBluescript II KS+ linearized with *Eco*RI and *Hind*III→p*rp49*EH

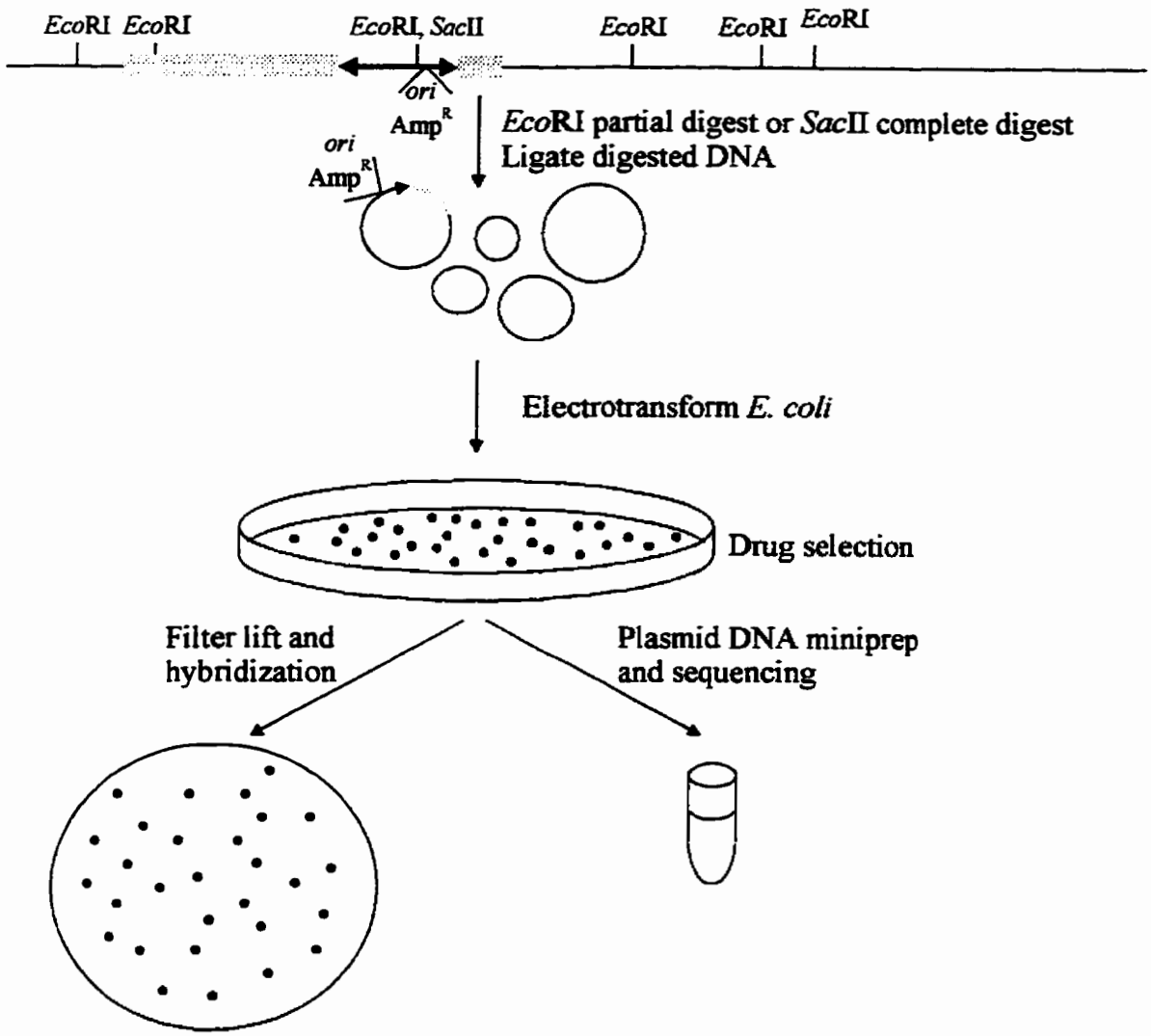


FIGURE 2.6. Schematic diagram of plasmid rescue using P[lac*W*]. Digestion of genomic DNA with the appropriate enzyme, circularization, and electrotransformation into *E. coli* results in the cloning of *Drosophila* DNA sequences adjacent to *P* element ends. Transformed colonies can then be used for either plasmid purification or filter hybridization procedures.

D. Polymerase Chain Reactions for *P* element Screens

PCR for screening P element insertions were carried out using standard protocols in 20 μL reaction volumes (Table 2.4).

TABLE 2.4. PCR screening for *P* element insertions.

Reaction Components	Cycling Profile
1X PCR buffer (GIBCO/BRL) 1.5 mM $MgCl_2$ 200 μM each dNTP (dATP, dCTP, dGTP, dTTP) 0.4 μM each oligodeoxynucleotide (Primers 1, 2, 3, 4, and P or Primers 2 and 2Rb) 0.5 μg genomic DNA 0.5 U *Taq* DNA polymerase (GIBCO/BRL)	94°C for 5 minutes --- 94°C for 1 minute 55°C for 1.5 minutes 40 cycles 72°C for 3 minutes --- 72°C for 10 minutes

The entire reaction mixture was then subjected to gel electrophoresis. For the second screen, each gel was Southern blot hybridized using DIG-labelled Pkc1 cDNA as a probe according to procedures outlined below.

E. DNA Probe Synthesis

Digoxigenin (DIG)-labelled probes were synthesized and quantitated using the materials and methods of the DIG Hybridization and Detection Kit (Boerhinger/Mannheim) according to manufacturer's instructions. Hybridization was performed with 10 ng of DIG-labeled probe per 1 ml of hybridization fluid.

F. Southern Transfer and Hybridization

Prior to transfer, gels were treated according to the membrane manufacturer's instructions. DNA was transferred to Hybond N+ nylon membrane (Amersham) by downward transfer using 10X SSC and a Turboblot apparatus (S&S) for 3 to 5 hours. DNA was fixed to the membrane by laying the membrane, DNA-side up, on a piece of filter paper saturated with 0.4 M NaOH for 20 to 30 minutes. Membranes were neutralized by washing briefly with 5X SSC and then air dried.

Prior to hybridization with DIG-labeled probes, membranes were prehybridized in DIG hybridization buffer without probe for 1 to 3 hours at 65°C. Hybridization buffer with probe was boiled for 10 minutes to denature probe, then cooled in an ice-water bath immediately prior to addition to the hybridization bag. Membranes were hybridized at 65°C 12 to 16 hours. Following hybridization, membranes were washed twice in 2X SSC/0.1% SDS at room temperature for 5 minutes, then twice in 0.1X SSC/0.1% SDS at 65°C for 15 minutes.

DIG probe detection was performed according to supplier's protocol (Boehringer/Manheim).

G. Manipulations of RNA

1. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and identify full-length RNA. Denaturing agarose gel electrophoresis was carried out using 1.4% agarose gels with 0.66 M formaldehyde and 1X MOPS buffer according to Sambrook *et al.* (1989).

2. RNA probe synthesis and purification

RNA probe synthesis: DNA templates were linearized and purified according to Ausubel *et al.* (1996). Probes were synthesized in a 20 μl volume from 1 μg DNA template in 1X transcription buffer (GIBCO/BRL), 10 mM dithiothreitol (DTT) (GIBCO/BRL), 20 to 25 U RNAguard (Pharmacia), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM UTP, 12.5 μM CTP, 5.0 μl α-^{32}P-CTP (10 μCi/μl, 400 mCi/mmol) and 25 U T3 or T7 RNA polymerase (GIBCO/BRL). The synthesis reaction was incubated at 37°C for 1 hour followed by digestion with 1 μl Amplification Grade DNAse I (GIBCO/BRL) for 15 minutes. The reaction mixture was made up to 50 μl volume with DEPC-treated water and 10 μg yeast tRNA. Unincorporated nucleotides were removed by PCI extraction followed by two consecutive precipitations with ½ volume of 7.5 M ammonium acetate and 3 volumes 100% ethanol or by gel purification.

Gel purification of RNA probes: When necessary, RNA probes were gel purified after the first ethanol precipitation by urea-PAGE according to the method of Ausubel *et al.* (1996).

Synthesis of radiolabelled RNA ladder: Radiolabelled RNA ladders were synthesized from 1 μg of an equimolar mixture of pBluescript II SK+ plasmid digested with one of *Xho*I, *Bam*HI, *Bss*HII, or *Pvu*II using T7 RNA polymerase and α-^{32}P-CTP, as described above for RNA probe synthesis. This produced a four-marker ladder with RNAs 25 nt, 76 nt, 149 nt, and 334 nt long. 2 μl of the synthesis reaction was mixed with 2 μl of loading dye (95% (v/v) formamide/0.25% (w/v) bromophenol blue/0.25% (w/v) xylene cyanol FF) prior to urea-PAGE (Ausubel *et al.*, 1996).

H. Northern transfer and hybridization

Prior to transfer, gels were soaked for 20 minutes in sterile ddH_2O to remove excess formaldehyde. RNA was transferred to Hybond N or Hybond N+ nylon membrane (Amersham) by downward transfer using 10X SSC and a Turboblot apparatus (S&S) for 3 hours. RNA was fixed to the membrane by exposure to short-wave UV light for 3 to 5 minutes.

Membranes were prehybridized in RapidHyb Buffer (Amersham) for 15 minutes to 1 hour. ^{32}P-labelled RNA probes were denatured at 80°C for 10 minutes and then 5 x 10^5 to 1 x 10^6 cpm per ml of hybridization buffer were directly added. Hybridization was carried out at 70°C for 1 to 2 hours. Membranes were washed twice in 2X SSC/0.1% SDS for 5 minutes at room temperature, then twice in 0.2X SSC/0.1% SDS at 45°C for 15 minutes.

Washed membranes were rinsed briefly in 0.2X SSC, blotted to remove excess SSC, and then sealed in hybridization bags. Autoradiography was performed at -70°C for 1 to 7 days with Kodak X-OMAT AR film and intensifying screens.

I. RNAse protection analysis

1. Hybridization

Aliquots of 10 or 15 μg of total *Drosophila* RNA were pelleted as described above. The pellet was thoroughly air dried and then resuspended in 30 μl of hybridization buffer (80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA) with 1 to 5 x 10^5 cpm probe. The RNA:probe mixture was heated to 80°C for 10 minutes, transferred immediately to 45°C, and hybridized 12 to 16 hours.

2. RNAse digestion and analysis

After hybridization, 0.35 ml of digestion solution (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 300 mM NaCl, 40 μg/ml RNAse A) was added to the hybridization mixture and incubated at room temperature for 1 hour. The RNA was then purified, pelleted, and analyzed by urea-PAGE as in Ausubel *et al.* (1996).

J. Isolation of *Pkc53E* cDNAs

1. cDNA synthesis

For 3'-RACE, cDNA was primed with 50 pmol of oligo$(dT)_{17}$-adapter primer (dTAP). For RT-PCR cDNA was primed with 50 pmol oligo$(dT)_{12-18}$. cDNA was synthesized from 5 μg of total RNA using the GIBCO/BRL Superscript Preamplification System for First Strand cDNA Synthesis according to manufacturer's protocol, unless otherwise indicated.

2. 3'-RACE PCR

For amplification of 3'-RACE products, 2 μl of the first strand cDNA reaction was used for amplification in a 100 μl PCR with *Taq* DNA polymerase under the conditions specified in Table 2.5 or in a 50 μl PCR with ELONGASE™ under the conditions specified in Table 2.6.

TABLE 2.5. 3'-RACE PCR conditions with Primers 4 or 4b.

Reaction Components	Cycling Profile
1X PCR buffer (GIBCO/BRL) 1.5 mM $MgCl_2$ 200 μM each dNTP (dATP, dCTP, dGTP, dTTP) 10 pmol Primer 4 or 4b 10 pmol Adapter Primer (AP) 2 μl First strand cDNA synthesis reaction or 1 μl Frist 3'-RACE reaction 2.5 U *Taq* DNA polymerase (GIBCO/BRL)	94°C for 3 minutes --- 94°C for 45 seconds 60°C for 30 seconds 35 cycles 72°C for 1.5 minutes --- 72°C for 10 minutes

TABLE 2.6. 3'-RACE PCR conditions with Primers 3 or 3b.

Reaction Components	Cycling Profile
1X ELONGASE™ reaction buffer (GIBCO/BRL) 1.8 mM $MgCl_2$ 200 μM each dNTP (dATP, dCTP, dGTP, dTTP) 10 pmol Primer 3 or 3b 10 pmol Adapter Primer (AP) 2 μl First strand cDNA synthesis reaction or 1 μl Frist 3'-RACE reaction 2 U ELONGASE™ (GIBCO/BRL)	94°C for 3 minutes --- 94°C for 45 seconds 56.5°C for 30 seconds 35 cycles 68°C for 3.5 minutes ---

For best results, two rounds of amplification were done. Primers 4 and AP, or 3 and AP, were used for the first round and primer 4b and AP, or 3b and AP, for the second round. 5% to 10% of the second 3'-RACE reaction was electrophoresed and Southern blotted and hybridized using Pkc1 cDNA DIG-labelled probe.

3. RT-PCR

RT-PCR for Southern hybridization: For RT-PCR, 2 μl of the first strand cDNA reaction was used for amplification in a 50 μl PCR reaction with ELONGASE™ under the conditions outlined in Table 2.7.

TABLE 2.7. RT-PCR conditions.

Reaction Components	Cycling Profile
1X ELONGASE™ reaction buffer (GIBCO/BRL) 1.8 mM $MgCl_2$ 200 μM each dNTP (dATP, dCTP, dGTP, dTTP) 10 pmol Primer 3 10 pmol Primer 4R 2 μl First strand cDNA synthesis or 2 μl Frist RT-PCR reaction 2 U ELONGASE™ (GIBCO/BRL)	94°C for 30 seconds --- 94°C for 30 seconds 64°C for 30 seconds[1] 30-35 cycles 68°C for 3 minutes[2] ---

[1]The annealing temperature (T_A) used was optimized for each primer pair as follows: 1b+3Rb or 1b+4Rb, T_A=53.8°C; 1c+4Rb, 1c+4Rd, or 1c+4Re, T_A=57.3°C; 2b+4Rc or 2b+4Rd, T_A=55.8°C; 3b+4Rd, T_A=55°C; 4b+4R, T_A=58°C. [2]Extension times were calculated based on n+1 minutes, where n=kbp to be amplified, to ensure that longer than predicted PCR products would not be excluded.

When necessary, two rounds of amplification were done. The same primers were used for each round. The first RT-PCR reaction was PCI extracted then precipitated with 160 mM LiCl, 20 μg glycogen and 2 ½ volumes of 100% ethanol at -20°C for ≥30 minutes. The cDNA was pelleted by centrifugation at 16,000 x *g* for 15 minutes, washed once with 70% ethanol, air dried, and resuspended in 50 μl of sterile ddH_2O before subsequent PCR reactions. 1% to 10% of the RT-PCR was electrophoresed, Southern blotted, and hybridized using Pkc1 cDNA DIG-labelled probe.

When sequencing was to be done, the RT-PCR used for electrophoretic analysis was PCI extracted and ethanol precipitated as above. The entire mixture was then resuspended in 20 μl sterile TE buffer, pH 7.5, and subjected to gel electrophoresis. The correct sized band(s) was then excised from the gel and purified with 20 μl of silica suspension as outlined above.

RT-PCR for sequencing or restriction analysis: For PCR product sequencing, 1 μl of the gel purified RT-PCR product was amplified as per the conditions in Table 2.7. Extension times were calculated as 1 minute per kbp to be amplified.

For gel purification, DNA was precipitated with 160 mM LiCl, 20 μg glycogen and 2 1/2 volumes of 100% ethanol. The DNA was pelleted by centrifugation at 16,000 x *g* for

15 minutes. The pellet was washed once with 100 μl 70% ethanol, air dried, and resuspended in TE buffer, pH 8.0. DNA loading dye was added to 1X final concentration and the entire mixture was subjected to agarose gel electrophoresis. The correct sized bands were excised and purified with 50 μl silica suspension, as outlined above.

4. 5'-RACE

cDNA for 5'-RACE was synthesized from 1 μg of polyA$^+$ RNA using primer 4Rc, 3Rb, or 2Rc to prime reverse transcription. All reactions were carried out using the 5'-RACE System for rapid amplification of cDNA ends, Version 2.0, from GIBCO/BRL, according to the supplied protocol. Reverse transcription reactions were incubated at 42°C as per the standard protocol or at 50°C according to the alternate protocol for reducing RNA secondary structure. Primer 4Rd, 3Rc, or 2Rd, respectively, was used in conjunction with the supplied abridged anchor primer (AAP) for amplification in a 50 μl volume according to the profile outlined in Table 2.8.

TABLE 2.8. 5'-RACE amplification conditions.

Reaction Components	Cycling Profile
1X ELONGASE™ reaction buffer (GIBCO/BRL) 1.8 mM $MgCl_2$ 200 μM each dNTP (dATP, dCTP, dGTP, dTTP) 10 pmol AAP 10 pmol Primer 4Rd 5 μl $(dC)_n$-tailed cDNA 2 U ELONGASE™ (GIBCO/BRL)	94°C for 30 seconds --- 94°C for 30 seconds 62°C for 30 seconds[1] 30-35 cycles 68°C for 3.5 minutes[2] ---

[1]The T_A used for 5'RACE was optimized for each primer pair as follows: 3Rc+AAP, T_A=59.6°C; 2Rd+AAP, T_A=62°C. [2]Extension times were calculated based on n+1 minutes, where n=kbp predicted to be amplified, to ensure that longer than predicted PCR products would not be excluded.

After amplification, 5 to 10 μl of each amplification reaction was analysed by agarose gel electrophoresis and Southern blot hybridization with Pkc1 cDNA DIG-labelled probe.

CHAPTER 3: *P* ELEMENT MUTAGENESIS

I. RESULTS

I did my first *P* element mutagenesis screen prior to having a full-length probe for Southern hybridization detection of PCR products. This screen therefore relied on visualization of unique PCR products on ethidium bromide-stained agarose gels. From approximately 400 vials of Cross 2, 936 w^- male progeny were screened by PCR and the results are summarized in Table 3.1. The w^+ phenotype confirms *P* element movement and represents a small pertinent subset of the total number of progeny screened. Of these, nine independent lines were established that gave unique PCR products from either primer 3 or primer 4 in conjunction with the *P* element primer (Table 2.2; Figure 3.1).

TABLE 3.1. Number of male progeny from Cross 2 screened following *P* element mutagenesis.

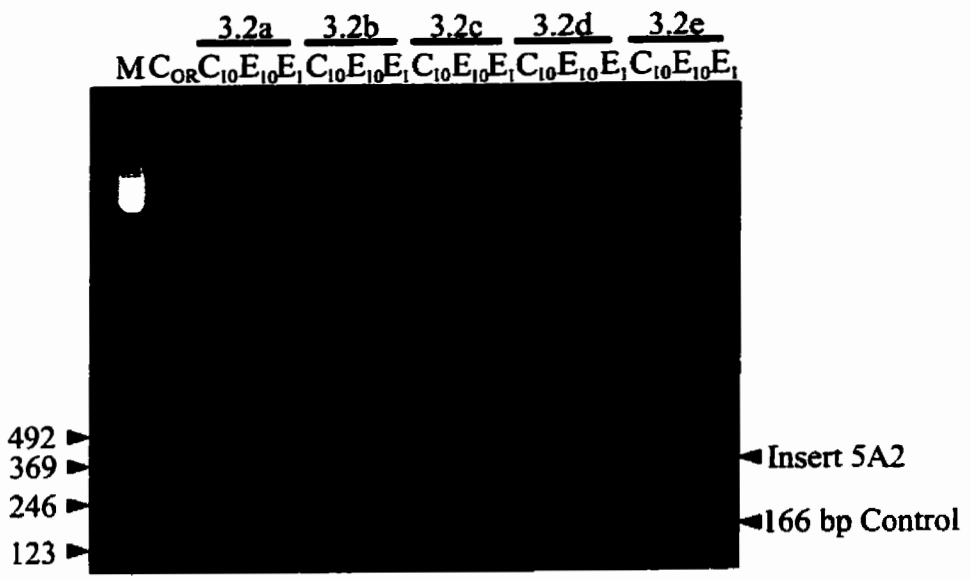
Screen	Number of w^+ ♂♂ PCR screened	Total number of w^+ ♂♂ screened	Total number of ♂♂ screened	Percent w^+
First	936	1,929	15,561	12.4 %
Second	8,673	16,990	102,634	16.5 %

Plasmid rescue and sequencing of *Drosophila* genomic DNA adjacent to the *P* element ends showed that these *P* elements were not within sequenced regions of *Pkc53E*. Figure 3.2A shows a comparison of the sequences obtained from plasmid rescue to each other. Sequences from strains 5E4a24-*Eco*RI and 5B4a3-*Eco*RI and 5F4a7-*Eco*RI and 5E4a7-*Eco*RI represent two pairs of rescued plasmids with overlapping sequence identity. These most likely represent insertions originating in siblings, as opposed to independent insertion events. Each of the other rescued sequences was unique.

To determine whether these insertions were located within known genes, the plasmid-rescue sequences were six-way translated and compared against protein databanks, most recently on February 15, 1997. Searches revealed only short stretches of weak similarity to some known sequences. These hits were not very long, nor particularly strong, often with one or more stop codons within the matched reading frame. This indicates that

FIGURE 3.1. **An example of *P* element insertional mutagenesis screening by PCR and agarose gel electrophoresis. A.** The appearance of a unique PCR band, 5A2, upon amplification with the screening primers from DNA sample 3.2a suggested that the *P* element was located near a *Pkc53E* primer. For each DNA sample, a parallel control PCR was done. **B.** PCR of DNA sample 3.2a using single experimental primer pairs indicates that the amplification product, 5A2, has sequences complementary to *Pkc53E* primer 3 and the *P* element primer. Lanes with control PCR samples are labelled C and those with screening (experimental) PCR samples are labelled E. Each experimental PCR was performed in duplicate with 500 ng or 40 ng of sample DNA (E_{10} or E_1, respectively). The appearance of a unique band with both PCRs was recorded as a positive result and isolation of the mutant chromosome in a balanced stock was done.

A.


3.2a
3.2b
3.2c
3.2d
3.2e
M C$_{OR}$ C$_{10}$E$_{10}$E$_1$ C$_{10}$E$_{10}$E$_1$ C$_{10}$E$_{10}$E$_1$ C$_{10}$E$_{10}$E$_1$ C$_{10}$E$_{10}$E$_1$
492
369
246
123
Insert 5A2
166 bp Control

B.

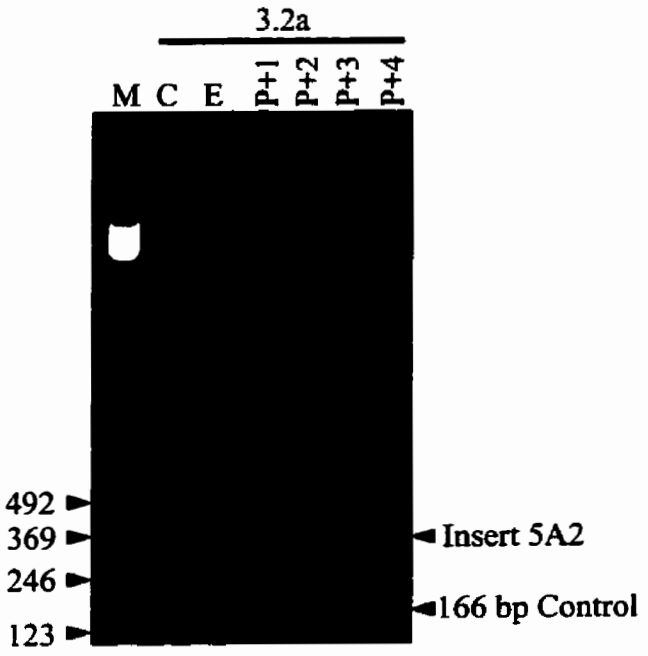
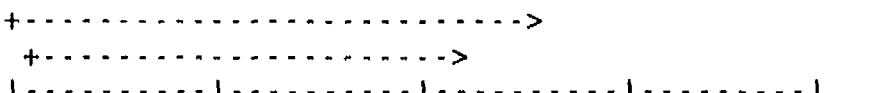

3.2a
M C E P+1 P+2 P+3 P+4
492
369
246
123
Insert 5A2
166 bp Control

FIGURE 3.2. Database search results for plasmid rescue sequences. A. A comparison among plasmid-rescue genomic sequences. Sequences are named for the strain designation from which they were isolated and the enzyme used for rescue. Regions of identity are indicated by the overlapping lines on the right hand side. The other rescued sequences were unique and are listed on the left. **B.** The alignment shown represents the best match of any of the plasmid rescue sequences and the only hit without stop codons within the matching region. 5E4a7-*Eco*RI shows only 24.7% similarity to the *D. melanogaster* cysteine-string protein 32 (*csp32*) mRNA over 73 amino acids. **C.** Three plasmid rescue sequences match with common repetitive sequences found in GenBank and EMBL databases.

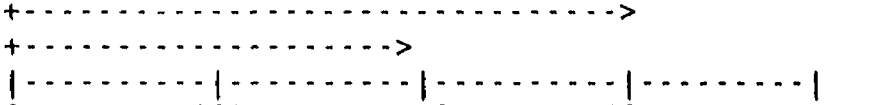
A.

5A2a12-*Eco*RI - no overlap
5B2a7-*Xba*I - no overlap
5A2a12-*Xba*I - no overlap
5B2a7-*Eco*RI - no overlap
5C4a1-*Eco*RI - no overlap
5C4a1-*Xba*I - no overlap
5E4a1-*Eco*RI - no overlap
5E4a1-*Xba*I - no overlap
5E4a53-*Eco*RI - no overlap

5B4a3-*Eco*RI
5E4a24-*Eco*RI

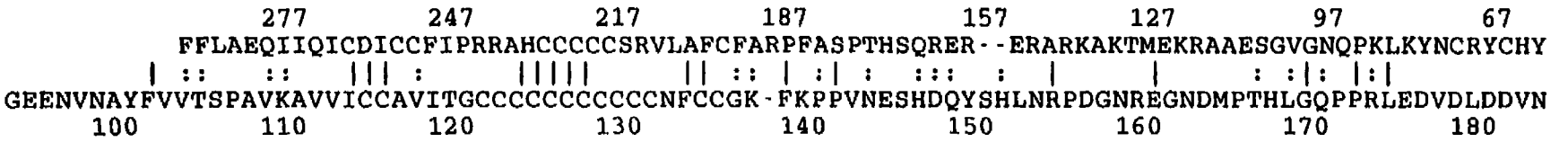


5E4a7-*Eco*RI
5F4a7-*Eco*RI



B.

5E4a7-*Eco*RI



Drosophila Cysteine String Protein (*csp32* mRNA)

C.

5B2a7-*Xba*I matched to ratrn55rp.seq, MMSQR2, DINUC-AG.SEQ
5E4a7-*Eco*RI matched to ratrn55rep.seq, DINUC-AG.SEQ
5F4a7-*Eco*RI matched to ratrn55rep.seq, DINUC-AG.SEQ

the hits are random and do not represent real homology or putative functional similarity. The alignment shown in Figure 3.2B represents the only hit without stop codons within the matching region. It is important to note however, that this same sequence (5E4a7-*Eco*RI) matches repetitive nucleotide sequences found during a search for common repetitive sequences. As shown in Figure 3.2C, two of the overlapping plasmid-rescue sequences also matched with common repetitive sequences found in the GenBank and EMBL databases.

The second *P* element mutagenesis screen was carried out using the same primers and crosses, however PCR products were run out on agarose gels, Southern blotted, and hybridized with a probe synthesized from the 3.2 kbp Pkc1 cDNA (Rosenthal *et al.*, 1987). For each PCR, a parallel positive control reaction, with primers 2 and 2Rb, was run to check DNA quality and hybridization efficiency. The positive control band hybridized on all 24 Southern blots for each control PCR reaction (*eg.* Figure 3.3). Of 8,673 male progeny, from about 1500 vials of Cross 2, subjected to PCR screening no inserts at *Pkc53E* were detected (Table 3.1). Taken together, a total of 118,195 males were screened for the w^+ phenotype and 9,609 mutagenized flies were further screened by PCR.

II. Discussion

Table 1.2 shows data from other reported screens. In the screen done by Dalby *et al.* (1995) mutations at the cytogenetic location nearest 53E, 54D, occurred at a frequency of 3/16,100 (or 1/5,367). The mutation frequency for all loci in this screen ranged from 0 to 4/16,100 (or 1/4,025). These data indicate that I screened a reasonable number of insertions.

In the screen performed by Pereira *et al.* (1992) a mutation frequency of 5/15,000 (or 1/3,000) was reported for a gene at 100EF, but no mutations were detected at three other loci screened in the same set of crosses. These five mutations were all located within 200 bp of each other, with four located within five base pairs of each other, and all were within the promoter region of the gene. Several other reports suggest that *P* elements often insert within the 5′ ends of genes upstream of the transcription unit or in the untranslated region of the transcription unit. Of 41 independent insertions at four different loci (*yellow*,

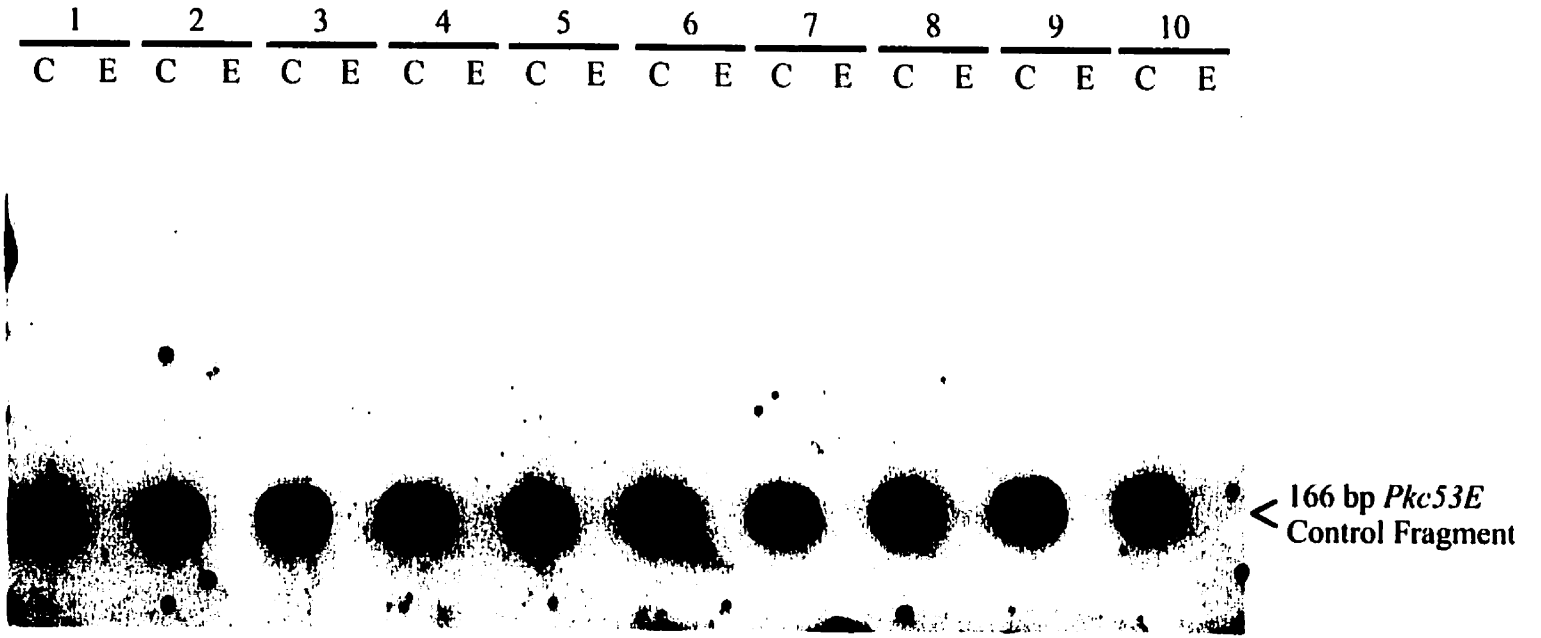


FIGURE 3.3. Typical result for *P* element insertional mutagenesis screening by PCR and Southern blot hybridization. Each pair of DNA samples represented 45 to 55 male flies. All DNA samples were suitable for amplification as shown by the appearance of a strongly hybridizing *Pkc53E* control band using primers 2 and 2Rb (lanes C). No insertions at *Pkc53E* were detected, as revealed by the absence of hybridization when the screening (experimental) primers were used (lanes E).

rudimentary, *RNA polymerase II-215 kDa subunit*, and *Notch*), 36 were located in the 5′ gene region (Chia *et al.*, 1986; Kelley *et al.*, 1987; Searles *et al.*, 1986; Tsubota *et al.*, 1985).

There are several possible explanations for the failure to isolate insertional mutants at *Pkc53E*. First, the chromatin structure at *Pkc53E* may be such that there is a much reduced transposition rate at this locus or *P* elements may not insert at all. If this is the case, other approaches to the genetic study of *Pkc53E* will be necessary. Some suggestions for alternative approaches are discussed below.

Secondly, it may be that insertions at *Pkc53E* could not be detected with the primers used in this screen. The location of the primers provided that only approximately 8 kbp (assuming the reliable amplification of up to 2 kbp in a PCR) is covered in the screen. The coding region of *Pkc53E* extends over approximately 20 kbp, therefore less than half of the gene was screened. Further, all primers were oriented such that only transcribed regions were screened. Since other reports suggest that promoter and 5′ non-coding regions may be likely sites of insertion, these primers may not have been the most appropriate for detecting *Pkc53E* insertional mutants. Screens which include promoter regions might improve the chances of detecting insertions at *Pkc53E*.

Third, insertions that did occur at *Pkc53E* resulted in a truncated PKC53E product that acted as a dominant negative lethal (antimorph) and so were not detected. A truncated PKC protein would contain part or all of the regulatory domain. This protein may bind up all of the available coactivators, cofactors, anchor proteins and/or binding proteins so that the native PKC may have been non-functional or mislocalized, or PKC substrates may have been sequestered by the mutant protein, thereby killing the mutant fly. This possibility could be tested by production of a transgenic line with a truncated PKC53E protein under the control of an inducible promoter.

Li *et al.* (1995) report that a mutated PKCδ with a nonfunctional ATP-binding site partially suppresses the phosphorylation of a PKC substrate peptide by wild type PKCδ *in vitro*. However, coexpression of mutant and wild type PKCδ in cell culture does not suppress PKCδ-mediated cell-specific functions, possibly due to incomplete suppression of

wild-type activity. Several other reports, discussed in Chapter 1, have indicated that kinase-negative PKCs can act as dominant negative regulators of PKC function, at least in cell culture (Diaz-Meco *et al.*, 1993; Hirai *et al.*, 1994; Hirano *et al.*, 1995; Li *et al.*, 1996).

These data suggest that point mutations affecting PKC activity might also function as dominant-negative lethals. In this case, suppressor and/or enhancer screens of genes that act within the PKC53E pathway(s) may not lead to the isolation of PKC mutants, thereby missing a component of the signal transduction pathway being studied. It seems then, that the most successful genetic approach to the study of *Pkc53E* should try to eliminate protein expression from the affected *Pkc53E* allele. This could be accomplished by a *P* element insertion, possibly followed by *P* element-induced deletions, in the *Pkc53E* promoter. Mosaic analysis could then be used to elucidate the function of *Pkc53E* gene products. Alternatively, antisense *Pkc53E* RNA could be used to reduce or eliminate PKC53E protein expression (discussed in detail below).

Provided that insertion does occur, an improved screening protocol may result in the detection of a *P* element insertion at *Pkc53E*. These improvements include screening a greater portion of the gene and using localized hopping. It has been shown that some mobilized *P* elements are more likely to reinsert at or near their location of origin (Engels, 1996). It is often possible to improve mutation frequency using local hopping protocols where the ammunition *P* element is located near the target gene. Furthermore, mutations are more likely to be detected if more of the gene is screened during the screening process. While the size of the *Pkc53E* gene and the size of the unsequenced introns make it difficult to cover the entire gene using PCR-based screens, a screen based on plasmid-rescue techniques might be successfully used in the future. After mutagenesis crosses in such a screen, DNA is extracted from the males collected for screening (Cross 3, Figure 2.5) and subjected to plasmid rescue (Figure 2.6). After transformation of *E. coli*, filter colony lifts are done and these filters are hybridized to genomic *Pkc53E* DNA probes covering the entire length of the gene, including promoter regions. Insertions at *Pkc53E* are identified by hybridizing colonies. True-breeding strains of insertional mutants can then be established

using the same series of crosses shown in Figure 2.5, Crosses 4 and 5 with plasmid-rescue based screens.

III. Future Directions

Drosophila is such a valuable research tool because of the powerful genetic techniques available for the study of gene and protein interactions. As such, it is highly desirable to have a mutant to access this avenue of study. An alternative to mutagenesis at the *Pkc53E* locus could involve the application of antisense RNA technology. This would entail the cloning of *Pkc53E* cDNA(s) antisense to an inducible promoter. This transgene could then be inserted into the genome by *P* element transformation. The use of an inducible promoter would ensure the survival of the transgenic fly, since *Pkc53E* is likely an essential gene. The *Drosophila hsp70* and a yeast GAL4-upstream activating sequence (UAS) construct are commonly used for these types of experiments (FlyBase, 1996; McGarry and Lindquist, 1985). The advantage of the GAL4-UAS is that several transgenic lines of *Drosophila* have been established that express the GAL4 transcriptional activator in tissue- and/or developmental stage-specific patterns. Straightforward crosses can be used to determine when and where *Pkc53E* gene products are necessary in *D. melanogaster*.

Similar approaches using dominant negative forms of PKC, or the exploitation of PKC*i* peptide expression, under the control of general inducible or tissue-specific promoters, could also prove useful. However, these approaches are less likely to be *Pkc53E*-specific, since cofactors and pseudo-substrate inhibitor peptide specificities are often shared by more than one PKC isoenzyme.

CHAPTER 4: MOLECULAR ANALYSES

I. RESULTS

A. Northern Blot Analysis

Northern blot analysis of total RNA isolated from adult heads and gonads shows that the 4.3, 4.0, and 2.4 kb transcripts of *Pkc53E* are expressed in adult heads and in adult gonads (Figure 4.1). This analysis shows the 2.4 kb transcript is more abundant than the 4.0 kb or 4.3 kb transcript.

Hybridization of the same blot with an rp49 probe (Wong *et al.*, 1981) demonstrates that the RNA is intact. The relative levels of rp49 expression are consistent with the report of Al-Atia *et al.* (1985).

B. cDNA Library Screening

The *Pkc53E* cDNA sequence reported by Rosenthal *et al.* (1987) was obtained by aligning two different overlapping cDNA clones isolated in a screen of *Drosophila* embryonic, early pupal, and adult cDNA libraries. The developmental stage from which each clone was isolated was not reported. Further, the size of this overlapping cDNA, 3.2 kbp, does not correlate well with the sizes of transcripts identified in adult flies.

It is known that in mammals, distinct PKC isoenzymes have discrete developmental functions. Thus, it seemed possible that *Pkc53E* could encode multiple PKC isoenzymes. The identification of each of the *Pkc53E* transcript sequences should reveal whether each transcript can encode a functional kinase and may reveal coactivator and isoenzyme properties based on the putative protein sequences. Further, this information could allow the design of appropriate molecular, biochemical, and genetic approaches for the study of *Pkc53E* function.

Screening of the adult head λgt10 cDNA library yielded three positive clones out of 6×10^5 plaques. The ovary λgt22A cDNA library yielded a single positive clone out of 1×10^6 plaques. The largest insert was only 0.8 kbp (data not shown), much smaller than a full-length cDNA would be. Given these results, I decided to approach the quest for

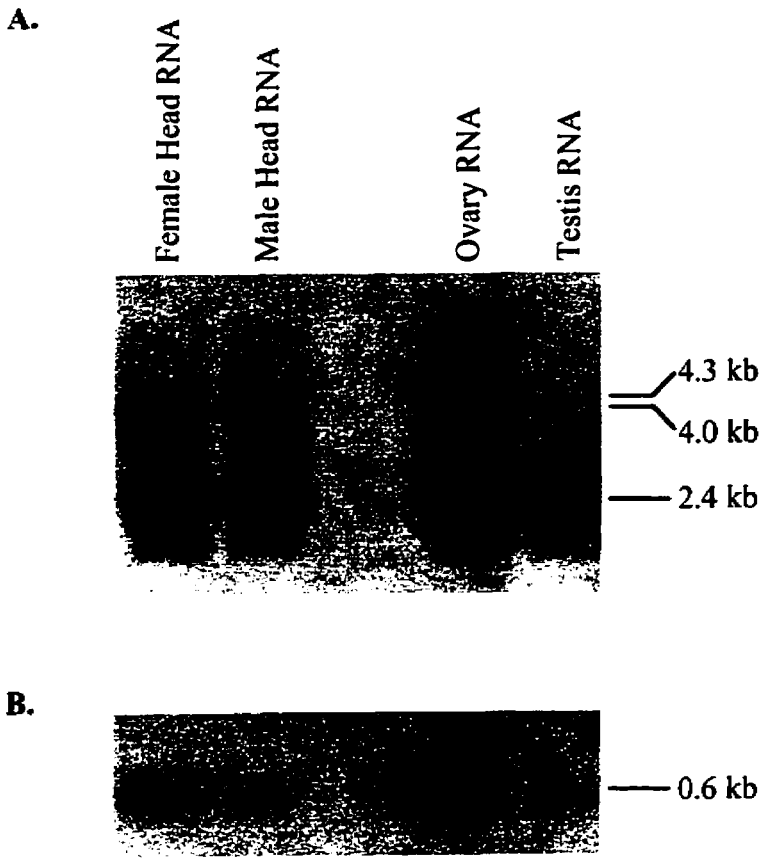


FIGURE 4.1. **Tissue-specific expression of *Pkc53E* transcripts in total RNA from adult heads and gonads.** RNA was extracted from the tissues of 75 adult females and 75 adult males. The complete RNA samples were electrophoresed on a 1.2% agarose-formaldehyde gel and Northern blotted according to the methods described. **A.** Hybridization to an antisense Pkc1 ^{32}P-labelled riboprobe corresponding to *Nco*I to *Xba*I of p*Pkc1*(SK). **B.** Hybridization to ^{32}P-oligolabelled rp49 probe (Wong *et al.*, 1981).

sequence information using reverse transcription-PCR (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques.

C. RNAse Protection Analysis

Examination of the gene structure of *Pkc53E* reveals the presence of five introns for which complete sequence information is unavailable. Two of these, introns 2 and 5, are quite large, approximately 4.1 kbp and 8 kbp, respectively. In order to optimize the probability of amplifying sequences from each message, I first examined the structure of *Pkc53E* RNAs using RNAse protection analysis (RPA).

1. RPA Probes

In order to synthesize antisense *Pkc53E* RNA probes, the Pkc1 sequence from plasmid p0161 had to be subcloned into a vector antisense to a RNA polymerase promoter. The pBluescript™ vectors are ideally suited for this purpose. The two plasmids, p*Pkc1*(KS) and p*Pkc1*(SK), represent probe vectors from which full-length antisense Pkc1 probes can be synthesized from the T3 RNA polymerase promoter (Figures 4.2 and 4.3).

It was desirable to have the Pkc1 sequence in both orientations with respect to the MCS for subsequent subcloning purposes. Normally, non-directional cloning results in the insertion of the cloned sequence into the vector in both orientations. With the Pkc1 sequences, all subclones (53 for the pBluescript™ II KS+ and 18 for the pBluescript™ II SK-) had the Pkc1 sequences inserted antisense to the *lacZ* transcription unit. This necessitated the use of two different plasmid cloning vectors.

Figure 4.4 is a map of p*rp49*, the plasmid used to synthesize antisense rp49 RNA probes (Wong *et al.*, 1981). Hybridization with antisense rp49 probes was used to verify RNA integrity (Al-Atia *et al.*, 1985).

For the synthesis of shorter Pkc1 probes, fragments of the Pkc1 sequence were removed by restriction digestion or exonuclease III deletions (see Table 2.3). Figure 4.5 shows the abridged plasmid maps for the nine Pkc1 probes used for RPA probe synthesis. T7 and T3 primers were used to sequence both strands of each insert to confirm the Pkc1

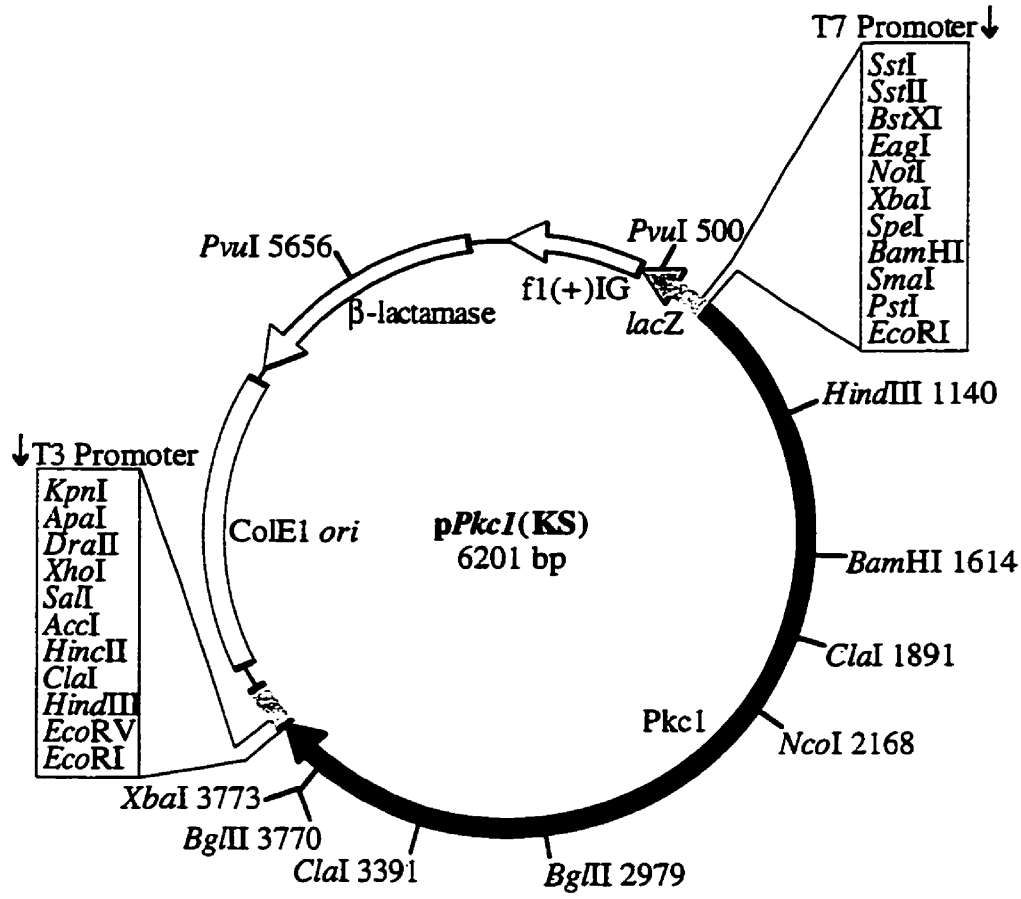


FIGURE 4.2. p*Pkc1*(KS). The complete 3.2 kbp Pkc1 fragment was subcloned into the *Eco*RI site of pBluescript™ II KS+. The Pkc1 fragment is cloned in the opposite orientation with respect to the MCS and M13 origin of replication (f1(+) IG) as compared to p*Pkc1*(SK) (Figure 4.3). Several restriction sites are indicated. When purifying the 3.2 kbp Pkc1 fragment, it is necessary to cleave the vector fragment first with *Pvu*I in order to separate the vector and insert fragments.

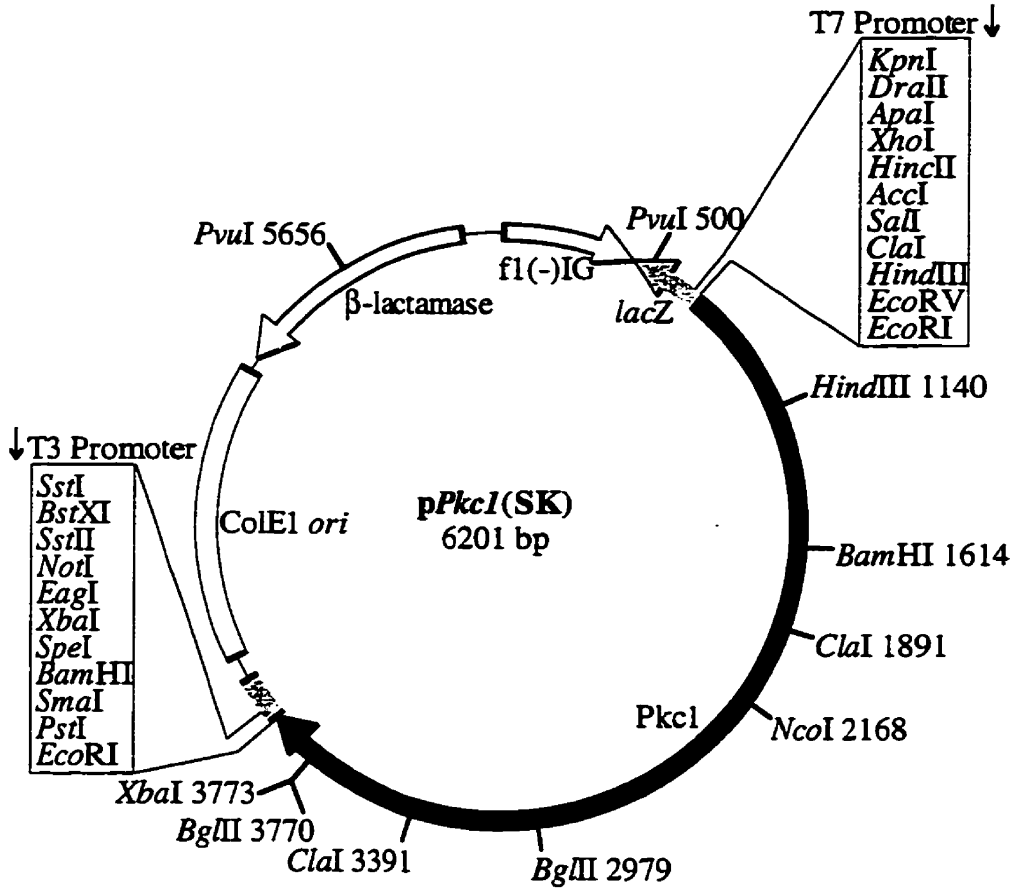


FIGURE 4.3. p*Pkc1*(SK). The complete 3.2 kbp Pkc1 fragment was subcloned into the *Eco*RI site of pBluescript™ II SK-. The Pkc1 fragment is cloned in the opposite orientation with respect to the MCS and M13 origin of replication (f1(-) IG) as compared to p*Pkc1*(KS) (Figure 4.2). Several restriction sites are indicated. When purifying the 3.2 kbp Pkc1 fragment, it is necessary to cleave the vector first with *Pvu*I in order to separate the vector and insert fragments.

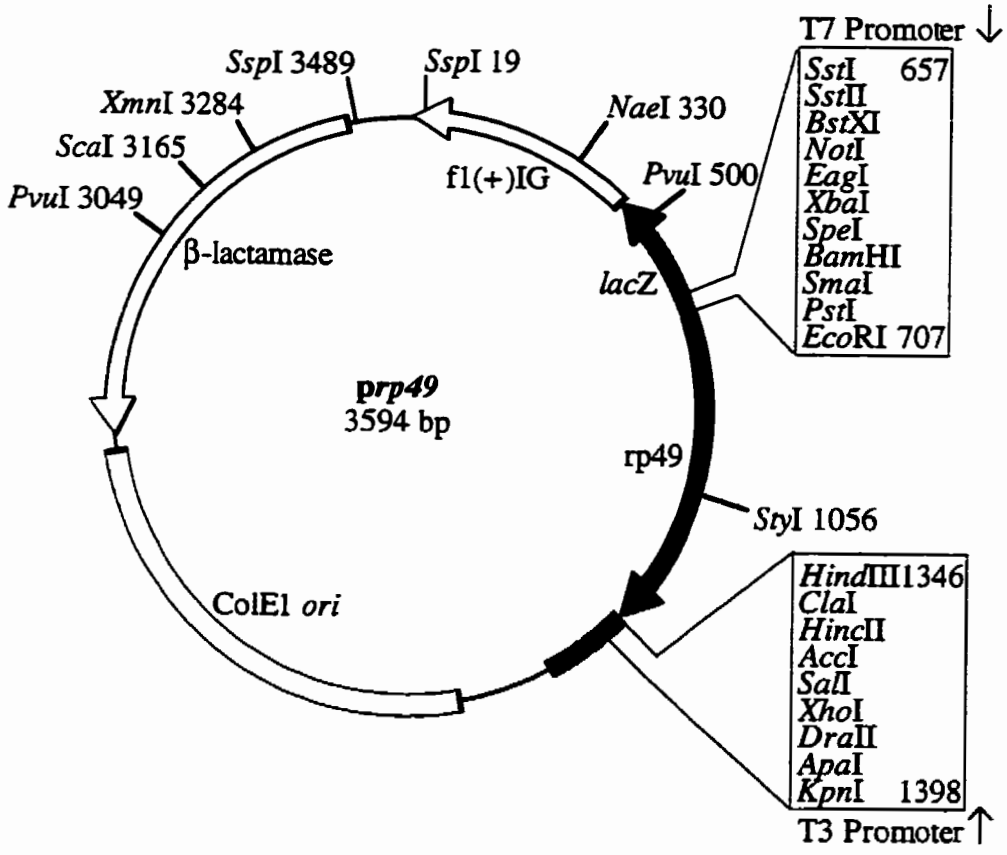
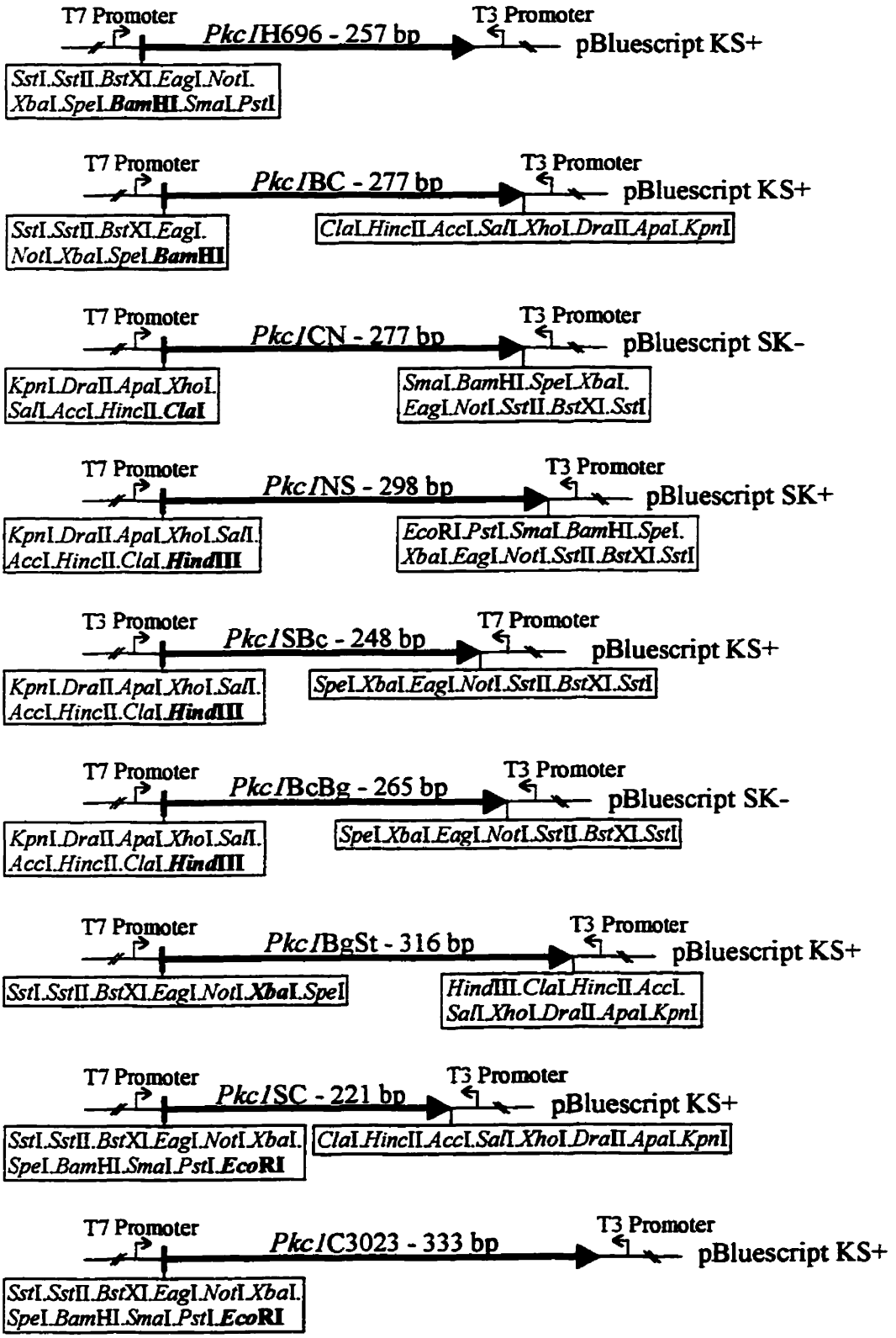


FIGURE 4.4. *prp49*. The *Eco*RI-*Hind*III genomic rp49 fragment from pHR0.6 (Wong *et al.*, 1981) was subcloned into pBluescript™ II KS+. Several restriction sites are indicated. For the synthesis of antisense rp49 riboprobes for RPA, *Sty*I was used to linearize the plasmid for run-off transcription using T3 RNA polymerase.

FIGURE 4.5. Plasmid maps of Pkc1 fragment subclones used for RPA probe synthesis. These plasmids were used to synthesize antisense Pkc1 riboprobes for RPAs. Arrowheads indicate the sense direction of transcription. The restriction enzyme sites remaining in the pBluescript™ MCS are indicated in boxes. Boldface type denotes the enzyme used for linearization of the plasmid prior to antisense riboprobe synthesis using run-off transcription with either T3 or T7 RNA polymerase. The plasmid name and the size of each insert, in base pairs, are indicated above each insert.


T7 Promoter
PkcIH696 - 257 bp
T3 Promoter
pBluescript KS+
SstI.SstII.BstXI.EagI.NotI.
XbaI.SpeI.BamHI.SmaI.PstI
T7 Promoter
PkcIBC - 277 bp
T3 Promoter
pBluescript KS+
SstI.SstII.BstXI.EagI.
NotI.XbaI.SpeI.BamHI
ClaI.HincII.AccI.SalI.XhoI.DraII.ApaI.KpnI
T7 Promoter
PkcICN - 277 bp
T3 Promoter
pBluescript SK-
KpnI.DraII.ApaI.XhoI.
SalI.AccI.HincII.ClaI
SmaI.BamHI.SpeI.XbaI.
EagI.NotI.SstII.BstXI.SstI
T7 Promoter
PkcINS - 298 bp
T3 Promoter
pBluescript SK+
KpnI.DraII.ApaI.XhoI.SalI.
AccI.HincII.ClaI.HindIII
EcoRI.PstI.SmaI.BamHI.SpeI.
XbaI.EagI.NotI.SstII.BstXI.SstI
T3 Promoter
PkcISBc - 248 bp
T7 Promoter
pBluescript KS+
KpnI.DraII.ApaI.XhoI.SalI.
AccI.HincII.ClaI.HindIII
SpeI.XbaI.EagI.NotI.SstII.BstXI.SstI
T7 Promoter
PkcIBcBg - 265 bp
T3 Promoter
pBluescript SK-
KpnI.DraII.ApaI.XhoI.SalI.
AccI.HincII.ClaI.HindIII
SpeI.XbaI.EagI.NotI.SstII.BstXI.SstI
T7 Promoter
PkcIBgSt - 316 bp
T3 Promoter
pBluescript KS+
SstI.SstII.BstXI.EagI.NotI.XbaI.SpeI
HindIII.ClaI.HincII.AccI.
SalI.XhoI.DraII.ApaI.KpnI
T7 Promoter
PkcISC - 221 bp
T3 Promoter
pBluescript KS+
SstI.SstII.BstXI.EagI.NotI.XbaI.
SpeI.BamHI.SmaI.PstI.EcoRI
ClaI.HincII.AccI.SalI.XhoI.DraII.ApaI.KpnI
T7 Promoter
PkcIC3023 - 333 bp
T3 Promoter
pBluescript KS+
SstI.SstII.BstXI.EagI.NotI.XbaI.
SpeI.BamHI.SmaI.PstI.EcoRI

sequence identity (data not shown). The positions of each probe within the reported cDNA are shown in Figure 4.6.

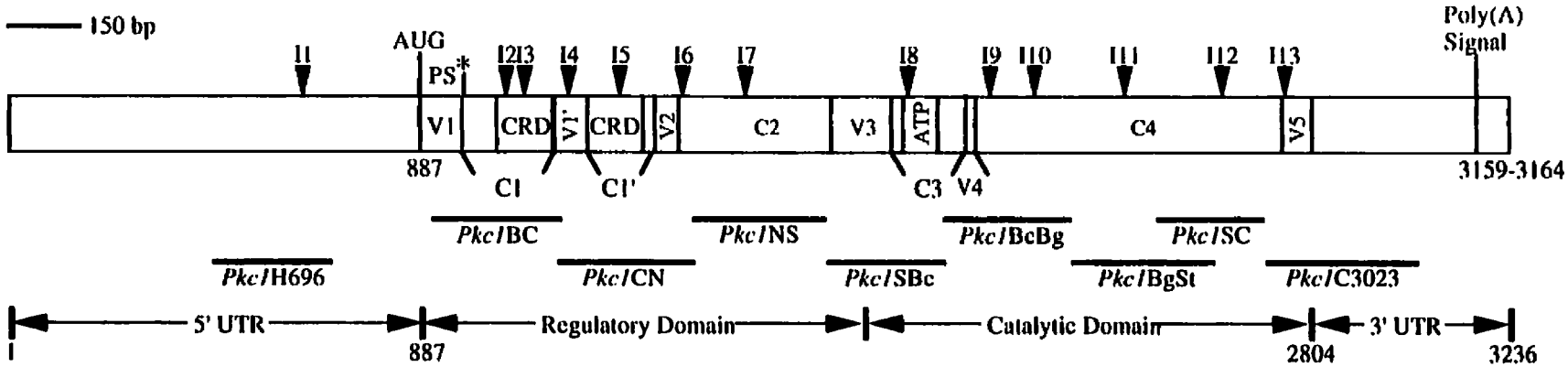
2. RPAs

The Pkc1 probes were designed to span reported intron-exon boundaries, with intron positions asymmetrically located so that exon fragments could be differentiated, when necessary. Figure 4.7 is a schematic diagram of each RPA probe used. The location of each intron-exon boundary is indicated, as are the sizes of each exon fragment within the probe. Hybridization of a probe to a *Pkc53E* transcript that has an altered exon composition would result in one or more of the exon fragments being unprotected. As a result smaller bands would be detected by urea-PAGE.

An example of an RPA gel is shown in Figure 4.8. The complete rp49 probe was protected in each RNA sample. The intensity of the rp49 signal varies considerably between samples, being most intense with ovary RNA and weakest with testis RNA. This is expected given the work of Al-Atia *et al.* (1985). These authors demonstrated that rp49 RNA was most abundant in adult ovaries, with much less in other adult tissues. While this signal cannot be used to determine the relative quantity of total RNA, it does indicate the that RNA is suitable for hybridization, *ie.* it is intact.

With the *PkcI*BC probe, three protected fragments are seen with each of the adult head RNAs and with ovary RNA. These fragments are 277 nt, 172 nt, and 105 nt, corresponding to the full-length protected probe, exon 2 sequences, and exon 3 plus exon 4 sequences, respectively. A fourth protected fragment of 33 nt, corresponding to exon 3, is seen only in female head RNA. When RNAse digestion time is extended, the faint 33 nt band seen with ovary RNA (denoted by double asterisks, **) disappears. The band denoted by a single asterisk (*) represents incompletely digested probe. This band also disappears when digestion time is extended from 30 minutes to 45 minutes.

The results of the RPAs are summarized in Figure 4.9. The protected fragments seen for each probe were identical with both male and female head RNA, except for the 33 nt fragment protected by the *PkcI*BC probe. This fragment is denoted by the symbol ♀ in Figure 4.9A. Several probes gave multiple protected fragments, except for probe

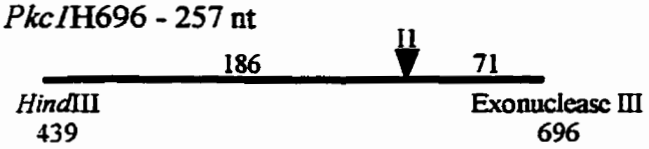


Domain	Range (nt)	Intron	Size	Location (nt)
V1	887-976	I1	~500 bp	625
C1	1001-1176	I2	~4100 bp	1085
Pseudosubstrate	977-1000	I3	<100 bp	1118
CRD	1060-1171	I4	~1000 bp	1198
V1'	1177-1246	I5	~8000 bp	1313
C1'	1247-1396	I6	~500 bp	1442
CRD	1256-1366	I7	<100 bp	1589
V2	1397-1435	I8	<100 bp	1938
C2	1436-1777	I9	<100 bp	2122
V3	1778-1903	I10	<100 bp	2214
C3	1904-2068	I11	<100 bp	2416
ATP-binding	1928-1996	I12	<100 bp	2605
V4	2069-2080	I13	<100 bp	2746
C4	2081-2737			
V5	2738-2804			

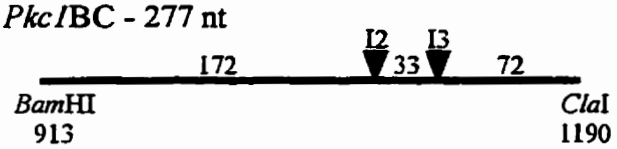
FIGURE 4.6. Schematic diagram of *Pkc53E* cDNA and RPA probe locations. The cDNA, as reported by Rosenthal *et al.* (1987), is shown by the open box. The putative domain locations are indicated within the box and their locations with respect to the published +1 of transcription are listed. Intron locations are designated by the closed arrowheads (▼). Their locations with respect to the published +1 of transcription and their sizes are listed. The locations of the Pkc1 probes used for RPAs are indicated by the solid lines.

FIGURE 4.7. Schematic diagram of the Pkc1 probes used for RPAs. Each probe is represented by a solid line. The locations of the intron-exon boundaries are indicated by the closed arrowheads (▼). The size of each exon fragment, in nucleotides (nt), is indicated immediately above each probe. Each probe end is labelled with the enzyme used, and it's cut site location relative to the published +1 of transcription, for subcloning the probe.

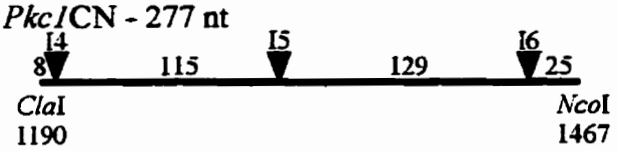
Probe *Pkc1*H696 - 257 nt



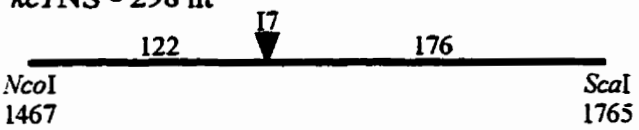
Probe *Pkc1*BC - 277 nt



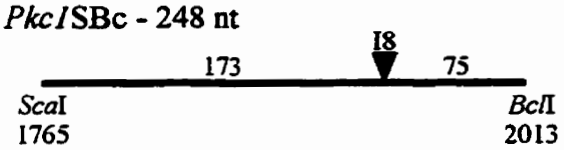
Probe *Pkc1*CN - 277 nt



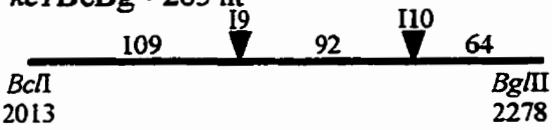
Probe *Pkc1*NS - 298 nt



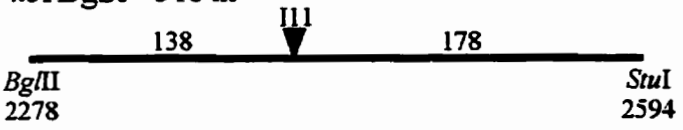
Probe *Pkc1*SBc - 248 nt



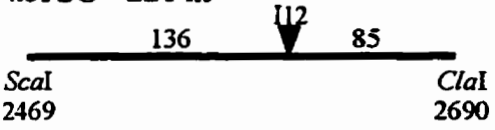
Probe *Pkc1*BcBg - 265 nt



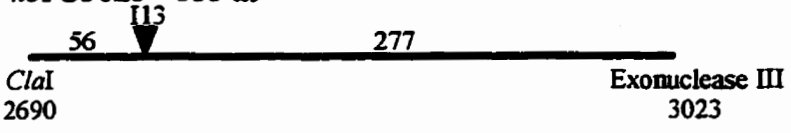
Probe *Pkc1*BgSt - 316 nt



Probe *Pkc1*SC - 221 nt



Probe *Pkc1*C3023 - 333 nt



Probe *rp49* - 291 nt

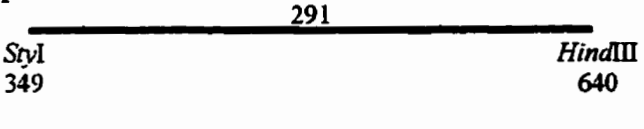


FIGURE 4.8. A representative autoradiograph of an RPA gel with probes rp49 and *Pkc1*BC. The lanes contain the following samples:

Lane	Target RNA	RNAse A
-ve	Yeast tRNA	none
+ve	Yeast tRNA	40μg/ml
FH	Female head RNA	40μg/ml
Ov	Ovary RNA	40μg/ml
MH	Male head RNA	40μg/ml
Ts	Testis RNA	40μg/ml

Lane	Target RNA	RNAse A
-ve	Yeast RNA	none
+ve	Yeast RNA	40μg/ml
FH	Female head RNA	40μg/ml
Ov	Ovary RNA	40μg/ml
MH	Male head RNA	40μg/ml
Ts	Testis RNA	40μg/ml

RP49 corresponds to hybridization with the rp49 probe spanning the 3′ *Sty*I-*Hind*III fragment of genomic rp49 DNA. *Pkc1*BC shows the results obtained with the *Pkc1*BC probe. MW denotes the molecular weight markers that were synthesized using T7 RNA polymerase from 1 μg of an equimolar mixture of pBluescript™ II SK+ digested with *Pvu*II (334 nt), *Bss*HII (149 nt), *Bam*HI (76 nt), or *Xba*I (25 nt). In panel *Pkc1*BC, both the bands marked with an asterisk (*), that is undigested probe, and the 33 nt band, in lane Ov marked with two asterisks (**), disappear when RNAse digestion time is extended from 30 to 45 minutes.

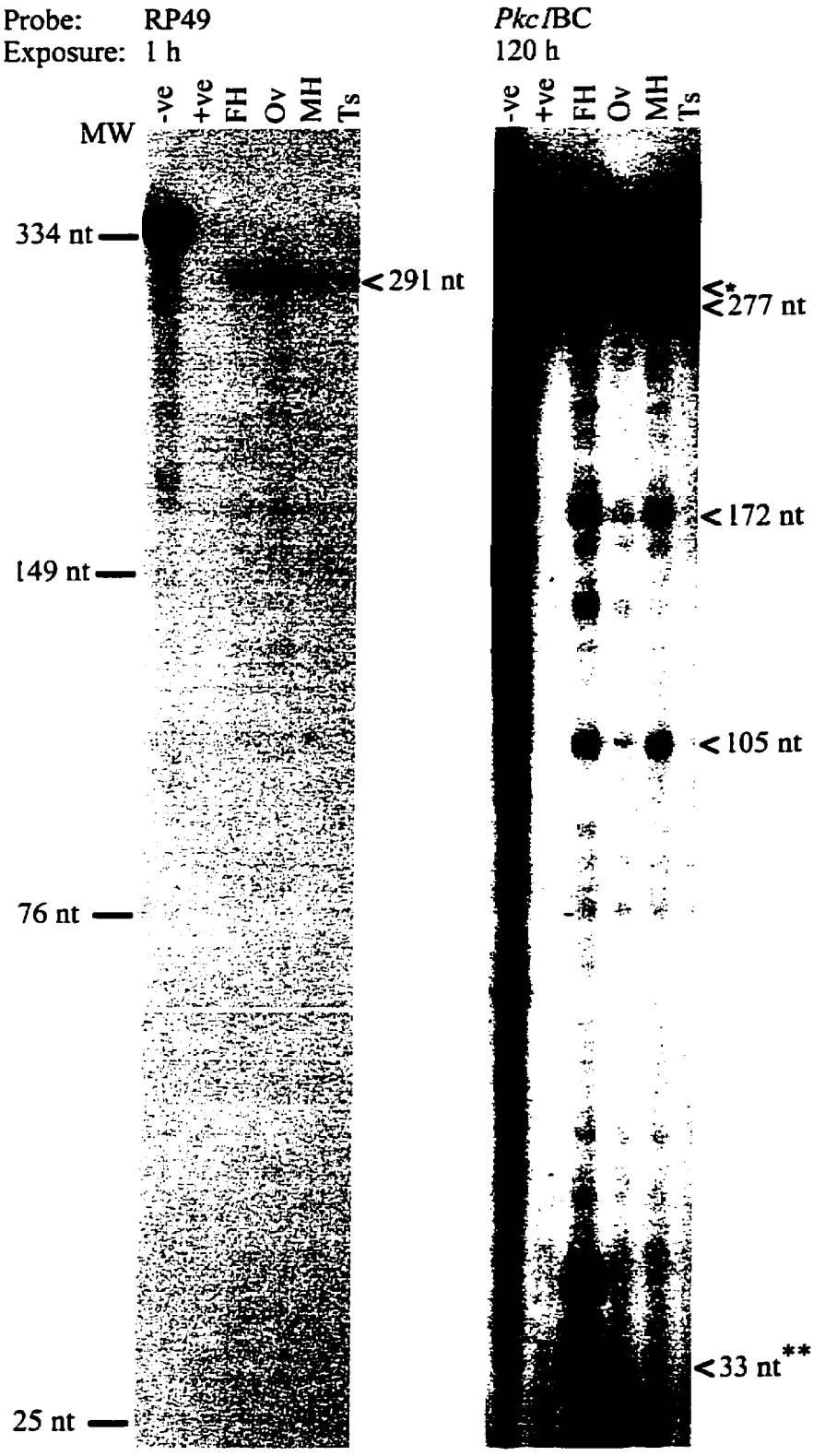
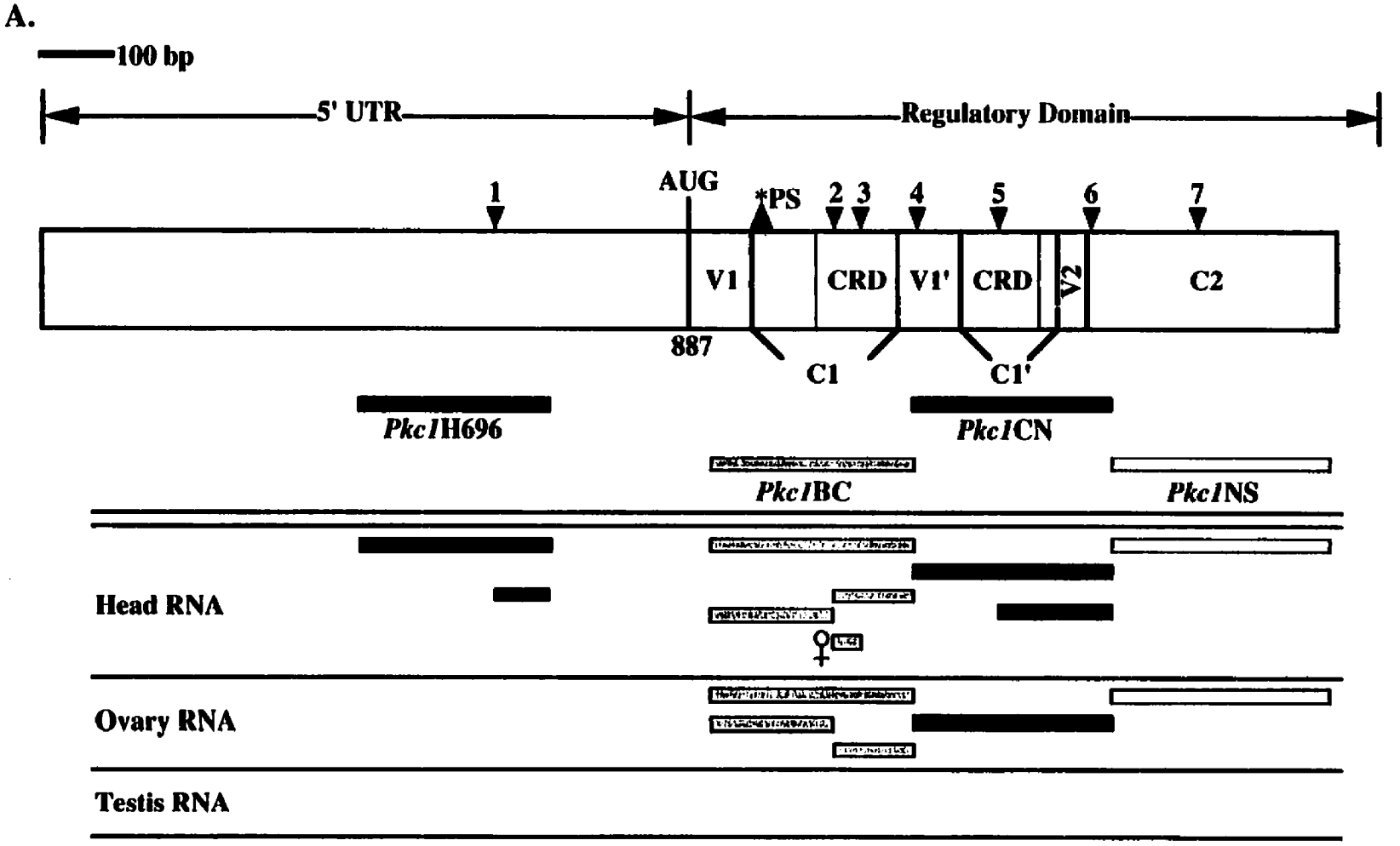
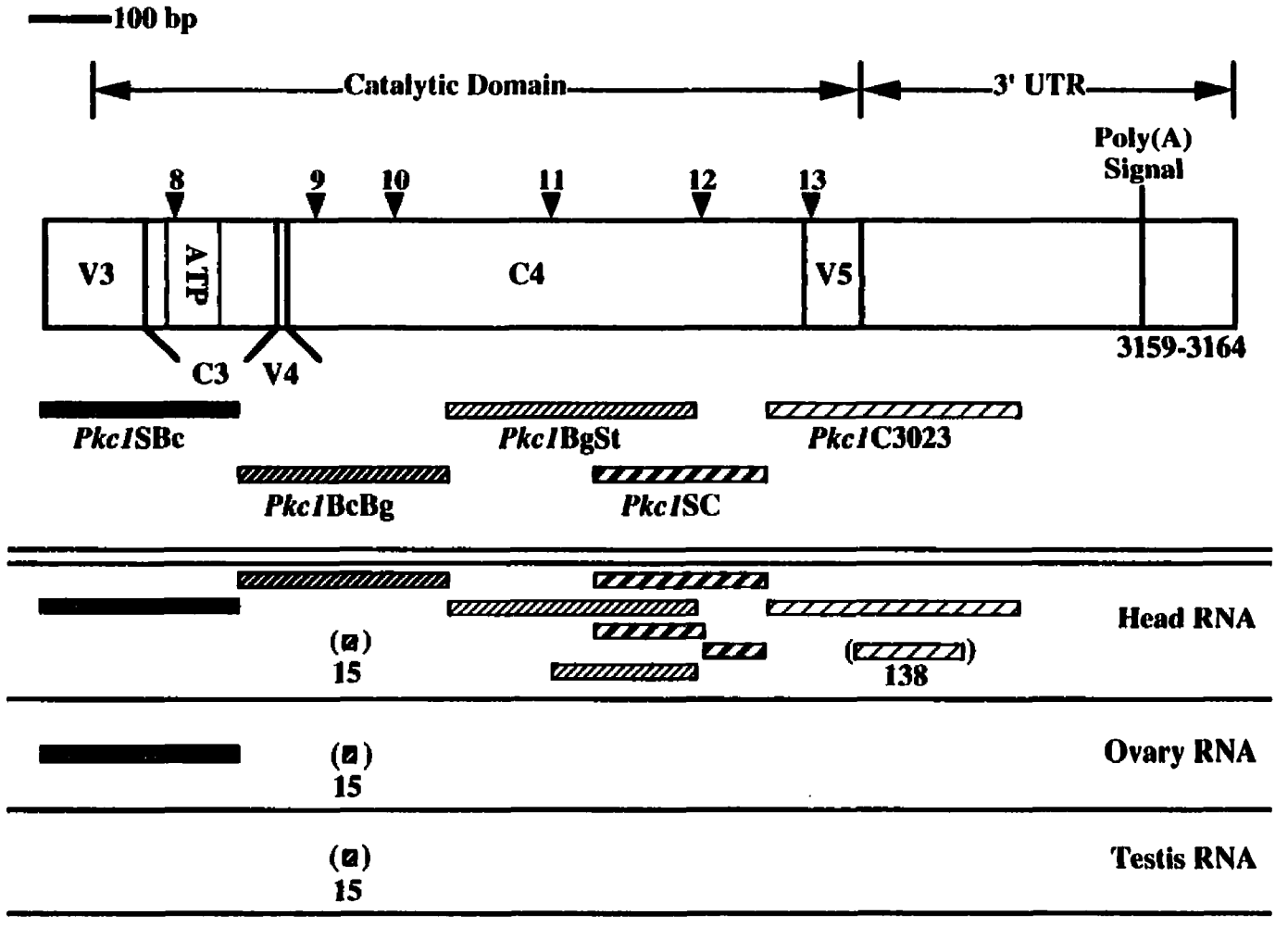

Probe: RP49
Exposure: 1 h
-ve
+ve
FH
Ov
MH
Ts
MW
334 nt
149 nt
76 nt
25 nt
291 nt
PkcIBC
120 h
-ve
+ve
FH
Ov
MH
Ts
277 nt
172 nt
105 nt
33 nt**

FIGURE 4.9. Summary of RPA results. The reported RNA sequence is represented by the open box with the putative domains delimited within the box. Intron locations are indicated by the closed arrowheads (▼). Full length probes are shown immediately below the box. Protected fragments are shown below the double horizontal line. **A.** Summary of RPA results using probes complementary to the putative 5' UTR and regulatory domain. ♀ denotes a 33 nt protected fragment present only when female head RNA is used from RPAs. **B.** Summary of RPA results using probes complementary to the putative catalytic domain and 3' UTR. Brackets indicate the presence of protected fragments that do not correspond to the size of known exons from within the probe.


A.
100 bp
5' UTR
Regulatory Domain
AUG
*PS
1
2
3
4
5
6
7
V1
CRD
V1'
CRD
V2
C2
887
C1
C1'
Pkc1H696
Pkc1CN
Pkc1BC
Pkc1NS
Head RNA
Ovary RNA
Testis RNA

B.


100 bp
Catalytic Domain
3' UTR
Poly(A)
Signal
8
9
10
11
12
13
V3
ATP
C4
V5
C3
V4
3159-3164
Pkc1SBc
Pkc1BgSt
Pkc1C3023
Pkc1BcBg
Pkc1SC
Head RNA
(-)
15
(-)
138
Ovary RNA
(-)
15
Testis RNA
(-)
15

*Pkc1*SBc that spans the ATP-binding domain and *Pkc1*NS that spans the C2 domain (Figure 4.9).

With testis RNA none of the predicted protected fragments were observed. It is possible that the levels of *Pkc53E* transcript(s) is too low for detection by the RPA probes. However, this does not preclude the presence of alternatively processed testis *Pkc53E* RNAs that do not contain known *Pkc53E* sequences and therefore do not show protection products with the probes used.

With ovary RNA, probes from either end of the published cDNA sequence (5′: *Pkc1*H696; 3′: *Pkc1*SC and *Pkc1*C3023) were not protected. These probes correspond to sequences within the 5′ and 3′ UTRs and the carboxyl terminus of the catalytic domain (see Figure 4.6).

A 15 nt fragment from *Pkc1*BcBg is protected in all RNA samples. This does not correspond to the size of any of the exon fragments from this probe. It is possible that it represents a short span of sequence identity with another transcript. There is more variability among protected fragments with head RNA than with ovary RNA, and more variability observed when using probes from the putative regulatory domain than from the putative catalytic domain.

Generally, the intensity of the full-length protected fragments was greater than that of the smaller fragments, but this may reflect the reduced amount of label in the shorter probe fragments. The exceptions are the 33 nt fragment derived from probe *Pkc1*BC and the 15 nt fragment derived from probe *Pkc1*BcBg.

D. PCR Analysis of RNA

Given that *Pkc1*SBc both spans the ATP-binding domain, a domain that is essential for an active kinase, and in RPAs reveals a single full-length protected fragment, I thought that primers complementary to this region would be likely to allow the amplification of the *Pkc53E* transcript sequences. 3′ RACE allows the amplification of polyA$^+$ mRNA using gene-specific 5′ primers and oligo(dT)-based sequences as non-gene-specific 3′ primers.

Hence, sequence information from only one part of the gene is necessary to amplify the 3′ ends of transcripts.

1. 3′ RACE

The results of 3' RACE reactions with two rounds of amplification using the nested gene-specific primers (GSPs) 4, and then 4b, are shown in Figure 4.10. On ethidium bromide-stained agarose gels, a major amplification product(s), approximately 1.2 kbp in size, is seen using cDNA from female and male head total RNA and testis total RNA. A doublet, running at approximately 1.2 kbp in size, is seen with cDNA from ovary total RNA and with cDNA from whole female polyA$^+$ RNA and ovary polyA$^+$ RNA. Southern blot hybridization with a DIG-labelled Pkc1 cDNA probe confirmed that bands in the 1.2 kbp size range contained *Pkc53E* sequences, with the notable exception of the band from testis cDNA. It was not clear whether both bands of the doublet from ovary cDNA hybridized.

To confirm that the hybridizing bands represent the amplification of *Pkc53E* sequences corresponding to the published sequence, each band was gel purified and subjected to restriction digestion analysis (Figure 4.11A and B). This revealed that each of the hybridizing bands gave rise to the predicted restriction fragments. As expected by the absence of hybridization signal, the band from testis cDNA was not cut by these enzymes.

As seen in Figure 4.12, the larger (upper ≈1.2 kbp) fragment of the doublet seen with 3′ RACE of whole female polyA$^+$ RNA is not cut with *Cla*I. Further, digestion with *Sca*I cut the upper ≈1.2 kbp fragment twice, instead of once as predicted by *Pkc53E* sequence information. The resulting smaller bands did not reproduce very well, however they can be readily observed on overexposed pictures of the gel (data not shown). The upper ≈1.2 kbp fragment, isolated from the lower fragment upon *Cla*I digestion, does not hybridize to a DIG-labelled Pkc1 cDNA probe (data not shown). This clearly demonstrates that only the smaller (lower ≈1.2 kbp) fragment of the doublet is a true *Pkc53E* 3′ RACE product. The same results are seen with the doublet fragment from ovary cDNA (data not shown).

RPAs suggested that there was some variability within the putative catalytic domain of *Pkc53E* RNAs, however no differences were revealed with 3' RACE using GSPs 4 and

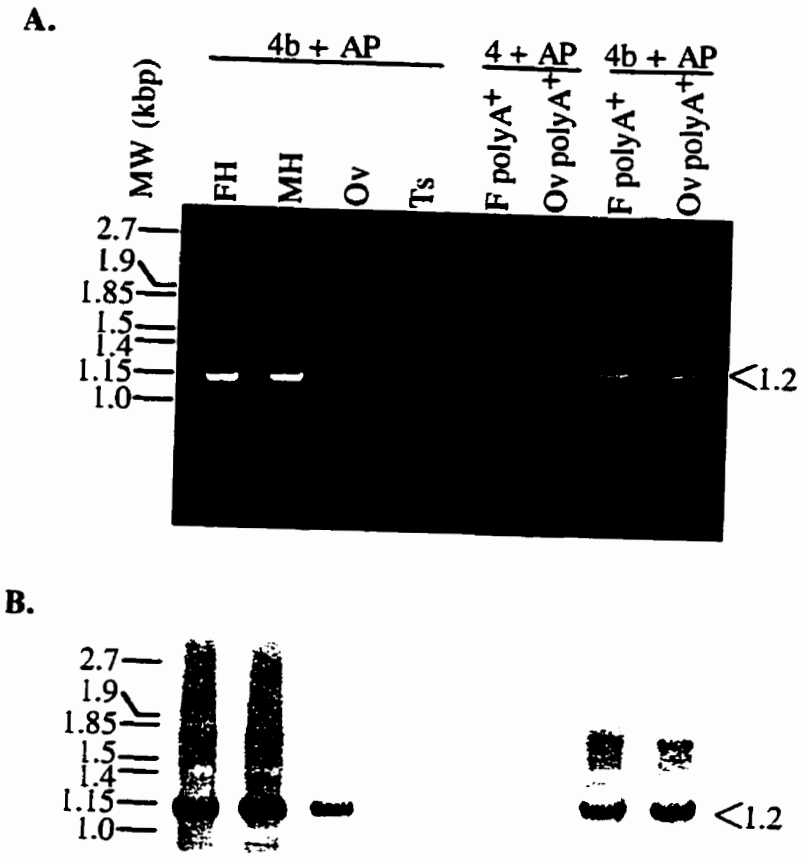


FIGURE 4.10. 3′ RACE using primers 4b and AP. A. Ethidium bromide-stained 1.2% agarose gel analysis of 3′ RACE products reveals the presence of a 1.2 kbp fragment after two rounds of amplification. A doublet is present in lanes where ovary cDNA was present as the amplification template. **B.** Southern blot hybridization of 3′ RACE products. The 1.2 kbp fragment hybridized to DIG-labelled Pkc1 probes in all lanes except with testis cDNA. The first round of amplification used primers 4 and AP, the second used primers 4b and AP. The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis. MW is the DIG-labelled molecular weight marker VII (Boerhinger/Manheim).

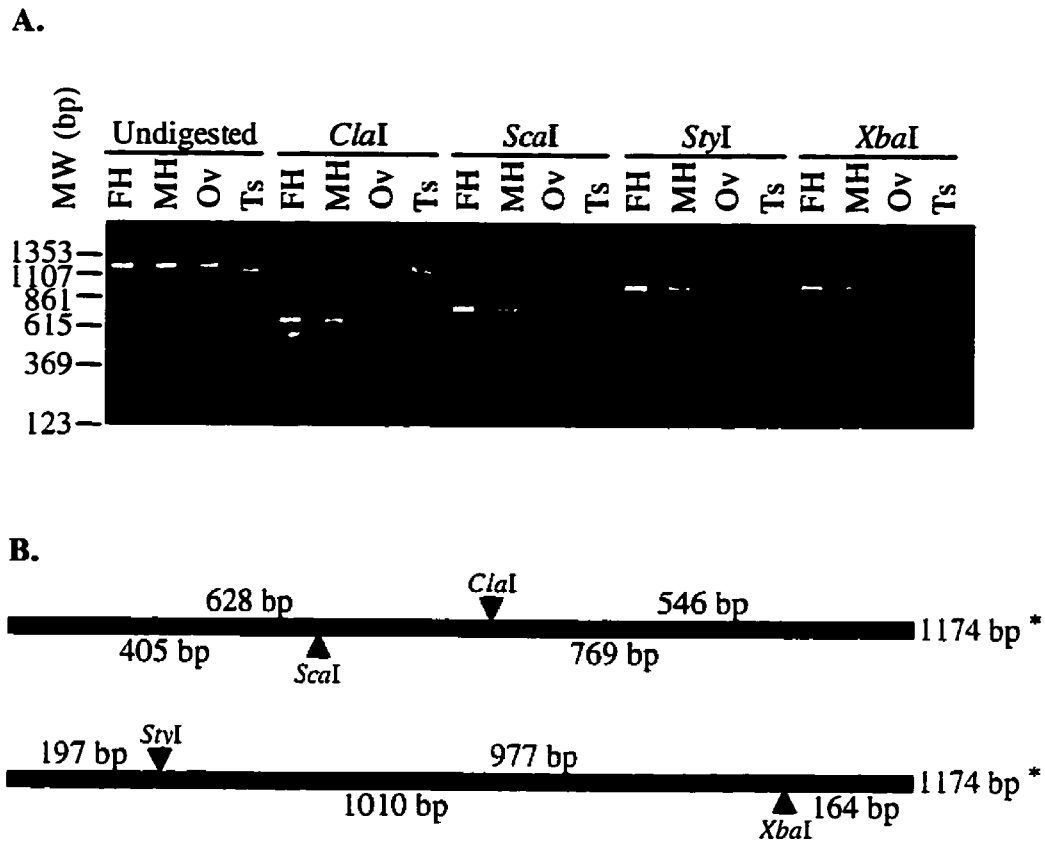


FIGURE 4.11. Restriction digest of 3′ RACE products from primers 4b and AP. **A.** Gel-purified 3′ RACE products were digested with *Cla*I, *Sca*I, *Sty*I, or *Xba*I. The restriction fragments isolated from the amplification of head and ovary cDNA are of the predicted size. The amplified testis cDNA fragment is not cut with these enzymes. The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis. MW is the 123 bp ladder (GIBCO/BRL). **B.** Schematic representation of the 3′ RACE product predicted from the published *Pkc53E* sequence (Rosenthal *et al.*, 1987). *The predicted size of the 3′ RACE product does not include polyA tail sequences.

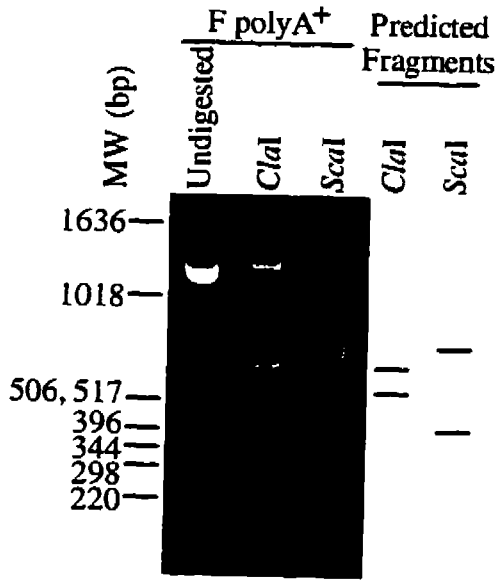


FIGURE 4.12. Restriction digest of 3′ RACE doublet. The ethidium bromide-stained agarose gel shows the lower band of the doublet digested by *Cla*I and *Sca*I, as predicted (Figure 4.11B) and shown by the fragment sizes at the right of the figure. The upper band of the 3′ RACE doublet is not digested by *Cla*I, but it is digested by *Sca*I, though into more fragments that are much smaller than predicted for *Pkc53E* sequences. PolyA$^+$ RNA isolated from whole female total RNA was used as the template for reverse transcription. Identical results are seen with ovary polyA$^+$ RNA (data not shown). MW is the 1 kb ladder molecular weight marker (GIBCO/BRL).

4b. Probe *Pkc1*NS was also fully protected in RPAs, so I attempted 3' RACE with GSPs 3 and 3b from this region to try to characterize potential variability within the putative catalytic domain of *Pkc53E* RNAs. Figure 4.13 shows the amplification of a single *Pkc53E* hybridizing band of 1.8 kbp with cDNA from adult heads and ovaries, but absent with cDNA from adult testes. Restriction analysis revealed that the structures of the hybridizing 3′ RACE products were as predicted (Figure 4.14). These results are summarized in Figure 4.15.

2. RT-PCR

RT-PCR uses two GSPs for the amplification of cDNA sequences. In further attempts to characterize differences among *Pkc53E* RNAs, I used pairs of GSPs from each of the putative conserved domains.

Amplification using primers 4b and 4R revealed the presence of a Pkc1-hybridizing 801 bp band in all lanes (Figure 4.16A). This band was amplified using cDNA samples from 3′ RACE experiments, confirming that the absence of a hybridizing band with 3' RACE of testis cDNA was not due to poor RNA quality, inefficient reverse transcription, nor the absence of primer 4b complementary binding sites. Restriction analysis confirmed that the structure of this 801 bp fragment corresponded to the published *Pkc53E* sequence. Gel purified 801 bp fragments were subjected to restriction digestion with *Cla*I and *Sca*I. Each 801 bp fragment was cut once and yielded the expected fragments (Figure 4.16B and C). Southern blot hybridization of this gel confirmed that each of the resulting fragments hybridized to DIG-labelled *Pkc53E* cDNA probe (data not shown).

RT-PCR results for each of the primer pairs used are summarized in Figure 4.17. Primers located 3′ of intron 1 all yielded the predicted size fragments (Figure 4.17A). The identity of each fragment was confirmed by Southern hybridization to a DIG-labelled Pkc1 cDNA probe and/or restriction analysis (data not shown).

Amplification of cDNAs using primers 3 and 4R yielded the expected 1.5 kbp fragment (Figure 4.18). Further, each sample also gave rise to a 0.8 kbp fragment and ovary cDNA revealed a 1.4 kbp fragment. Each of these amplified fragments hybridized to DIG-labelled Pkc1 probes. Complete direct cycle sequencing of the 1.4 kbp and 0.8 kbp

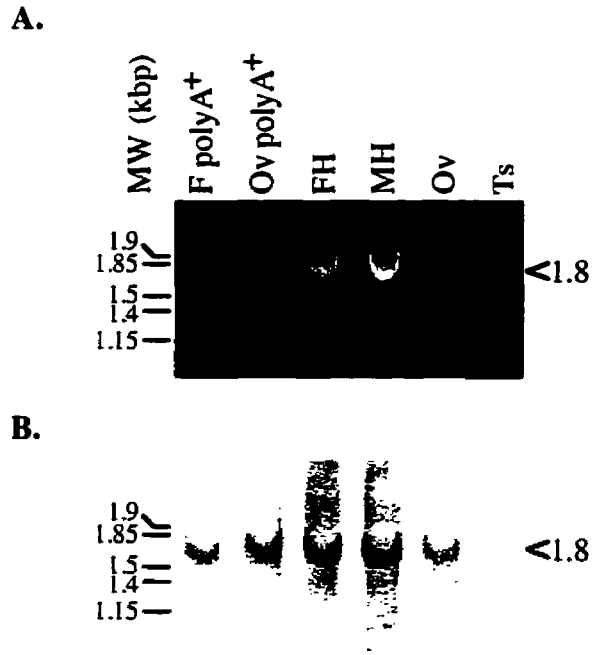


FIGURE 4.13. 3′ RACE using primers 3b and AP. A. Ethidium bromide-stained 1.2% agarose gel analysis of 3′ RACE products reveals the presence of the predicted 1.8 kb fragment after two rounds of amplification, the first with primers 3 and AP and the second with primers 3b and AP. **B.** Southern blot hybridization of 3′ RACE products. The 1.8 kb fragment hybridizes to *Pkc53E* sequences. The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis. MW is the DIG-labelled molecular weight marker VII (Boerhinger/Manheim).

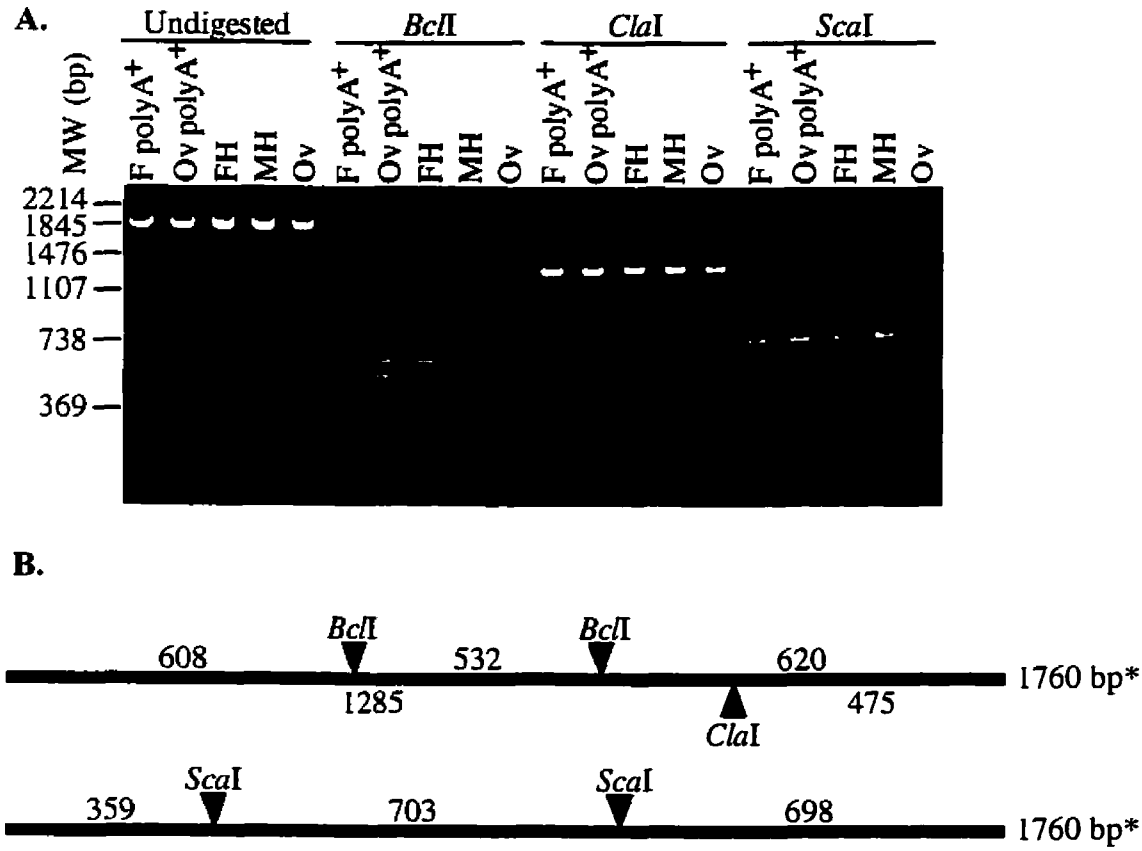


FIGURE 4.14. Restriction digest of 3′ RACE products from primers 3b and AP. **A.** 3′ RACE products were digested with *BclI*, *ClaI*, or *ScaI*. The restriction fragments isolated from the amplification of head and ovary cDNA are of the predicted size. The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis. MW is the 123 bp ladder (GIBCO/BRL). **B.** Schematic representation of the 3′ RACE product predicted from the published *Pkc53E* sequence (Rosenthal *et al.*, 1987). *The predicted size of the 3′ RACE product does not include polyA tail sequences.

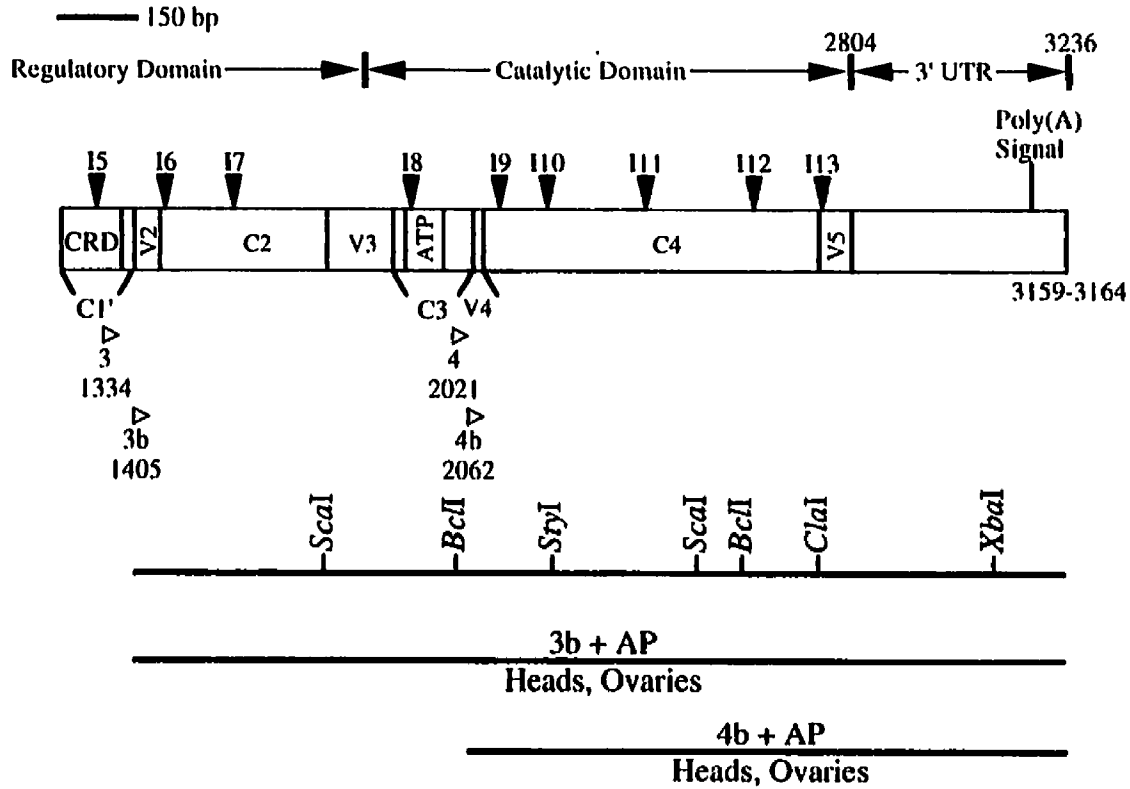


FIGURE 4.15. Schematic representation of 3′ RACE data. The sequences derived from published cDNAs are represented by the open boxes in which the putative domains are labelled (Rosenthal *et al.*, 1987). Immediately below, represented by the solid lines (▬) are schematic representations of the restriction digest sites found in that region of the published cDNA and the 3′ RACE products. Each 3′ RACE product is labelled with the primers used for amplification above and the source of RNA from which it was amplified below.

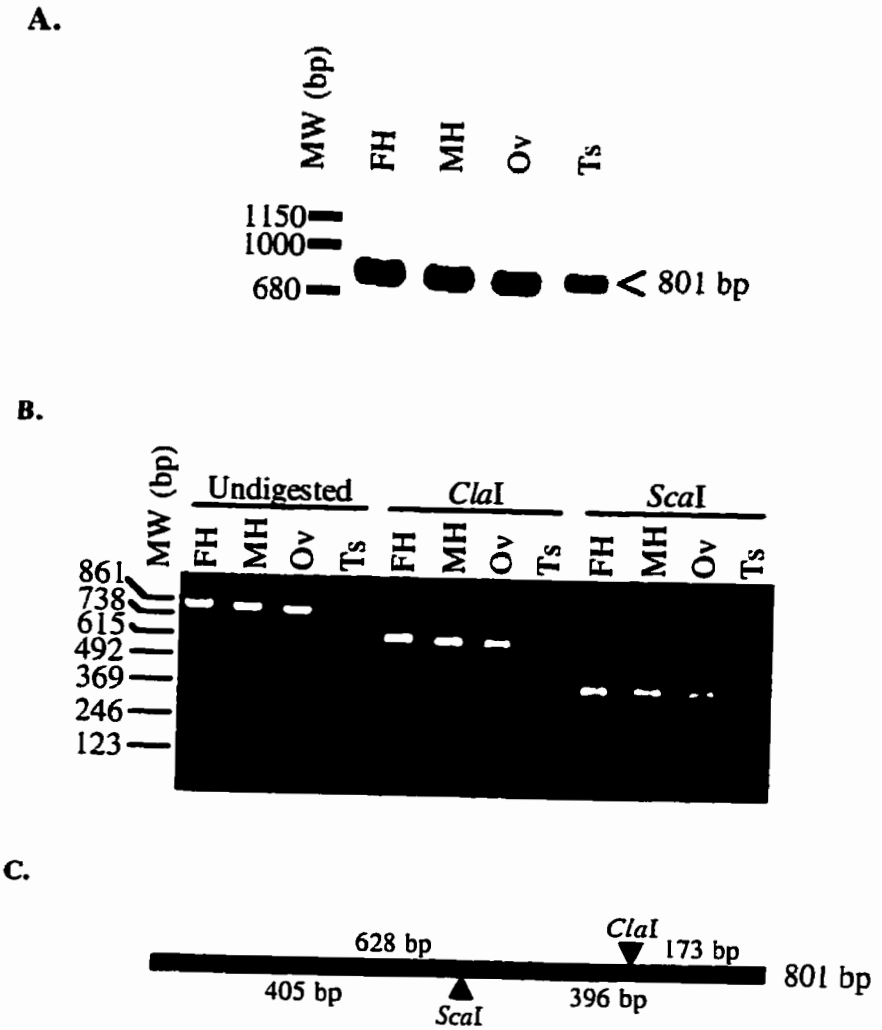
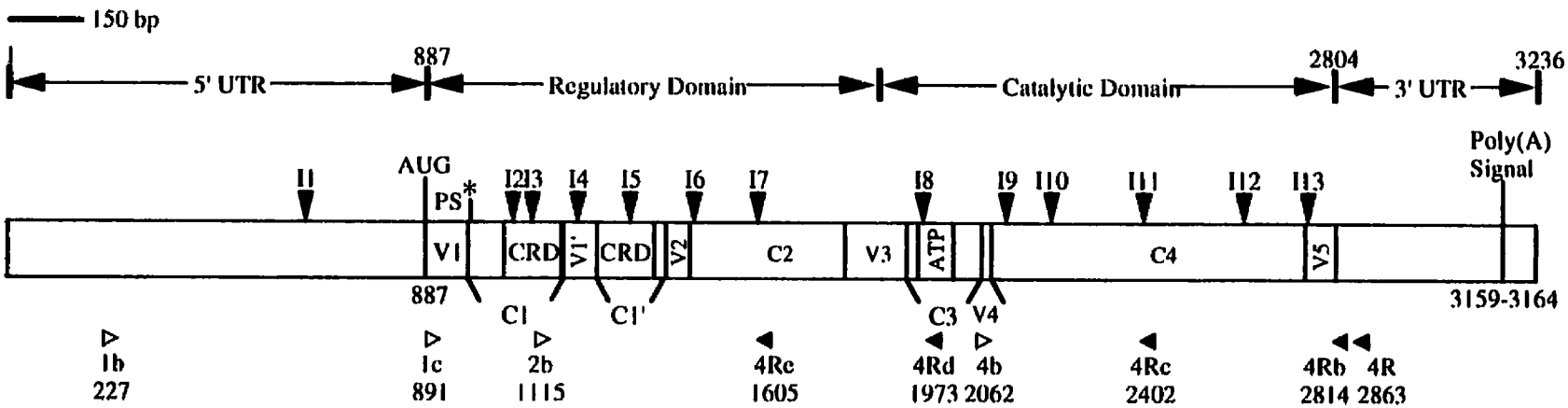


FIGURE 4.16. Results of RT-PCR using primers 4b and 4R. A. Southern blot analysis of RT-PCR fragments from cDNA synthesized from adult head and gonad total RNA. MW is the DIG-labelled molecular weight marker VII (Boerhinger/Manheim). The larger band seen with head cDNA disappears when only 30 cycles of PCR are used (data not shown) and is likely artifactual. **B.** Gel-purified 3′ RACE products were digested with *Cla*I or *Sca*I. The restriction fragments are of the predicted size. MW is the 123 bp ladder (GIBCO/BRL). **C.** Schematic representation of the RT-PCR product predicted from the published *Pkc53E* sequence (Rosenthal *et al.*, 1987). The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis.

FIGURE 4.17. Schematic representation of RT-PCR data. The sequences derived from published cDNAs are represented by the open boxes in which the putative domains are labelled (Rosenthal *et al.*, 1987). Immediately below, represented by the solid lines (▬) are the schematic representation of the RT-PCR products. **A.** RT-PCR yielded only the predicted bands for primer pairs with their 5′ primer 3′ to intron 1. **B.** RT-PCR using primers 1b and 3Rb or 1b and 4Rb yielded the predicted products as well as a larger product that contained intron 1. Brackets indicate that when the oligo$(dT)_{12\text{-}18}$ primer annealing temperature for cDNA synthesis was raised to 50°C amplification products from testis cDNA are no longer observed.

A.



1c + 4Re
Heads, Ovaries, (Testes)

1c + 4Rd
Heads, Ovaries, (Testes)

1c + 4Rb
Heads, Ovaries, (Testes)

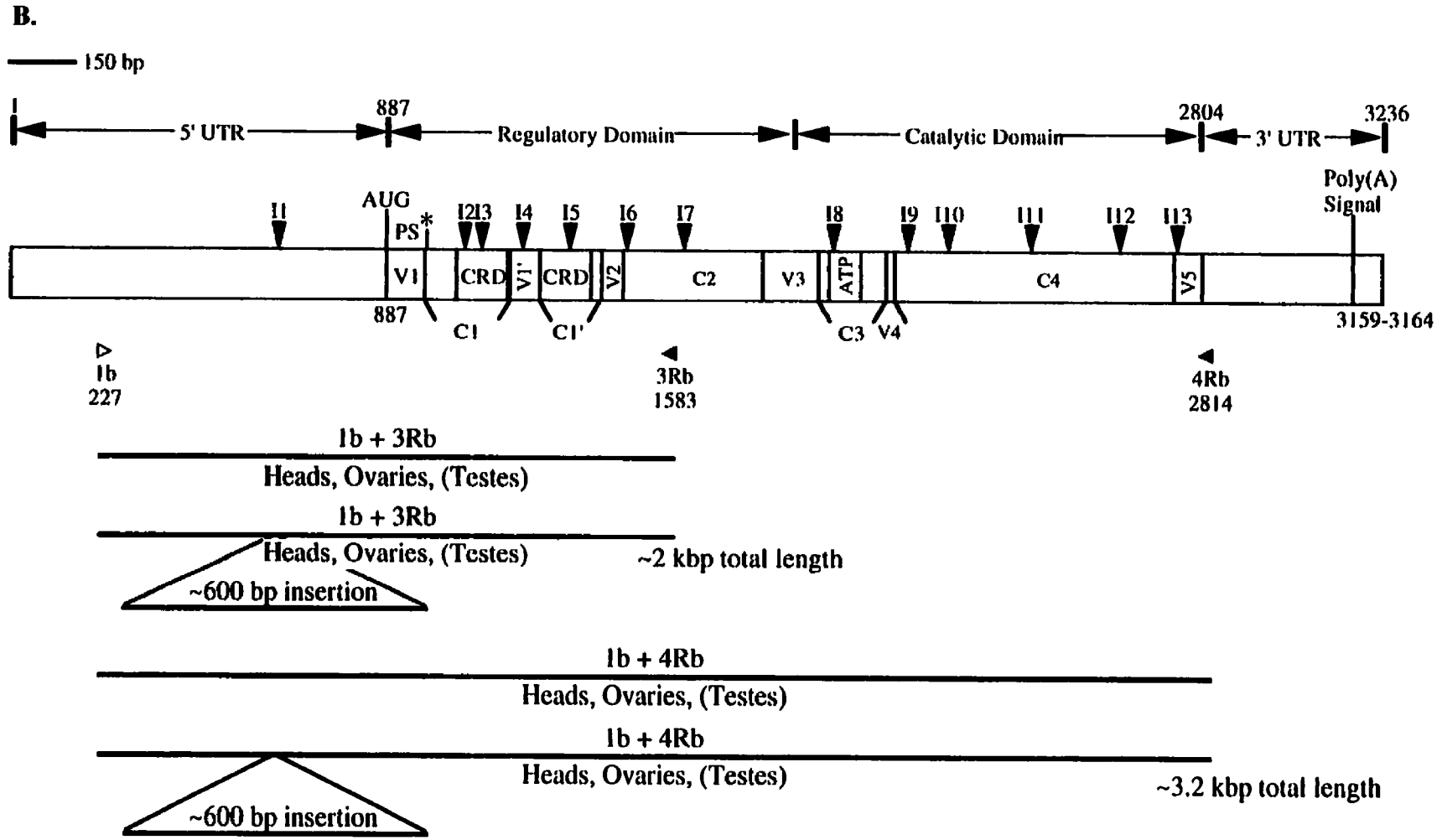
2b + 4Rd
Heads, Ovaries, (Testes)

2b + 4Rc
Heads, Ovaries, (Testes)

3 + 4R
Heads, Ovaries, (Testes)

3b + 4Rb
Heads, Ovaries, (Testes)

4b + 4R
Heads, Ovaries, (Testes)


B.
150 bp
1
887
2804
3236
5' UTR
Regulatory Domain
Catalytic Domain
3' UTR
Poly(A) Signal
AUG
PS*
I1
I2I3
I4
I5
I6
I7
I8
I9
I10
I11
I12
I13
V1
CRD
V1'
CRD
V2
C2
V3
ATP
C4
V5
887
C1
C1'
C3
V4
3159-3164
1b
227
3Rb
1583
4Rb
2814
1b + 3Rb
Heads, Ovaries, (Testes)
1b + 3Rb
Heads, Ovaries, (Testes)
~600 bp insertion
~2 kbp total length
1b + 4Rb
Heads, Ovaries, (Testes)
1b + 4Rb
Heads, Ovaries, (Testes)
~600 bp insertion
~3.2 kbp total length

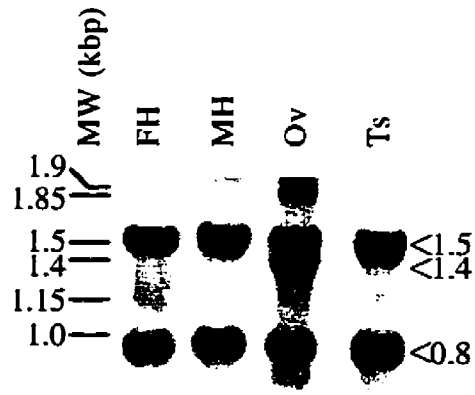


FIGURE 4.18. Southern blot of RT-PCR using primers 3 and 4R. Southern blot analysis of RT-PCR fragments shows both the predicted 1.5 kbp fragment and a novel 0.8 kbp fragment. In addition, a novel 1.4 kbp fragment is found with amplification of ovary cDNA. Reverse transcription was primed with dTAP and the cDNA amplified with primers 3 and 4R. The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis. MW is the DIG-labelled molecular weight marker VII (Boerhinger/Manheim).

fragments showed that they are truncated *Pkc53E* sequences (data not shown), however it is not clear from where they arose since sequencing using primers 3 or 3b was not successful. Amplification of each cDNA sample with primer 3 or primer 4R alone did not give rise to any amplification products (data not shown). These shorter fragments are now thought to be artifactual for two reasons. First, RT-PCR using the nested primers 3b and 4Rb yields a single product of the predicted length (Figure 4.17A). Second, two rounds of amplification were necessary to see any of these bands, suggesting that primers 3 and 4R are very inefficient for RT-PCR, possibly due to promiscuity of primer hybridization.

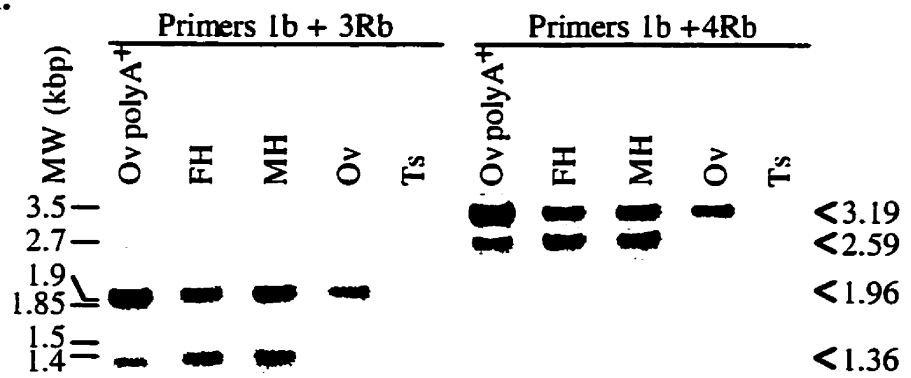
RT-PCR with primers 1b and 3Rb or 1b and 4Rb each yielded two *Pkc53E*-hybridizing amplification products, one of the predicted size and one approximately 600 bp longer (Figures 4.17B and 4.19A). Restriction analysis showed that these fragments were collinear with published *Pkc53E* sequences 3′ of the *Stu*I site (data not shown) and located the insertion between the *Apa*I site at nt 611 and the *Stu*I site at nt 637 (Figure 4.19B and 4.19C). These sites surround the location of intron 1, a previously incompletely sequenced intron of approximately 600 bp.

In order to determine if the insertion was intron 1, primer 1Ra was used for direct cycle sequencing of the 3′ end of the insert. The sequence of intron 1 was derived by cycle sequencing of both strands of pSB29ASt, an *Apa*I-*Stu*I genomic fragment of *Pkc53E* containing all of intron 1 (Figure 4.20A). Sequence comparison using BLAST (see Methods) showed that the 600 bp insert in the 1.96 kbp 1b+3Rb fragment was intron 1. The identical size increase, insert location, and restriction map of the 3.19 kbp 1b+4Rb fragment identified it as containing intron 1 as well. The BLAST search engine was used to compare intron 1 nucleotide sequences, and all six translation frames, with the public databases on April 13, 1997. No significant similarities to any sequences were found using the default search parameters.

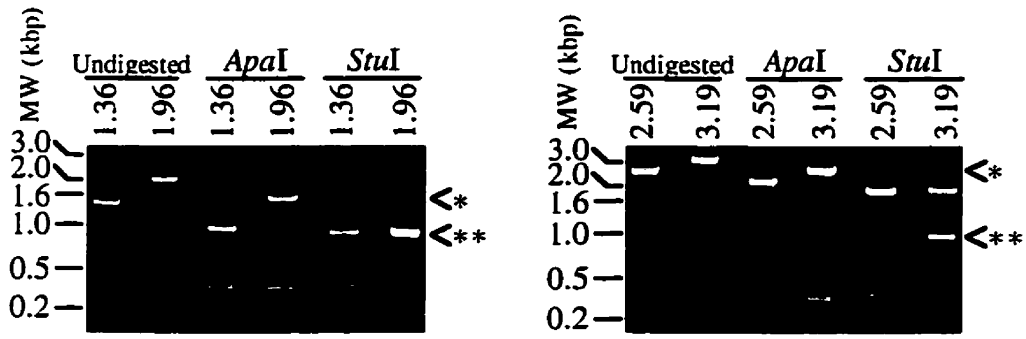
When the intron sequence is inserted into its proper relative position within the published cDNA (immediately after nt 625), an ORF of 125 amino acids, with its ATG start codon found in exon 1 at nt 576, is generated (ORFA; Figure 4.20B). The sequence surrounding this ATG, GCATATG, does not agree with the consensus *Drosophila*

FIGURE 4.19. RT-PCR using primers 1b and 3Rb or 1b and 4Rb. A. Southern blot analysis of RT-PCR using primers 1b and 3Rb or primers 1b and 3Rb. Each reaction yielded the predicted sized product, 1.36 and 2.59 kbp respectively, as well as an additional product about 600 bp longer than predicted, 1.96 and 3.19 kbp respectively. **B.** Gel-purified RT-PCR products were digested with *Apa*I or *Stu*I. The 3′ *Apa*I fragments, marked with single asterisks (*), from the larger (1.96 and 3.19 kbp) bands conatined all of the insert DNA, as indicated by the size increase compared to those from the smaller (1.36 and 2.59 kbp) bands. The 5′ *Stu*I fragments, marked with double asterisks (**), from the larger bands contained all of the insert DNA, as indicated by the size increase compated to those from the smaller bands. This localized the insert between *Apa*I and *Stu*I restriction sites. **C.** Schematic diagram of the locations of the *Apa*I and *Stu*I cut sites relative to intron 1 (not to scale). Intron 1 was reported to be approximately 500 bp (Rosenthal *et al.*, 1987), very near the size increase seen with the larger fragments. This suggested, but did not prove, that the size increase was due to the inclusion of part or all of intron 1 in some *Pkc53E* transcripts. The source of RNA used for reverse transcription is indicated above each lane. Abbreviations are FH=female head; MH=male head; Ov=ovary; Ts=testis. MW is the DIG-labelled molecular weight marker VII (Boerhinger/Manheim; panel A) or the 1 kb ladder (GIBCO/BRL; panel B).

A.


Primers 1b + 3Rb
Primers 1b +4Rb
MW (kbp)
Ov polyA+
FH
MH
Ov
Ts
3.5
2.7
1.9
1.85
1.5
1.4
3.19
2.59
1.96
1.36

B.


MW (kbp)
Undigested
ApaI
StuI
1.36
1.96
2.59
3.19
3.0
2.0
1.6
1.0
0.5
0.2
*
**

C.

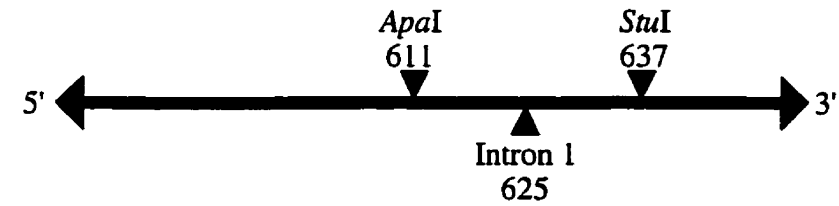

ApaI
611
StuI
637
5'
3'
Intron 1
625

FIGURE 4.20. *Pkc53E* intron 1 sequence and localization within transcript sequences. **A.** Both strands of intron 1 (as defined by Rosenthal *et al.*, 1987) were sequenced from pSB29ASt. The boldface type represents sequences from exon 1 and exon 2 that are also found in pSB29ASt flanking intron 1 sequences. The arrows (⇓) indicate the end of intron sequences previously reported by Rosenthal *et al.* (1987). The single-underlined sequences represent intron 1 sequences that were identified by direct cycle sequencing of the larger RT-PCR product amplified with primers 1b and 3Rb, and sequenced with primer 1Ra. **B.** Representation of ORFA. An open reading frame, designated ORFA, is generated when intron 1 is inserted in the appropriate location relative to the published +1 of transcription (Rosenthal *et al.*, 1987). The junction occurs at nt 623 relative to the published +1 and is underlined. ORFA begins within exon 1 and extends for 126 amino acids before terminating. It is unlikely that this ORF is actually translated (see text for details).

A.

```
1     CCTGCACAGG TGAGAGCAGT GGCCAGCAAG AACGTGTCTG TTAGCGAAGC
      GGACGTGTCC ACTCTCGTCA CCGGTCGTTC TTGCACAGAC AATCGCTTCG
                 ⇓
51    ACACAAAAAT TGGCGTTGCA AATATTTCAA TAAAGCTGAA AAACAAAAGC
      TGTGTTTTTA ACCGCAACGT TTATAAAGTT ATTTCGACTT TTTGTTTTCG

101   AAAACATTGA AATAAAAATT ATTTCGTTTG CACATCGCTT TTTTATTTGC
      TTTTGTAACT TTATTTTTAA TAAAGCAAAC GTGTAGCGAA AAAATAAACG

151   ACGCGCGCTC AATCTTCGTC ACATTTTTTT CGCTTTTTCA TTTTTTCTGC
      TGCGCGCGAG TTAGAAGCAG TGTAAAAAAA GCGAAAAAGT AAAAAAGACG

201   GCCTCTCATT CAACAAGCTG GGAAAAATTA TATTATTTGC CAGTTATTAT
      CGGAGAGTAA GTTGTTCGAC CCTTTTTAAT ATAATAAACG GTCAATAATA

251   TTGGCCCATG TTCCGCCGAA AAGGTGCCCC GTCCACCCAA CCACCCAAAA
      AACCGGGTAC AAGGCGGCTT TTCCACGGGG CAGGTGGGTT GGTGGGTTTT

301   CCCCTTCGGA AATTCTTTTA TATATTTAAC CGTTGAGGCA TTAAAATTAA
      GGGGAAGCCT TTAAGAAAAT ATATAAATTG GCAACTCCGT AATTTTAATT

351   TGCTAATTAT GAGCTTGTTT GTCGCCAGTG ACATGTGCTT GAGTTTTTGT
      ACGATTAATA CTCGAACAAA CAGCGGTCAC TGTACACGAA CTCAAAAACA

401   TTAATTGCTG CACTTGTTAT GAGAAAAATC AATTGAAATC GATGCGTCGG
      AATTAACGAC GTGAACAATA CTCTTTTTAG TTAACTTTAG CTACGCAGCC
                                       ⇓
451   ACGCGAGTGA AAAATAATTA AGCAAACGGC GAACGTGCGG CCAGTGATTA
      TGCGCTCACT TTTTATTAAT TCGTTTGCCG CTTGCACGCC GGTCACTAAT

501   ATAAAATTAT GCAAATCGCC TTTTGGGCAA ATTAAAGCGA AATTCTCAAG
      TATTTTAATA CGTTTAGCGG AAAACCCGTT TAATTTCGCT TTAAGAGTTC

551   TCCAGTTTGG TTTTATTATT TTCGCTCTCT CTATCTCTCT CTCTCTCGCC
      AGGTCAAACC AAAATAATAA AAGCGAGAGA GATAGAGAGA GAGAGAGCGG

601   CATTATTAAC GTTAGATGGC ATTTAAGGC
      GTAATAATTG CAATCTACCG TAAATTCCG
```

B.

```
1     GAATTCTAATAATACCCCTCGGGATAATAACGAAAAAGCAGTCATAGCCA

51    TTATGGCCATTAAGCACAAAAAAGTGACATGAAAACGTGAAGGGAAATGT

101   GTTCCACAAATGGAGAAGAAAAAAAAAACGCGCTAACGCAAGTCGTTCGG

151   AAATGATAGTAACTGTTGCATTTCGCTCACAAAGACTATGATAGTTTTGC

201   TAACACAGAGTTTCAGTCGAGTCCTGAATTGTCCTTTTTGTGGTCTAGTT

251   TGCGGTTGCAATCAATGCTGAATATGTTGTATGCAGGGTAGCAATAGAGT

301   TGTACTTACTGCTGATTAGATAATTAAATGTACATCTTGGATAGATGAAA

351   ATGTTCTTGCTTTAAAATGTTTCAATTAGTTCATTGATTGAGTTGAACCA

401   ATTATTTTACAATTAGCCAACATTTCTTGATATAATTAAAGCTTAAATTG

451   GGACTAAATTGACTAAGGATTTATTTATTTTAAAATTCTCAGAGTGTTAT

501   CATTTTATAGCTGAATTAAATCAAATTATTTGAGTGTTTGCTTTAGTAAG

551   TTGTCAGCCACATAGCAGACTGCATATGTTTACAGGGTATGGCACCATCG
                                    M  F  T  G  Y  G  T  I  V  -

601   TATCCCAAGTGGGCCCACCTGCACAGGTGAGAGCAGTGGCCAGCAAGAAC
        S  Q  V  G  P  P  A  Q  V  R  A  V  A  S  K  N

651   GTGTCTGTTAGCGAAGCACACAAAAATTGGCGTTGCAAATATTTCAATAA
      V  S  V  S  E  A  H  K  N  W  R  C  K  Y  F  N  K  -

701   AGCTGAAAAACAAAAGCAAAACATTGAAATAAAAATTATTTCGTTTGCAC
       A  E  K  Q  K  Q  N  I  E  I  K  I  I  S  F  A  H  -

751   ATCGCTTTTTTATTTGCACGCGCGCTCAATCTTCGTCACATTTTTTTCGC
        R  F  F  I  C  T  R  A  Q  S  S  S  H  F  F  R

801   TTTTTCATTTTTTCTGCGCCTCTCATTCAACAAGCTGGGAAAAATTATAT
      F  F  I  F  S  A  P  L  I  Q  Q  A  G  K  N  Y  I  -

851   TATTTGCCAGTTATTATTTGGCCCATGTTCCGCCGAAAAGGTGCCCCGTC
       I  C  Q  L  L  F  G  P  C  S  A  E  K  V  P  R  P  -

901   CACCCAACCACCCAAAACCCCTTCGGAAATTCTTTTATATATTTAACCGT
        P  N  H  P  K  P  L  R  K  F  F  Y  I  F  N  R

951   TGA
      *
```

translation initiation sequence of (C/A)AA(A/C)ATG (Cavener, 1987). Examination of the context of the other ATG sequences within intron 1 did not reveal any consensus translation start sequences. In contrast, the sequence surrounding the ATG of the putative *Pkc53E* kinase ORF, AACAATG, agrees much better with the *Drosophila* consensus (Rosenthal *et al.*, 1987). Furthermore, comparison of this amino acid sequence predicted from ORFA with the public databases using the default BLAST peptide search protocol (April 13, 1997) revealed no similarities with known sequences. Nor are there any similarities to known protein motifs (*eg.* PKC pseudosubstrate domain, C2 Ca^{2+}-binding domain, *etc.*). Therefore, it is unlikely that ORFA encodes a translated protein.

3. 5′ RACE

Natesan (1990) used an antisense primer complementary to nt 210 to 230 of the published cDNA to define four transcription start sites around the published +1. RPA indicated that additional transcription start sites may be present within some of the incompletely sequenced introns. For example, an RPA probe spanning intron 1 gave rise to an incompletely protected fragment with head RNA (Figure 4.9). To analyse the 5′ ends of *Pkc53E* transcripts, I used 5′ RACE in conjunction with three pairs of nested GSPs: 4Rc and 4Rd; 3Rb and 3Rc; and 2Rc and 2Rd.

Initially, 5′ RACE was performed with primer 4Rc for priming cDNA synthesis from 1 μg of polyA$^+$ RNA and primers 4Rd and AAP for amplification. Southern blot hybridization of the 5′ RACE reactions with whole female RNA showed a Pkc1-hybridizing band of about 1.55 kbp (Figure 4.21A). Two major bands, approximately 1.55 and 1.1 kbp, are seen upon amplification of ovary cDNA (Figure 4.21A). All of these bands are smaller than that predicted by the published sequence (1.97 kbp). The fuzziness of the observed bands may be due to premature termination of the reverse transcription reaction, multiple transcription start sites, or to unequal addition of the oligo(dC) to the cDNAs.

5′ RACE with primer 3Rb to prime cDNA synthesis and 3Rc and AAP for PCR, or primer 2Rc to prime cDNA synthesis and 2Rd and AAP for PCR, each yielded one predominant *Pkc53E*-hybridizing band (Figure 4.21B). When aligned with the published cDNA based on size, these bands appeared to terminate at the same 5′ site as the 1.55 kbp

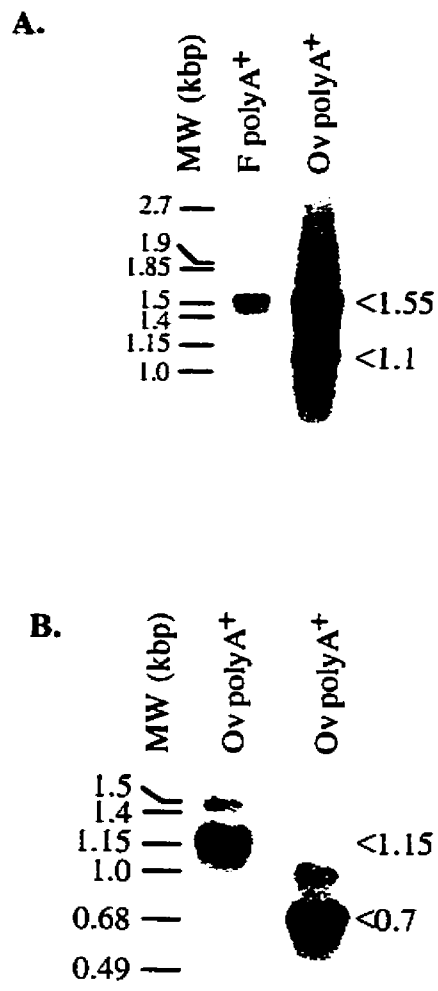


FIGURE 4.21. 5′ RACE analysis of polyA⁺ RNA. A. Southern blot hybridization shows the presence of a 1.55 kbp band amplified from both whole female and ovary cDNA and an additional 1.1 kbp band from ovary cDNA, using primer 4Rc for cDNA synthesis and primer 4Rd for PCR. Both bands hybridize to DIG-labelled Pkc1 probes. **B.** Southern blot hybridization shows the presence of a 1.15 kbp band amplified from ovary cDNA with primer 3Rb for cDNA synthesis and primer 3Rc for PCR. A 0.7 kbp band is amplified from ovary cDNA with primer 2Rc for cDNA synthesis and primer 2Rd for PCR. The source of polyA⁺ RNA is indicated above each lane. MW indicates the DIG-labelled molecular weight marker VII (Boehringer/Manheim).

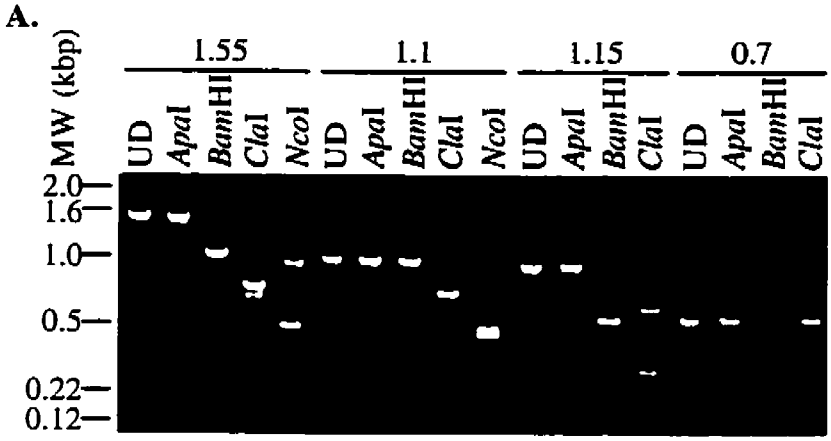
fragment amplified with primers 4Rd and AAP. This suggested a site of very stable secondary structure, a transcription start site 3′ from the published +1, or a novel transcription start site 5′ to the published +1 and spliced to exon 2. These bands were present even when the cDNA synthesis reaction was increased to 50°C, arguing against premature reverse transcription termination due to the presence of secondary structure (Figure 4.21B and data not shown).

To differentiate between these possibilities, restriction mapping of the 5′ RACE products was done (Figure 4.22). Restriction enzyme digestion showed that the 1.1 kbp 5′ RACE product terminated near the *Bam*HI site at nt 913 of the published cDNA. Direct cycle sequencing of this product using primer 2Rc showed that this fragment is a truncated cDNA, ending at nt 952 of the published cDNA (data not shown). This region may contain stable secondary structure or primary sequences that lead to the attenuation of the reverse transcription reaction. Since this 1.1 kbp product is still seen when reverse transcription is performed at 50°C, the latter possibility seems more likely.

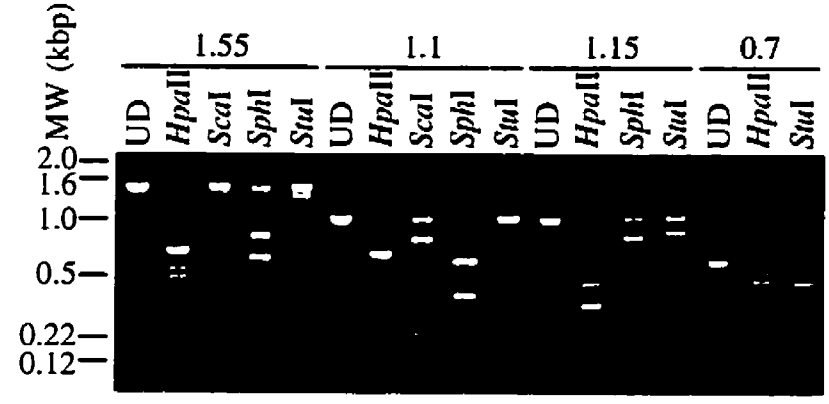
Restriction analysis of the other 5′ RACE products revealed them to be collinear with the published cDNA 3′ of the *Stu*I site at nt 637. However, the *Apa*I site at nt 611 of the published cDNA was absent from these fragments. This suggested that these transcripts originated either within intron 1 or upstream of the published +1, with at least both exon 1 and intron 1 being spliced out of the mature transcript. *Apa*I activity was confirmed in parallel restriction digests of pSB29, which contains a 2.9 kbp *Eco*RI fragment of *Pkc53E* from the published +1 to an *Eco*RI site within intron 2. This fragment includes the *Apa*I site at nt 611 of the published genomic and cDNA sequences (data not shown). The 1.55 kbp fragment was sequenced using primer 2Rc (to confirm collinearity with the published cDNA; data not shown) and primer 1Ra to identify the sequence at the 5′ end (Figure 4.23 and summarized in Figure 4.24). The 5′ ends of these RACE products contain novel sequences spliced onto exon 2 of the published *Pkc53E* cDNA. Comparison of this sequence with the public database sequences using the default settings of the BLAST search engine (April 13, 1997), revealed no similarities with other sequences. These sequences were also compared with those of Natesan (1990) and Hecker (1996), sequences of

FIGURE 4.22. Restriction digest analysis of 5′ RACE products. A. & B. Restriction analysis of the 5′ RACE products. The enzymes used for digestion are marked on above each lane, UD=undigested. 5' RACE products are indicated by the size of the undigested amplification product on agarose gels (see Figure 4.21). **C.** Schematic representation of the restriction digest sites within the published cDNA. Restriction digestion sites are shown above the bar and amplification primer locations below. Comparison of the actual digest with the predicted digest results shows that the *Apa*I site (*) is absent from the 5′ RACE products. The *Hpa*II sites are also different from those predicted with an extra doublet showing up when fragments 1.55, 1.15, and 0.7 are digested. The source of these doublets is not known. They may represent fragments for two adjacent transcription start sites, as is indicated by the noisy signal produced from sequencing the 5′ RACE products.

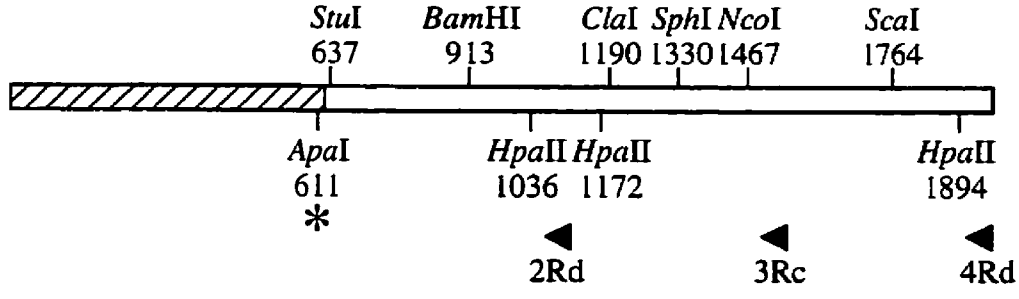
A.


MW (kbp)
1.55
1.1
1.15
0.7
UD
ApaI
BamHI
ClaI
NcoI
UD
ApaI
BamHI
ClaI
NcoI
UD
ApaI
BamHI
ClaI
UD
ApaI
BamHI
ClaI
2.0
1.6
1.0
0.5
0.22
0.12

B.


MW (kbp)
1.55
1.1
1.15
0.7
UD
HpaII
ScaI
SphI
StuI
UD
HpaII
ScaI
SphI
StuI
UD
HpaII
SphI
StuI
UD
HpaII
StuI
2.0
1.6
1.0
0.5
0.22
0.12

C.


StuI
637
BamHI
913
ClaI
1190
SphI
1330
NcoI
1467
ScaI
1764
ApaI
611
HpaII
1036
HpaII
1172
HpaII
1894
2Rd
3Rc
4Rd

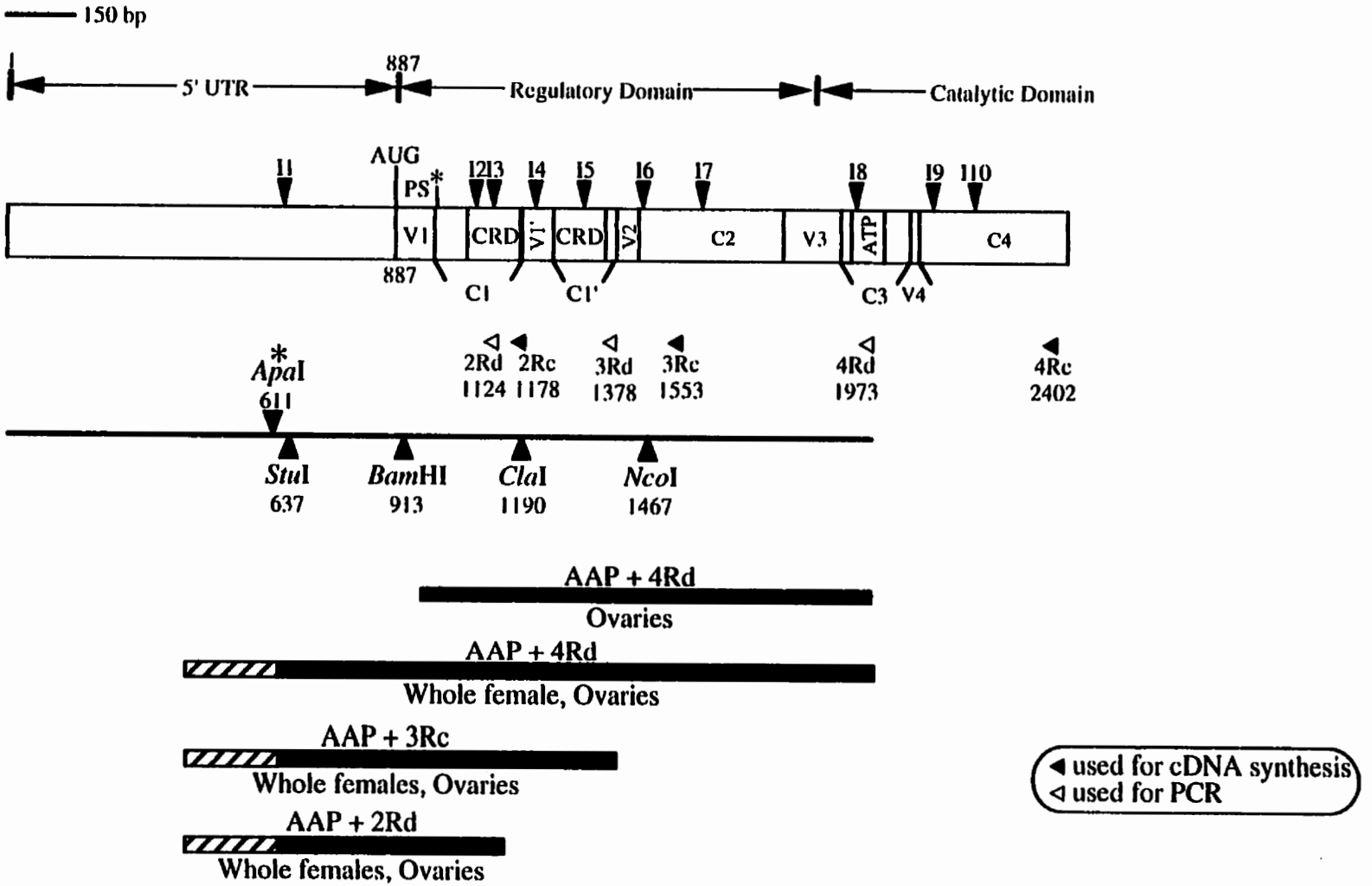
```
1     AGAACGcATT TGTGTgTTGA GCGAAGCACA CtTaCCTGTG GACAgCGAAA
                                          g
51    CacATTCTTT CTTCCAGTCa TTCGCGACCG CGAAgAGCTA GAGATAcACT

101   ACCCTCACtT AGTCCAGcCA cTACtACTAC TACTCATGcA GATGGCATTT
                        t   t   a

151   A
```

FIGURE 4.23. Sequence of the 5′ end of the 1.55 kbp 5′ RACE product. Boldfaced, double-underlined sequences represent the 5′ end of exon 2 of the published cDNA. Small letters represent sites of ambiguity within the chromatograph from the ABI373 sequencer. The 5′ end shown here represents the sequence ending at the first string of oligo(dC), representing a site of oligo(dC) addition during cDNA tailing. Some weak, noisy signal continued past this point for approximately another 40 nt, ending in another string of oligo(dC). This indicates that there may be multiple transcription start sites for this exon. This would be consistent with multiple transcription start sites being part of *Pkc53E* regulation as shown with multiple transcription start sites around the published +1 of exon 1 (Natesan, 1990).

FIGURE 4.24. Schematic representation of the 5′ RACE products. The sequences derived from published cDNAs are represented by the open boxes in which the putative domains are labelled (Rosenthal *et al.*, 1987). Immediately below, represented by the solid line is the schematic representation of the restriction digest sites found in that region of the published cDNA and below this, represented by the solid and striped bars, are the 5′ RACE products. Each 5′ RACE product is labelled with the primers used for amplification above and the source of RNA from which it was amplified below. The solid bar represents sequences that are collinear with the published sequence. The striped bar represents the novel sequences spliced onto exon 2 in these transcripts. *The *Apa*I site is not present in 5′ RACE products.


150 bp
5' UTR
887
Regulatory Domain
Catalytic Domain
I1
AUG
PS
I2
I3
I4
I5
I6
I7
I8
I9
I10
V1
CRD
V1'
CRD
V2
C2
V3
ATP
C4
887
C1
C1'
C3
V4
2Rd 1124
2Rc 1178
3Rd 1378
3Rc 1553
4Rd 1973
4Rc 2402
ApaI 611
StuI 637
BamHI 913
ClaI 1190
NcoI 1467
AAP + 4Rd
Ovaries
AAP + 4Rd
Whole female, Ovaries
AAP + 3Rc
Whole females, Ovaries
AAP + 2Rd
Whole females, Ovaries
◀ used for cDNA synthesis
◁ used for PCR

5′ RACE products and of 5′ genomic sequences of *Pkc53E*, respectively. Fasta (GCG) revealed no regions of similarity or identity. Thus these sequences represent a novel exon(s) originating at least 1.2 kbp upstream of the published +1 site of transcription (Rosenthal *et al.*, 1987).

II. Discussion

A. Northern Blot Analysis

Northern analysis of total RNA showed the 2.4 kb transcript was more abundant than either the 4.0 or 4.3 kb transcript (Figure 4.1). These results differ from those of Rosenthal *et al.* (1987) that showed approximately equal expression of the three transcripts. A possible explanation for this difference is that Rosenthal *et al.* (1987) used polyA$^+$ RNA for their Northern analysis, as opposed to total RNA used here. This may indicate that some *Pkc53E* RNA(s) is not polyadenylated (see below).

B. cDNA Library Screening

Natesan (1990) reported the isolation of three cDNA clones with inserts of 4.0, 3.8, and 2.5 kbp each from the adult head λgt10 cDNA library used here. Unfortunately these clones were lost. In an attempt to reisolate and further analyse these clones, I rescreened this head library. Screening 6 x 10^5 plaques (about twice the complexity of the library) resulted in the isolation of three Pkc1 hybridizing clones, the largest of which contained only a 0.8 kbp insert. Screening of about two times the complexity of an ovary cDNA library yielded a single positive clone. Again the size of the insert was less than 1 kbp. Since both libraries were synthesized using oligo(dT) primers, the insert sequences likely represented sequences adjacent to the polyA tail. Given that much of the variability of the mammalian PKCs occurs in the regulatory domain, we were primarily interested in isolating full-length cDNAs. Further, the isolation of full-length clones from a single developmental stage avoids the potential problem of overlapping cDNAs from different transcripts. The clones isolated were much less than full-length. Given these results, alternative approaches were employed.

C. RNAse Protection Analyses

1. RPA Probes

The absence of Pkc1 fragment insertions in the sense orientation with respect to *lacZ* transcription in pBluescript™ is unusual for non-directional cloning procedures. This suggests that Pkc1-encoded products in this context are lethal to *E. coli*. Insertion of the Pkc1 fragment in both directions with respect to *lacZ* was possible for pTZ18U vectors (New England Biolabs; data not shown), suggesting that the observed lethality is due to the particular *lacZ*-Pkc1 fusion, rather than inherent to sense Pkc1 transcripts in *E. coli*.

2. RPAs

The results of the RPAs showed that the variability among head RNAs is greater than that among ovary RNAs (Figure 4.9). This correlates well with Northern blot analyses which reveal the presence of a prominent *Pkc53E* hybridizing band in ovaries, with three transcripts identified in adult heads. A 2.4 kb transcript is also present in total testis RNA. However, RPA probes were not protected with testis RNA. It is possible that the level of *Pkc53E* transcript(s) is too low for detection by the RPA probes. It is also possible that the testis 2.4 kb transcript detected with Northern analysis contains as yet unidentified *Pkc53E* exons that are not sufficiently similar to give rise to protected RPA fragments. Alternatively, an endogenous message complementary to *Pkc53E* messages may be present and might compete with the RPA probes for *Pkc53E* transcript(s) binding.

The variability observed among protected fragments from the putative regulatory domain was greater than among those from the putative catalytic domain. This suggests that, like mammalian PKCs, *Pkc53E*-encoded PKCs may differ from each other most in the regulatory domain. These differences can result in PKC isoenzymes with altered cofactor/coactivator requirements as is seen in other systems (see Figure 1.1; Newton, 1996b). Differences within the catalytic domain suggest the possibility that an altered catalytic site may be present, which may reveal substrate specificity differences when isolated and tested, as seen with the PKCβ isoenzymes (Blobe *et al.*, 1996).

D. PCR Analysis of RNA

1. 3′ RACE

As mentioned above, 3′ RACE takes advantage of the polyA$^+$ tail on mRNA to allow the amplification of mRNA using oligo(dT)-based primers for cDNA synthesis and amplification. As such, limited gene-specific sequence information can be used to amplify 3′ sequences. RPAs demonstrated a single, full-length protected fragment using *Pkc1*SBc, a probe spanning the ATP-binding domain, a domain essential for active kinases. Given this, I reasoned that amplification using primers specific for this region would likely enable the amplification of the 3′ RNA sequences of *Pkc53E*-encoded kinases.

3′ RACE using C2 or C3 domain-specific primers yielded the predicted products for each cDNA sample, except for testis cDNA (Figures 4.10 to 4.14 and summarized in Figure 4.15). The amplification of a *Pkc53E* sequence with primers 4b and 4R, but not with 3′ RACE, using testis cDNA suggests that while there are *Pkc53E* transcripts within the testes, these transcripts are not polyadenylated. This is further supported by the observation that increasing the oligo(dT)$_{12\text{-}18}$ primer annealing temperature and reverse transcription reaction temperature to 50°C eliminates *Pkc53E* sequence amplification from testis cDNA. This explains why the Northern analyses reported by Rosenthal *et al.* (1987) using polyA$^+$ RNA differs from those reported here using total RNA. With some of the 2.4 kb transcript not polyadenylated, the relative abundance of the 2.4 kb transcript on Northerns with total RNA compared to the equality of *Pkc53E* transcripts on polyA$^+$ Northerns is explained.

The sharpness of the hybridizing 3′ RACE bands suggests that only one, or perhaps multiple very proximate, polyadenylation sites are used with *Pkc53E* transcripts that contain C2 and/or C3 domain sequences (primers 4 and 4b and primers 3 and 3b, Figure 2.3). Natesan (1990) did RPA with a probe spanning the polyadenylation signal at 3159-3164 of the published transcript. He observed the presence of two protected fragments, one corresponding to transcript polyadenylation using the signal at 3159-3164 of the published sequence and another extending beyond this point. These two fragments were present throughout development. He did not differentiate between tissues or sexes in these experiments. These differences may be reconciled if, rather than the use of alternative

polyadenylation sites, some transcripts are not truncated by polyadenylation. This explains the observed protection of a probe that extended past the published polyadenylation signal at 3159-3164 (Natesan, 1990).

The doublet seen with ovary cDNA (and therefore present in cDNA from whole females) with 3′ RACE using primers 4 and 4b has only a single *Pkc53E* hybridizing band (Figure 4.12). The origin of the non-*Pkc53E* band is not known. It is possible that it originates from the same transcript as the non-hybridizing band seen with testis cDNA (Figure 4.10), representing a gonad-specific transcript with limited sequence similarity to *Pkc53E*.

The amplification of only the 3′ RACE products predicted from the published cDNA (summarized in Figure 4.15) is somewhat unexpected given the RPA results. Each RPA probe from the catalytic domain was fully protected with head RNA (Figure 4.9). This suggested, but did not confirm, the presence of a transcript corresponding to the published cDNA. However with probes, *Pkc1*BgSt, *Pkc1*SBc, and *Pkc1*C3023, shorter protected fragments were also seen. This indicated that some alternative processing within the putative catalytic domain might occur. The failure of 3′ RACE to detect such transcripts might mean that primer binding sites for 4, 4b, 3, and/or 3b are absent from these transcripts. This may in turn mean that there are *Pkc53E* transcripts that lack part or all of the C3 domain and/or C2 domain. Alternatively, these protected probe fragments may have arisen from hybridization to incompletely processed RNAs, pseudogene transcripts, or non-polyadenylated RNAs.

While there are sequence polymorphisms within these regions between the Oregon-R cDNA and Canton-S genomic sequences (Rosenthal *et al.*, 1987), Oregon-R was the source of both the cDNA sequences for probe synthesis and the total RNA targets. Thus, there is no evidence for sequence polymorphism as the source of the smaller protected probe fragments in RPA.

In addition, it was somewhat surprising to see amplification of the predicted 3′ RACE products with ovary cDNA (Figures 4.10 and 4.13). Of the catalytic domain probes, only probe *Pkc1*SBc was protected by ovary RNA (Figure 4.9B). This suggested

that this catalytic domain transcript was not present. However 3′ RACE, relying as it does on the exponential amplification of DNA, is much more sensitive than RPA. The full-length transcripts may be very rare in ovaries and therefore not detected during RPA. This increased sensitivity likely also accounts for the amplification of a *Pkc53E* product from testis RNA using primers 4b and 4R (Figure 4.16) when no protected fragments were seen using testis RNA in RPA (Figure 4.9).

2. RT-PCR

RT-PCR using primer pairs spanning each of the putative conserved domains of *Pkc53E* RNA resulted in the amplification of only the fragments predicted from the published cDNA (summarized in Figure 4.17A). These results suggest that each of the *Pkc53E* RNAs detected by Northern hybridization contains the ORF encoding a DG-, PS-, and Ca^{2+}-dependent PKC. This result correlates well with the finding that a single protein band is detected by anti-PKC antibodies made against a glutathione-*S*-transferase (GST)-*Pkc53E* V1/pseudosubstrate fusion protein (A. Piekny, unpublished data).

RT-PCR with primer 1b, from the *Pkc53E* 5′ UTR, and either primer 3Rb or primer 4Rb resulted in the amplification of the fragments predicted from the published cDNA, as well as fragments that were about 600 bp larger (summarized in Figure 4.17B). Restriction analyses localized the insertion and sequencing confirmed that the insertion was intron 1 (Figures 4.19 and 4.20A). Analysis of the *Pkc53E* sequence generated by this insertion shows that while this does not extend the previously predicted *Pkc53E* ORF, a short ORF, designated ORFA, of 126 amino acids can be predicted (Figure 4.20B). This peptide sequence does not resemble any peptide sequences in the public databases as determined with the BLAST search engine (searched April 13, 1997). Furthermore, the sequence surrounding the start site of ORFA differs from the start site of the predicted *Pkc53E* ORF, AACAATGT, the latter of which is in good agreement with the cannonical *Drosophila* translation start site (Rosenthal *et al.*, 1987). Thus, it is unlikely that ORFA actually results in protein synthesis.

This begs the questions, why is intron 1 present in some *Pkc53E* transcripts? The UTRs of several *Drosophila* RNAs have been shown to be important in post-transcriptional

regulation of translation (*eg. bicoid, nanos*; FlyBase, 1996). While BLAST analysis (done April 13, 1997) of the *Pkc53E* intron 1 sequence does not reveal any similarity to known transcripts, this sequence may play a role in post-transcriptional regulation of the *Pkc53E* messages in which it is present. For example, it has been shown that bicistronic mRNAs in *Drosophila* show differential translation of the two ORFs when translation is initiated in a CAP-dependent manner, with the 3′ ORF translated much less efficiently than the 5′ ORF (*eg.* LINE retrotransposons, Bouhidel *et al.*, 1994). ORFA, if translated, could function to reduce expression of the *Pkc53E* kinase ORF. Alternatively, intron 1 may regulate translation by secondary structure interactions with other regions of the transcript, such as seen with alcohol dehydrogenase transcripts in *Drosophila* (Parsch *et al.*, 1997). Further experiments would be necessary to determine the effect of intron 1 on *Pkc53E* expression.

3. 5′ RACE

5′ RACE analysis was used to try to extend the transcript structure information upstream from the sequences isolated by 3′ RACE and RT-PCR. 5′ RACE revealed that there are novel 5′ sequences spliced onto exon 2 of the published *Pkc53E* RNA (summarized in Figure 4.24). These sequences did not match any known sequences using the BLAST search engine (search performed April 13, 1997) nor any *Pkc53E* sequences previously identified in this laboratory (Hecker, 1996; Natesan, 1990). Furthermore, these sequences do not originate from intron 1. This suggests the presence of an as yet unidentified exon 5′ of the published +1.

Several other bands are revealed upon Southern hybridization of the ovary cDNA 5′ RACE reaction using primers 4Rd and AAP for amplification. Some of this may be accounted for by the differences in length of the oligo(dC) tail added during the 5′ RACE procedure. Some may also represent other, less uniform premature terminations of the reverse transcription. Finally, these other bands may represent less prevalent transcript start sites. Natesan (1990) used primer extension analysis to identify four transcription start sites within an approximately 270 nt region surrounding the published +1 site of transcription from -224 to +43. These may be the result of alternative splicing within the 5′ UTR of *Pkc53E* transcripts and/or the use of different transcriptional start sites within the promoter

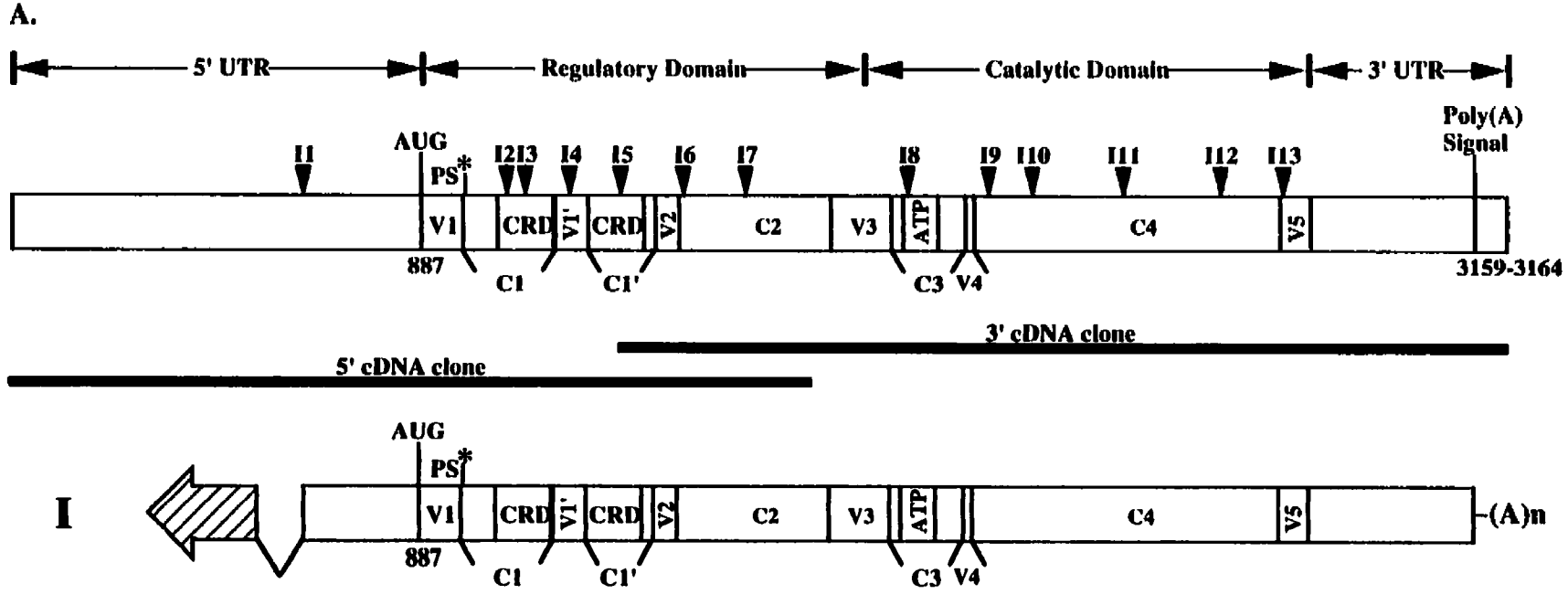
of *Pkc53E*. These start sites were identified using a primer complementary to nt 210 to 230 of the published *Pkc53E* cDNA and represent transcript sequences collinear with exon 1. It would therefore not be surprising to see multiple transcription start sites used at another *Pkc53E* promoter.

III. Conclusions

Figure 4.25 is a schematic diagram of putative *Pkc53E* transcripts, derived from the available data. The published cDNA sequence is represented by the open box with putative domain structures indicated within. These domains were derived from computer translation of the cloned and published cDNA nucleotide sequence and comparisons with known PKC protein domains (Schaeffer *et al.*, 1989). Known intron locations are indicated by the closed arrowheads. The two solid overlapping lines immediately beneath the cDNA schematic represent two cDNA clones from which the published cDNA was compiled (Rosenthal *et al.*, 1987). The 3′ fragment encompassed the complete known 3′UTR, putative catalytic domain and part of the regulatory domain, terminating at the boundary of intron 5, an approximately 8 kbp unsequenced intron. The 5′ fragment extends from near the carboxyl terminus of the C2 domain 5′ to the published transcription start site. The putative transcripts are shown below and are labelled with roman numerals I, II, III, and IV.

Transcript I (Figure 4.25A) represents a polyadenylated, 2.7 kb transcript present in adult heads and ovaries. This transcript corresponds to the smallest of the *Pkc53E* transcripts detected on Northern blots and previously estimated to be 2.4 kb. The 3′ RACE and RT-PCR data suggest that polyadenylated transcripts with C3 domain sequences (primers 4 and 4b binding sites), are contiguous with the second CRD (primers 3 and 3b binding sites) and the V5 domain (primers 4R and 4Rb binding sites). These data correlate well with the observed 3′ cDNA clone isolated by Rosenthal *et al.* (1987). These transcripts are present in adult heads and ovaries, but polyA$^+$ transcripts are absent from testes (Figures 4.10 and 4.13; summarized in Figures 4.15 and 4.17). Further RT-PCR data suggests that sequences 3′ of intron 1 are collinear with the published *Pkc53E* cDNA and that these sequences are present in adult heads, ovaries, and testes.

FIGURE 4.25. Schematic diagram of hypothetical *Pkc53E*-encoded transcripts. The sequences derived from published cDNAs are represented by the open boxes in which the putative domains are labelled. Immediately below, represented by the solid lines (▬) are the cDNA clones used to compile the cDNA sequence (Rosenthal *et al.*, 1987). The hypothetical transcripts are labelled with roman numerals, I, II, III, and IV, respectively. Novel sequences are indicated by the striped boxes. Lines connecting exon boxes are indicative of splicing events. Arrowheads at the 5′ ends of transcripts suggest transcription from multiple transcription start sites, resulting in variable 5′ terminal sequences. **A.** Transcript I is a polyadenylated 2.7 kb RNA species found in adult heads and ovaries. **B.** Transcript II is a non-polyadenylated 2.7 kb species found in adult testes and possibly also in adult heads and ovaries. **C.** Transcript III is polyadenylated and found in adult heads and at very low levels in ovaries. It contains intron 1 in the mature transcript. This transcript may be present in a non-polyadenylated form in testes. **D.** Transcript IV encodes a protein very similar to that predicted by Rosenthal *et al.* (1987). This is a 3.4 kb polyadenylated transcript expressed in larval and early pupal stages of development. See text for further details.



3' RACE: sequences within the catalytic domain correspond to the published sequence
transcript is polyadenylated in adult heads and ovaries

RT-PCR: sequences 5' of primer 1c correspond to the published sequence

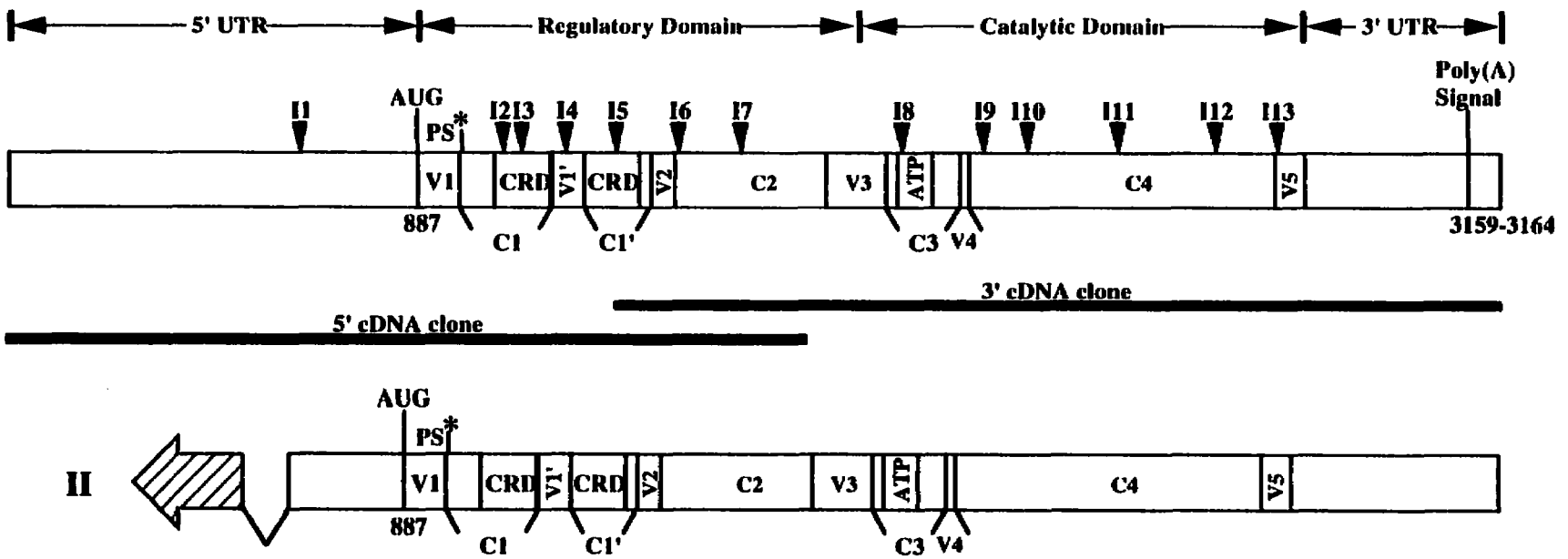
5' RACE: transcription start site is closer to primer 4Rd site than in published sequence

Sequence: novel sequences are spliced to exon 2

RPA: protected fragments indicate variability in the region of intron 1

This represents a polyadenylated 2.7 kb *Pkc53E* transcript expressed in adult heads and ovaries.

B.



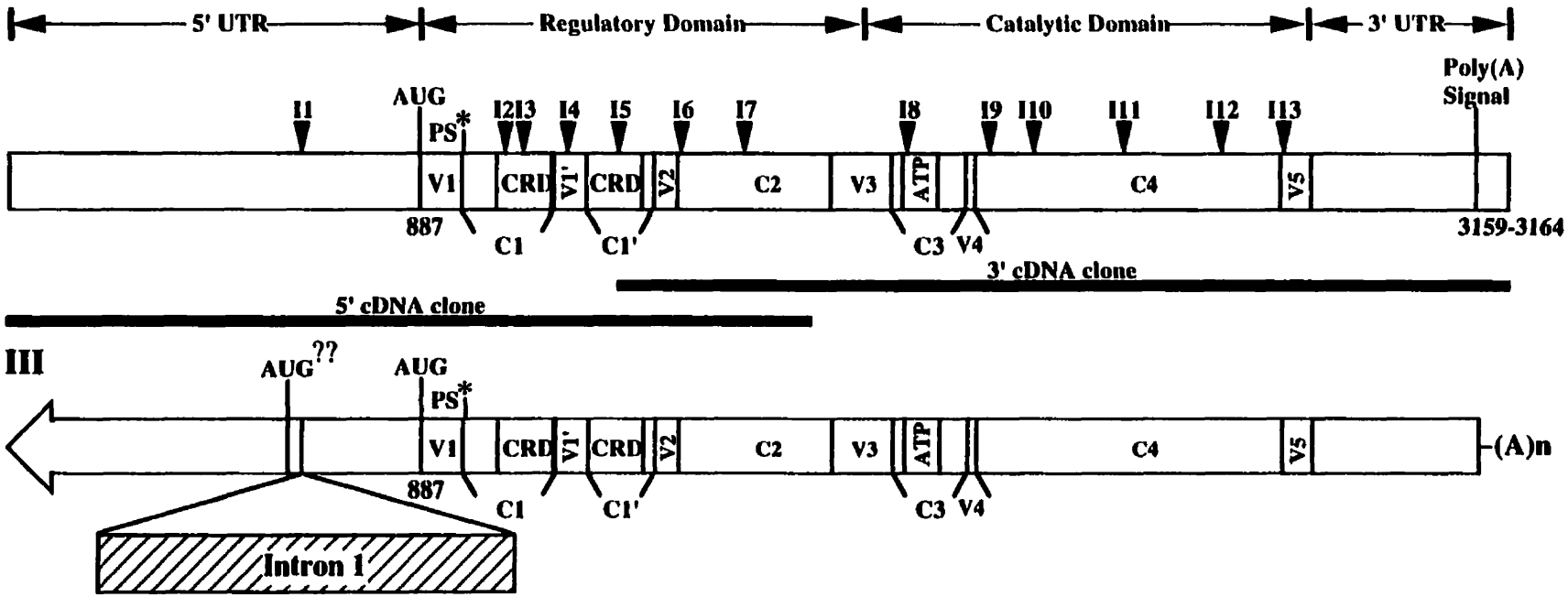
Northern Analysis: hybridization indicates the presence of an abundant message of approximately 2.4 kb

3' RACE: no polyadenylated *Pkc53E* transcripts in testes

RT-PCR: high temperature annealing of cDNA synthesis oligo(dT) primers (50°C) eliminates RT-PCR products from testis RNA

This represents a non-polyadenylated 2.7 kb *Pkc53E* transcript expressed in adult heads, ovaries, and testes. All *Pkc53E* transcripts in testes are non-polyadenylated.

C.



3' RACE: transcripts containing the published catalytic domain sequences are polyadenylated in adult heads and ovaries

RPA: there is some variability in the region of intron 1

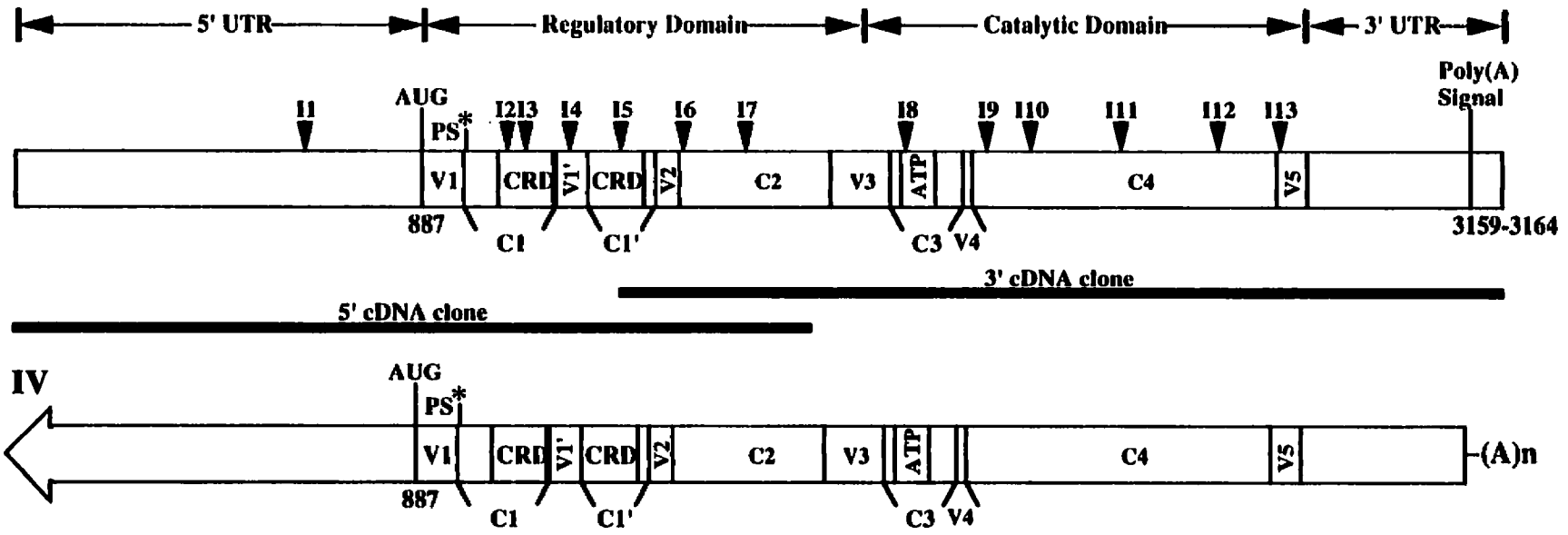
Northern Analysis: there are two larger transcripts in adults of 4.0 and 4.3 kb that contain sequences not in the published sequence

Sequence: sequencing of the insert within the larger RT-PCR products from amplification with primers 1b and 3Rb demonstrated the presence of intron 1 sequences within *Pkc53E* transcripts

Primer extension: Natesan (1991) showed the presence of multiple transcription start sites up to about 250 nt 5' of the published +1

This represents the polyadenylated 4.0 and 4.3 kb transcripts expressed in adults.

D.



Northern Analysis: a 3.4 kb transcript is expressed in late larval and early pupal stages

cDNA library clonestwo overlapping clones isolated from early pupal and/or adult head libraries give a 3.2 kb transcript that contains an open reading frame predicted to encode a cPKC

3.2 kb correlates well with the larval transcript detected on Northerns

Primer extension: Natesan (1991) showed the presence of multiple transcription start sites up to about 250 nt 5' of the published +1

This represents a polyadenylated 3.4 kb transcript expressed in late larval and early pupal stages.

RPA suggests that in adult heads and ovaries, there is a transcript that truncates at the intron 1/exon 2 boundary (probe *Pkc1*H696, Figure 4.9A). 5′RACE revealed the presence of a transcript with about 150 bp of novel sequences spliced onto exon 2 (summarized in Figure 4.24). These 5′ RACE reactions were done with polyA$^-$ RNA isolated from ovaries and whole females, but given the presence of RT-PCR products in all tissues tested, are likely present in male heads as well.

Hence, Transcript I (Figure 4.25A) is a polyadenylated 2.7 kb message found in adult ovaries and heads, with exon 1 absent and replaced by novel sequences spliced directly to exon 2.

Transcript II (Figure 4.25B) is a non-polyadenylated 2.7 kb transcript found in adult testes, ovaries, and heads. The presence of a non-polyadenylated message is suggested by different relative intensities of *Pkc53E* transcripts when comparing polyA$^+$ and total RNA by Northern blot hybridization (Rosenthal *et al.*, 1987; Figure 4.1). Furthermore, RT-PCR with testis cDNA demonstrated the presence of *Pkc53E* transcripts spanning the published sequences (summarized in Figure 4.17). However, these transcripts are likely not polyadenylated since 3′ RACE amplification of *Pkc53E* sequences was not successful using testis cDNA (Figure 4.10 and 4.13).

The regulation of polyadenylation may serve as a post-transcriptional tissue-specific regulator of PKC53E protein expression. The abundance of the previously identified 2.4 kb transcript on total RNA Northern blots suggests that polyadenylation of this transcript may be very inefficient. Further, anti-PKC53E V1/pseudosubstrate antibodies do not recognize protein from testes with Western blotting or immunofluorescence, suggesting that these epitopes (V1 and pseudo-substrate domains) are not present (A. Piekny, unpublished data).

Therefore, Transcript II (Figure 4.25A) is a non-polyadenylated 2.7 kb transcript found at least in ovaries and testes, and possibly heads as well. Further, this transcript is translationally inactive in testes.

Transcript III (Figure 4.25C) is proposed to encode the 4.0 and/or 4.3 kb polyadenylated transcripts found primarily in adult heads, as demonstrated by Northern analysis (Figure 4.1). The 4.0 and 4.3 kb transcripts are larger than the cDNA sequence

reported by Rosenthal *et al.*, (1987) and suggested the presence of as yet unidentified exon sequences. RT-PCR using primer 1b from the published 5′ UTR of *Pkc53E* cDNA and 3′ primers 3Rb or 4Rb revealed that larger *Pkc53E* transcripts retain intron 1 (Figures 4.19 and 4.20). The size of the cDNA (without the polyA$^+$ tail) as reported here is 3.8 kbp. The use of alternate transcription start sites, as defined by Natesan (1990) accounts for the detection of two closely sized bands on Northern blots. Polyadenylation of these transcripts results in the detection of transcripts estimated at 4.0 and 4.3 kb on Northern blots.

Intron 1 sequences do not resemble any sequences within the public databases. The function of this alternative exon is not known, but may involve post-transcriptional regulation of *Pkc53E*. In *C. elegans*, it has been shown that levels of PKC1B protein are regulated post-transcriptionally and/or post-translationally (Land *et al.*, 1994). Further, the alternative splicing of mammalian PKCβ mRNA regulates isoenzyme expression (Blobe *et al.*, 1993; Chalfant *et al.*, 1995). Therefore it would not be surprising if some or all *Pkc53E* transcripts are post-transcriptionally regulated.

Thus, Transcript III (Figure 4.25C) represents the 4.0 and/or 4.3 kb message found in adult heads, ovaries, and testes. It is likely not polyadenylated in testes, as indicated by the 3′ RACE results (see discussion of Transcript II).

Transcript IV encodes the open reading frame reported by Rosenthal *et al.* (1987; Figure 4.25D). Furthermore, on Northern analysis of larval and polyA$^+$ RNA, a 3.4 kb *Pkc53E* transcript has been identified (Natesan, 1990). The size of the sequenced cDNA is 3.2 kbp. The addition of a polyA$^+$ tail accounts for the larger estimated size on Northern blots. These data allow me to state that Transcript IV represents a polyadenylated 3.4 kb transcript expressed in late larval and early pupal stages of *Drosophila* development.

IV. Future Directions

Novel 5′ *Pkc53E* exon sequences have been identified with 5′ RACE. Cloning of this exon from genomic DNA would help complete *Pkc53E* gene structure. The promoter and transcriptional regulatory signals controlling *Pkc53E* expression remain to be elucidated. These experiments could involve the use of DNA cloned from the 5′ region of

Pkc53E linked to reporter genes. These types of experiments would identify the sequences important in regulating *Pkc53E* expression in response to both developmental and environmental cues. Once identified, these promoter/enhancer sequences could be used to identify the transcription factors that regulate *Pkc53E* expression and biochemical and functional analyses of these factors may lead to the identification of the normal signal transduction events that activate *Pkc53E* expression.

Instead of encoding different isoenzymes, the different *Pkc53E* transcripts differ within their UTRs, possibly representing differences in post-transcriptional or translational regulation. Experiments using chimeric (*Pkc53E*-5′ UTR)-(reporter-ORF) RNAs may help elucidate 5′ UTR function, if promoter and protein expression analyses suggest that these experiments are warranted.

The types of experiments described above are of a primarily descriptive nature. While they would be useful in defining the cellular circumstances that activate *Pkc53E* expression, they would not likely reveal the function of PKC53E protein in *Drosophila*. Description of the function of PKC53E protein will require a "brute force" biochemical approach coupled with genetic analyses. While this thesis describes one attempt at producing a *P* element insertional mutant at *Pkc53E*, better screen design (*ie.* primer location, ammunition *P* element selection, and/or screening approach) and/or alternative mutagenesis approaches will likely be necessary for the production of a *Pkc53E* mutant. The myriad of *P* element lethals being produced by the *Drosophila* genome sequencing project may fortuitously yield a *Pkc53E* mutant. With this, the elegant experimental options and genetic proofs available to *Drosophila* geneticists become available.

CHAPTER 5: SUMMARY

This project began with a goal towards using the tools available with *Drosophila* to examine the genetics of *Pkc53E*. Due to the importance of PKCs in several cellular processes, this necessitated the use of a screen that enabled the screening of heterozygous mutants without a known phenotype. The type of *P* element mutagenesis screen first described by Ballinger and Benzer (1989), and used by many others (Dalby *et al.*, 1995; Hamilton *et al.*, 1991; Kaiser and Goodwin, 1990; Pereira *et al.*, 1992), is ideally suited to this purpose. Unfortunately, I was unable to isolate a mutant at *Pkc53E*, possibly due to a non-permissive gene structure and/or to dominant-negative lethality of truncated PKC53E proteins encoded by insertional mutant alleles. This forced the contemplation of other approaches to elucidate *Pkc53E* gene function. One such approach could include the use of antisense-*Pkc53E* RNA transgenes.

Several reports have shown that when antisense transcripts are present within a cell, they prevent translation of the cognate sense transcript, thereby decreasing or obliterating protein levels (*eg.* Jongens *et al.*, 1992). When multiple transcripts from a gene differ in their 5′ sequences, it may be possible to target single transcripts, and therefore single protein products, from a gene. This could allow the differentiation of the function(s) of individual gene products. This approach appears promising for a gene like *Pkc53E* that is known to encode multiple transcripts.

The first step toward such an approach is the elucidation of the transcript-specific sequences so that the appropriate antisense transgenes can be designed. With this in mind, I decided to examine the transcript structure of *Pkc53E*-encoded RNAs toward the determination of complete transcript sequences. The approaches of RPA, 3′ and 5′ RACE, and RT-PCR generated data that allowed predictions as to *Pkc53E* transcript structures (Figure 4.25).

Four types of transcripts are predicted. The 2.7 kb Transcript I (formerly estimated to be 2.4 kb) contains the published ORF with a truncated 5′ UTR. The extreme 5′ end of this transcript originates from a novel exon, likely 5′ of the published exon 1. Transcript II is a non-polyadenylated 2.7 kb transcript with the *Pkc53E* encoded sequences found in

Transcript I. These two transcripts may be transcribed from multiple, closely located transcription start sites. Transcript III contains the published intron 1 and has bariable transcript start sites surrounding the published +1. This transcript is polyadenylated in all tissues tested, except testes. Transcript IV is a larval- and pupal-specific transcript corresonding to the published cDNA, with multiple transcription start sites.

This molecular data indicates that in spite of multiple *Pkc53E*-encoded transcripts, only a single PKC53E isoenzyme exists. This correlates well with immunohistochemical and Western blot analyses (A. Piekny, unpublished data). In order to elucidate *Pkc53E* function both biochemical and genetic analyses must be employed.

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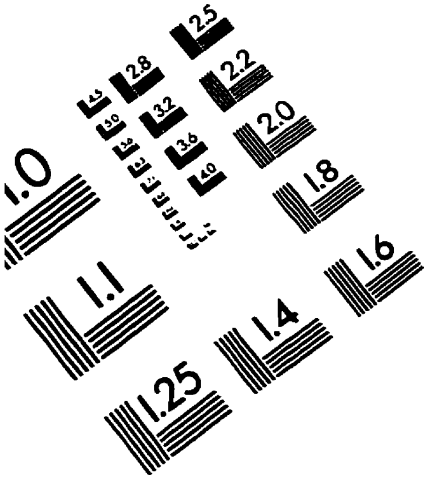

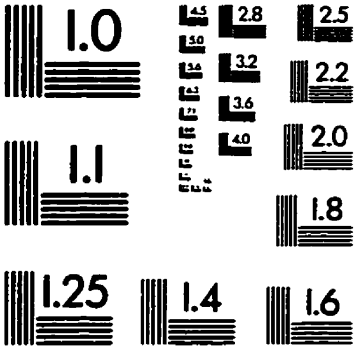

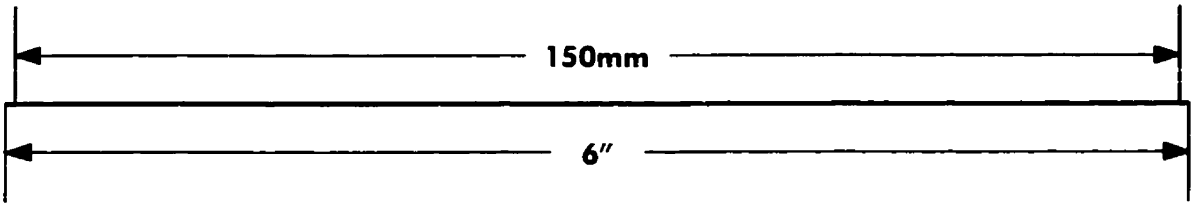

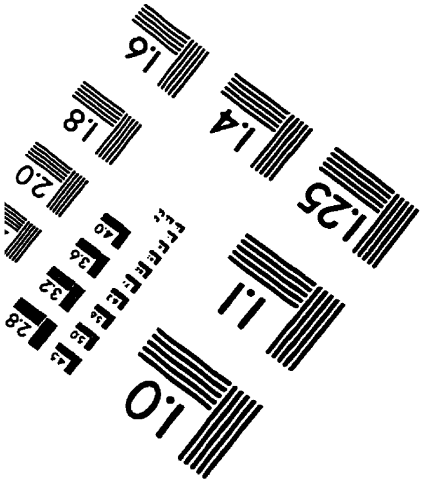

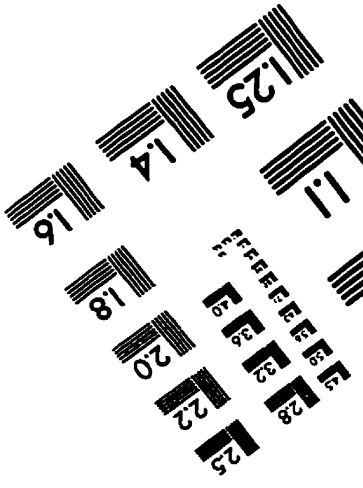

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