The Pan Gu Protein Kinase:
Regulator of the Early Embryonic Cell Cycle in Drosophila
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ABSTRACT

In Drosophila, fertilization is required for the embryo to undergo the first 13 rapid cell cycles, which consist of alternating DNA replication and mitosis without gap phases. In contrast, eggs from females mutant in the pan gu (png) locus no longer require fertilization to initiate DNA replication, and both fertilized embryos and unfertilized eggs from png mutant mothers have giant polyploid nuclei, indicating that S phase is not linked to mitosis and nuclear division in this mutant. png mutations fall into two classes: fertilized embryos from some alleles never undergo mitosis, while fertilized embryos from a second class of alleles are able to complete a few mitoses before the nuclei stop dividing and over-replicate. Four new mutations in the png gene were characterized, including new mutations of the multinuclear class, confirming that this phenotype results from loss-of-function of png and indicating that png functions during the first five divisions. To understand the function and mechanism by which the png gene product inhibits S phase, the gene was cloned. A screen for new alleles of png was completed, and a deficiency was isolated that delineated the gene to a 20 kb region. Smaller fragments that rescue png mutations were isolated, and the region was sequenced. All eight alleles of png have mutations in a single open reading frame, and a fragment containing only this transcription unit was capable of rescuing png mutants. The PNG protein shows strong homology to Ser/Thr protein kinases. The position and nature of the amino acid changes caused by the png mutations show that the severity of mutant phenotype correlates with the predicted degree of reduction of kinase activity. Phylogenetic analysis suggests that PNG represents a new family of protein kinases. Analysis of expression of png showed that the protein and mRNA are specifically expressed in mature oocytes and during early development, and ectopic expression of PNG later in development had no effect, indicating that PNG is a specific regulator of the early cell cycles. PNG protein co-immunoprecipitates with the PLUTONIUM protein, which also regulates the early embryonic cell cycles.
To my parents

for their endless love and support
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# TABLE OF CONTENTS

## Chapter One:
### Introduction 7
- Control of the Cell Cycle 10
- Conserved Controls 10
- Dependency of S Phase on Mitosis 11
- The Centrosome 14
- Fertilization: the Egg to Embryo Transition 18
  - Vertebrates 18
  - Early Embryogenesis in Drosophila 19
- *pan gu* and Early Embryogenesis 22
- References 27

## Chapter Two:
### Materials and Methods 35
- Mutations and Strains 36
- Unfertilized Eggs 36
- Embryo Fixation and Staining 37
- Microscopy 37
- Quantitative Southern Blots 37
- Construction of Transgenes and P Element Transformation 38
- Test for Rescue of *png* Mutations 40
- Isolation of cDNAs 40
- Sequencing 40
- Developmental Northern Blot 41
- *In situ* Hybridization to Ovaries 41
- Polyclonal Antibodies Against PNG 41
- Immunoblots 41
- Protein Extracts 42
- Immunoprecipitations 43
- Kinase Assays 43
- References 46

## Chapter Three:
### Screen for New Alleles of *pan gu* Using P Element Mutagenesis 48
- Purpose of the Screen 49
- Genetic Design of the Screen 49
- Potential Isolation of Zygotic Alleles 49
- Use of *lozenge* Mutations to Reduce Labor 49
- Test of Female Sterility 53
- Results of the Screen 53
- Southern Blot Analysis to Detect Rearrangements 55
- Conclusion 67
- References 68
Chapter One

Introduction
The cell cycle is the ordered execution of events that allows a cell to grow, replicate, and divide into two cells. The typical eukaryotic cell cycle consists of S phase, when the chromosomes are replicated, followed by mitosis or M phase, when the chromosomes are partitioned between two progeny cells. A G1 gap phase normally separates the end of mitosis from the beginning of DNA replication, and a G2 gap phase separates the end of DNA replication from the beginning of mitosis. An important aspect of cell cycle regulation is the coordination of cell cycle events to ensure proper and complete replication of the genome, division of the cell, and stable maintenance of genome content. DNA replication must alternate with mitosis, and during S phase the entire genome must be copied exactly once.

The cell cycle has been studied in many diverse organisms ranging from the single-celled yeasts to multicellular fruitflies, frogs, and mammals. Variations of the canonical G1-S-G2-M cell cycle can be found in different tissues and at different times during the development of these organisms, either in response to external signals, or to coordinate cell cycle events with ongoing developmental processes. One example of coordination between the cell cycle, development, and external signals occurs in multicellular organisms during fertilization of the egg and entrance into the embryonic cell cycles. The quiescent egg must be inhibited from undergoing cell cycle events until fertilization, after which it must divide and develop into a complete multicellular organism.

In some organisms, including Xenopus and Drosophila, the early embryonic divisions immediately following fertilization are simpler than during later development, consisting of rapid synchronous alternations of S phase and M phase without gap phases (Foe et al. 1993; Murray and Hunt 1993).
Control of these cell cycles is also simpler, because there is little or no transcription, so regulation is post-transcriptional (Foe, et al. 1993; Murray and Hunt 1993).

Previous work in *Drosophila melanogaster* has shown that the *pan gu* (*png*) gene is necessary for inhibition of DNA replication in unfertilized eggs, as well as coordination of DNA replication and mitosis during early development (Shamanski and Orr-Weaver 1991). I have chosen to study *png* and its role during early Drosophila embryogenesis as a model for understanding the regulation of S phase and M phase, and the coordination of the cell cycle with development. To learn more about the role of *png* during these events and possible roles later during development, I completed a screen for new alleles of *png* (chapter 3) and characterized new *png* alleles given to us by other researchers (chapter 4). To understand the nature of the mutations and their effects on the *png* gene product, we molecularly isolated the gene (chapter 4). We found using molecular and biochemical experiments that *png* functions specifically at fertilization and during development of the early embryo (chapter 5). These studies also showed that the PNG protein directly interacts with the Plutonium protein, and that the C-terminus of PNG may mediate interaction with other proteins.

This introductory chapter is organized into three parts. The first part discusses cell cycle regulation and regulators in general, including the dependence of S phase on M phase. The second part discusses how the cell cycle is coordinated with fertilization and early embryogenesis in different organisms. The last section describes previous work on *png* and the other genes that function at the time of fertilization in the fruitfly, *Drosophila melanogaster*. 
Control of the Cell Cycle

Conserved Controls

Studies of the eukaryotic cell cycle in different organisms have led to the conclusion that many of the regulators and mechanisms are highly conserved through evolution. In retrospect this is not really surprising, because cell cycle regulation is so basic to a cell’s survival and proliferation. In this section I survey many of the conserved regulators and mechanisms.

The convergence of three different approaches to studying the cell cycle led to the first indications of its universality (Murray and Hunt 1993). Analysis of cell division cycle (cdc) mutants in budding and fission yeast led to the discovery of the CDC28 and cdc2 genes, respectively. Cells mutant for these genes arrest at Start, which is the commitment point in G1 for entrance into the cell cycle. The cdc2 gene is also required for entrance into mitosis (Nurse and Bissett 1981). CDC28 and cdc2 were eventually shown to be homologous to each other in structure and function, encoding a protein kinase (Beach et al. 1982). Meanwhile, cell biologists purified maturation promoting factor (MPF), an unstable component of Xenopus mature oocyte extracts that could promote meiotic progression in immature oocytes (Masui and Markert 1971). Later, cyclins A and B were discovered to oscillate with the synchronous cleavage divisions of the sea urchin embryo, peaking at G2/M and disappearing by anaphase (Evans et al. 1983). Several laboratories discovered simultaneously that MPF consisted of p34, which is the CDC28/cdc2 kinase, and its regulatory subunit cyclin B (Dunphy et al. 1988; Gautier and Maller 1991; Gautier et al. 1988; Labbe et al. 1989). Confirmation of the conserved role of p34cdc2 in eukaryotes came with the discovery that human cdc2+ can complement a fission yeast cdc2 t.s. mutation (Lee and Nurse 1987).
Like many protein kinases, p34 is regulated by a combination of activating and inhibitory phosphorylations (Murray and Hunt 1993). Phosphorylation of Tyr-15 (and in some organisms Thr-14) inhibits p34 activity, whereas phosphorylation of Thr-161 is required for activity. The inhibitory phosphorylation is dominant to the activating one. Interestingly, the kinases and phosphatases that regulate p34 are also conserved. The Wee1 and Mik1 protein kinases phosphorylate Tyr-15, and the phosphatase Cdc25 removes this phosphorylation. p34 activation is also dependent on binding to cyclin: in Xenopus, cyclin protein is required to initiate mitosis (Murray and Kirschner 1989) and must be degraded via the ubiquitin degradation pathway for exit from mitosis (Glotzer et al. 1991; Murray et al. 1989). Cyclins may also have important roles beyond activating the kinase: they are thought to target p34 to specific substrates, either directly or by affecting subcellular localization (Morgan 1997). These multiple controls of kinase activity, substrate specificity, and localization may allow a single kinase to coordinate multiple events throughout the cell cycle.

In multicellular organisms, Cdc2 represents one member of a family of cyclin dependent protein kinases (CDKs) that bind to one of a growing family of activating cyclin subunits (Morgan 1997). Different CDK complexes act at different points in the cell cycle, including the G1/S transition and the G2/M transition. These CDKs are regulated by the same mechanisms mentioned for p34: binding to cyclin subunit, inhibitory phosphorylation, and activating phosphorylation. Cdk inhibitory subunits (CKIs) can also bind and inactivate some CDK/cyclin complexes.

**Dependency of S Phase on Mitosis**

During a normal cell cycle, S phase must always follow M phase of the previous cycle, to ensure that cells do not undergo an extra round of S phase,
doubling their ploidy. Studies in several different organisms have identified some of the factors required for this dependency.

Examples exist in which DNA is replicated more than once between mitoses, so that some component of S phase control is missing. In fission yeast, the p34cdc2 kinase plays an important role in the dependency of S phase on M phase (Broek et al. 1991; Hayles 1994). Cells that are t.s. mutant for cdc2 or cdc13 (which encodes cyclin B) arrest during G2 at the non-permissive temperature. When these cells are released at the permissive temperature, they bypass M phase and enter S phase. In addition, cells deleted for cdc13 undergo repeated rounds of S phase without mitoses. Since normally cyclin B is destroyed at the end of mitosis, allowing a cell to enter G1 and subsequently S phase, these results suggest that the activity of p34cdc2 complexed with cyclin B tells a cell that it is in G2, and destruction of this complex tells a cell that it is in G1, and can enter S phase. Overexpression of two other S. pombe genes, rum1 and cdc18, also induces multiple rounds of DNA replication without mitosis (Moreno and Nurse 1994; Nishitani and Nurse 1995). The product of the rum1 gene is thought to inhibit p34cdc2. cdc18 is the homolog to the S. cerevisiae CDC6 gene, which is required for initiation of DNA replication (Bell et al. 1995; Muzi-Falconi and Kelly 1995).

Studies in mammalian cells have also identified mutants or treatments that allow multiple rounds of S phase. The Chinese hamster ts41 mutant also blocks mitosis and allows successive S phases without intervening mitoses (Handeli and Weintraub 1992). In addition, treatment of mammalian cells with staurosporene, a protein kinase inhibitor, induces extra S phases (Usui et al. 1991).

In Xenopus, permeabilization of sperm nuclear membranes followed by addition to egg extracts allows the DNA to rereplicate without undergoing
mitosis (Blow and Laskey 1988). The interpretation of this result is that “licensing” or cytoplasmic factors are required by the nuclei to initiate DNA replication and then are inactivated during S phase. Normally these factors cannot enter the nucleus until mitosis, when the nuclear membrane breaks down, preventing S phase until M phase has already occurred. The discovery that Xenopus MCM3 is required for DNA replication and is localized to DNA during the initiation of S phase suggested that it was a component of licensing factor (Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995).

Work in recent years has greatly increased our understanding of the proteins that interact with replication origins at the molecular level. The MCM (mini chromosome maintenance) proteins were originally identified in S. cerevisiae in a screen for genes required at autonomously replicating sequences (ARSs) (Maine et al. 1984). The origin recognition complex (ORC) consists of 6 proteins identified as potential initiators of DNA replication because they bind chromatin near ARS sequences (Bell and Stillman 1992). ORC is also required for initiation of DNA replication (Fox et al. 1995; Liang et al. 1995). It has been found that chromatin structure changes during the process of initiation, from a prereplicative (pre-RC) complex in G1 to a postreplicative complex during S, G2, and M (Diffley et al. 1994). ORC remains bound at origins throughout the cell cycle, where it can recruit other initiation factors (Liang and Stillman 1997). The first step in pre-RC formation is recruitment of Cdc6 by ORC, followed by loading of MCM proteins, allowing the origin to be licensed for replication in the subsequent S phase (Tanaka et al. 1997; Aparicio et al. 1997). Cdc6 is required for loading of the MCMs. The multiple steps during initiation of DNA replication suggests multiple control points at which inappropriate firing of an origin can be prevented.
In some animals, rereplication can occur in variant cell cycles during the normal course of the organism’s development. One example is *Drosophila melanogaster*’s polytene larval tissues which undergo an endo cell cycle that consists of repeated rounds of S phase without mitosis (Smith and Orr-Weaver 1991). Understanding how the difference in such cells allows DNA to be repeatedly replicated without mitosis may help explain the mechanisms of S phase control. These cells shut off mitotic functions during the normal course of development. *Drosophila* endo cycles do not require *cdc2* (Smith et al. 1993; Stern et al. 1993), and mitotic functions appear to be shut off in polytene and polyploid cells. Mitotic cyclin B has not been detected in endo cycle tissues (Lehner and O’Farrell 1990; Lilly and Spradling 1996). Moreover, mutations in *cdc2* cause normally diploid tissues to become polytene, suggesting that the downregulation of *cdc2/cyclin B* may be sufficient to drive repeated rounds of S phase (Hayashi 1996).

Many factors may play a role in preventing inappropriate DNA replication. Phosphorylation is one mechanism by which cell cycle events are coordinated to prevent S phase without a previous mitosis, and control of proteins at origins of replication are likely to prevent repeated initiation of the same origin within a single S phase.

**The Centrosome**

Centrosome regulation is uncoupled from the nuclear cell cycle in *png* mutants. Embryos mutant for *png* undergo DNA replication without nuclear division. The centrosomes dissociate from the nuclei in this mutant, however, and centrosome duplication and formation of the mitotic spindle continues.

The centrosome nucleates the growth of microtubules, directing the events of mitosis in an animal cell (Kellogg et al. 1994). The duplication of the centrosome is coordinated with the cell cycle. The centrosomes start to
duplicate during S phase, and during prophase the duplicated centrosomes move to opposite sides of the nucleus, forming the spindle poles. During mitosis the centrosomes are segregated so that each resulting cell only receives one. The precise duplication and segregation of the centrosome is required for accurate segregation of the chromosomes.

The centrosome consists of a pair of centrioles surrounded by pericentriolar material, from which the microtubules grow. Duplication of the centrosome is semi-conservative: the paired centrioles split and a new centriole forms in association with each, creating two centrosomes (Kellogg, et al. 1994).

Budding yeast cells contain an analogous organelle called the spindle pole body that organizes the microtubules. When cells are arrested before Start in G1, using either mating pheromone or cdc28 t.s. mutations, the spindle pole body is unduplicated (Byers and Goetsch 1975). It does, however, have a satellite on its bridge structure, suggesting that spindle pole body duplication has already started, but requires a signal from the Cdc28 kinase to continue. It is unclear what the nature of this signal is.

Yeast cells mutant for cdc31, kar1, mps1, and mps2 are defective in spindle pole body duplication and arrest with large buds after completing DNA replication, indicating that DNA replication can continue in the absence of spindle pole body duplication (Rose and Fink 1987; Schild et al. 1981; Winey et al. 1991). Other cell cycle mutants can complete spindle pole body duplication in the absence of DNA synthesis and bud formation, indicating that the converse is also true (Bender and Pringle 1989; Hollingsworth and Sclafani 1990).

It is likely that these spindle pole body mutants arrest before mitosis due to feedback loops that prevent mitosis until assembly of a normal spindle
(Kellogg, et al. 1994). Interestingly, cells carrying the mps1-1 mutation cannot undergo spindle pole body duplication, but still go through multiple rounds of DNA replication, suggesting a role for MPS1 in the feedback control that detects unduplicated spindle pole bodies (Winey, et al. 1991). More recent work has shown that Mps1, a protein kinase, phosphorylates Mad1, and overexpression of Mps1 constitutively activates the spindle assembly checkpoint in wild-type cells (Hardwick et al. 1996). MAD (mitotic arrest defective) genes are required for the checkpoint that prevents completion of mitosis with defective spindles.

The centrosome cycle and other cell cycle events can be uncoupled in the early embryo as well. The nucleus of the fertilized sea urchin embryo can be removed and the remaining centrosome will continue to undergo several rounds of duplication, although the cycle time is slightly longer (Sluder et al. 1986). Similarly, if the sea urchin embryo is injected with aphidicolin to block DNA replication, the centrosome also continues to divide, dissociating from the arrested nucleus (Sluder and Lewis 1987). This experiment has also been performed on starfish and Drosophila embryos, with the same result (Picard et al. 1988; Raff and Glover 1988), showing that neither DNA replication nor a nucleus is required for centrosome duplication in the early embryo. The centrosome cycle does arrest in sea urchin embryos if they are arrested before the start of S phase, suggesting that the centrosome cycle can only continue during S phase (Hinchcliffe et al. 1998). The centrosome cycle does arrest in Drosophila and starfish embryos if protein synthesis is inhibited, although this is not true in frog or sea urchin embryos (Foe, et al. 1993; Gard et al. 1990; Picard, et al. 1988; Sluder et al. 1990). The latter result is surprising, since all other events of the cell cycle arrest when the embryo is treated with protein synthesis inhibitors, but it may mean that in the absence of protein synthesis
frog and sea urchin embryos arrest at a point in the cell cycle when the signal
to activate centrosome duplication is turned on (Kellogg, et al. 1994).

Recent results in *Xenopus laevis* embryos suggest that cyclin E/cdk2
plays a role in the centrosome cycle. Both cyclin E and cdk2 are present at
constant levels throughout the early embryonic divisions in Xenopus (Hartley
et al. 1996). If embryos are treated with cycloheximide and then injected with
p21 or the N-terminus of p27, which inhibit cdk2 activity, the centrosomes
stop duplicating (Lacey et al. 1999). If cyclin E is injected with p21, the
centrosomes are able to continue replicating, confirming that active cdk2 is
required for centrosome duplication. Similarly, the N-terminus of p27 can
inhibit centrosome duplication in vitro in Xenopus extracts, and addition of
cdk2/cyclin E can restart the centrosome cycle (Hinchcliffe et al. 1999).

Further work showed that p21 blocks centriole separation, which is the first
observable step of the centrosome duplication cycle (Lacey, et al. 1999).

Immunodepletion of cdk2 also inhibited centriole separation. Immunodepletion
of either cyclin E or cyclin A had only minor effects, while immunodepletion of
both greatly reduced centriole separation. These results suggest that cdk2
activity is required for the start of the centrosome cycle, but cyclin A or cyclin
E are partially redundant. Both cyclin E and cyclin A can bind to cdk2 in vitro,
but during early Xenopus embryogenesis cyclin E is found associated with cdk2
(Rempel et al. 1995).

In summary, a cell or an embryo needs to coordinate the mitotic
machinery of the centrosome with other cell cycle processes such as DNA
replication, in order to allow proper segregation of the chromosomes. Some of
this control may be from conserved regulators. The controls may also be
minimal in early embryos, since it is easy to decouple the nuclear cycle from
the centrosome cycle.
Fertilization: the Egg to Embryo Transition

Vertebrates

A unique and critical time during embryonic development is the fertilization of a mature oocyte and the activation of the early embryonic cell cycle. In organisms such as Drosophila, Xenopus, sea urchin, and starfish, the embryo develops independently of the mother, so the egg is provided with large quantities of maternal gene products necessary for the first several embryonic cell cycles. These gene products must be held inactive during the completion of meiosis until fertilization and the resumption of the mitotic cycle.

In many animal species, the mature oocyte arrests at metaphase of meiosis I or meiosis II before fertilization (Sagata 1996). In most vertebrates the arrest is at metaphase II. In Xenopus, the hormone progesterone stimulates the meiotic divisions and maturation of the oocyte, after which the egg arrests in metaphase of meiosis II until fertilization. In amphibian eggs, the cytoplasmic factor responsible for the arrest is called cytostatic factor (CSF) (Masui and Markert 1971). A similar activity was discovered in mouse oocytes, suggesting a conserved mechanism (Balakier and Czolowska 1977). The product of the c-mos proto-oncogene, which encodes a Ser/Thr kinase, has been shown to be an activator or component of CSF in Xenopus eggs (Sagata et al. 1989). Mos is expressed during oocyte maturation and disappears after fertilization, and is capable of arresting two-celled embryos in metaphase (Sagata et al. 1988; Sagata, et al. 1989; Watanabe et al. 1989). Furthermore, immunodepletion of Mos removes CSF activity from unfertilized egg cytoplasm (Sagata, et al. 1989). A Mos homolog is also found in mice, and oocytes from c-mos knockouts progress through metaphase II without arrest, resulting in parthenogenesis (Colledge et al. 1994; Hashimoto et al. 1994). It is thought that CSF functions through MPF, because injection of Mos into Xenopus
embryos stabilizes MPF by inhibiting cyclin degradation (Sagata et al. 1989). It is possible that stabilization of cyclin is direct, because Mos can phosphorylate cyclin B2 in vitro, but ectopic expression in oocytes does not cause cyclin B2 phosphorylation (Roy et al. 1990). Another possibility is that Mos inhibits the cyclin degradation machinery. Mos does not have a role during the early embryonic divisions, because it is destroyed immediately after fertilization (Sagata, et al. 1989). A Mos homolog has not been found in invertebrates.

Fertilization releases the metaphase arrest of the unfertilized egg, coupling the start of the embryonic cell cycles with the presence of the male pronucleus. In many organisms, including starfish, Xenopus, sea urchin and mouse, fertilization stimulates a transient increase in calcium (Sagata 1996). In Xenopus, the increase in calcium concentration is thought to be released from the endoplasmic reticulum (Terasaki and Sardet 1991). Studies in Xenopus cell-free extracts suggest that calcium/calmodulin-dependent kinase II (CaMK II) may play a role in early cell cycles. Constitutively active CaMK II can induce cyclin degradation and sister chromatid separation, while a specific inhibitor of CaMK II prevented these events (Morin et al. 1994). Inositol triphosphate also fluctuates at fertilization in sea urchin embryos, and might be involved in the calcium signal (Ciapa et al. 1994). It is unknown if calcium plays a role in Drosophila fertilization and restart of the cell cycle.

**Early Embryogenesis in Drosophila**

At the completion of oogenesis in Drosophila, the fully developed egg arrests in metaphase I of meiosis (Foe, et al. 1993). Ovulation, and probably hydration, stimulates the completion of meiosis I and II (Mahowald et al. 1983). Fertilization occurs during completion of meiosis, while the egg is in the uterus. At the completion of both meiotic divisions, the egg still contains all
four maternal meiotic products because the polar bodies are not budded off, but remain in the cytoplasm. The maternal nucleus, which is innermost in the egg, becomes the female pronucleus and fuses with the male pronucleus from the sperm.

The fertilized embryo is a syncytium during the first thirteen cell cycles: the nuclei divide and replicate synchronously in a common cytoplasm, without cell membranes, with the exception of the germ cells which cellularize during cycle 10 at the posterior end of the embryo. These cycles are very rapid S-M cell cycles, because mitosis and DNA replication immediately follow each other without intervening gap phases. These cell cycles are controlled post-transcriptionally by maternal protein and RNA placed into the oocyte by the nurse cells during oogenesis, because zygotic transcription does not occur during the first 9 cell cycles, and is not required for the first 13 cell cycles (Edgar et al. 1994). During the syncytial cycles the nuclear envelope breaks down only partially, near the spindle poles, perhaps because the mitotic cycles are so rapid, or to protect the chromosomes from neighboring spindles (Stafstrom and Staehelin 1984).

During the first cell cycle, nuclear membranes form around the two haploid pronuclei and they decondense and undergo DNA replication (Foe, et al. 1993). The polar bodies also decondense and probably replicate their DNA, and then recondense when the pronuclei do. The centrosome is brought into the egg by the sperm, and divides into two as the pronuclei decondense. The chromosomes then condense and the centrosomes form a bipolar mitotic spindle, which attaches to the chromosomes of the two pronuclei. The other three maternal meiotic products, or polar bodies, arrest in a condensed state during the first mitotic division. The polar bodies fuse together to form a star pattern, and remain arrested until they eventually degrade during
embryogenesis (Foe, et al. 1993). The fact that the polar bodies remain condensed during the early cell cycles indicates that the entire syncytium is not under the same control – in fact, localized regulators within the embryo must act to maintain condensation of the polar body nuclei while coordinating the S-M cell cycles of the embryonic nuclei.

If the egg is not fertilized, it still completes meiosis following ovulation. After completing the meiotic divisions, the four maternal meiotic products decondense, and then condense and remain condensed, similar to the polar bodies of the fertilized embryo. The fact that nuclei of unfertilized eggs remain arrested instead of entering mitosis suggests that something brought in by the sperm activates the mitotic cell cycle. This could be the centrosome, the presence of the male pronucleus, or some other signal.

During the first nine cycles, the nuclei remain in the interior of the embryo (Zalokar and Erk 1976). Starting at cycle 8, the nuclei begin to move outward, toward the surface of the embryo (Foe, et al. 1993). By cycle 10, all of the nuclei are at the surface, forming a syncytial blastoderm, except for about 100 nuclei which have fallen to the center of the embryo and become part of the yolk. Cycles 2 through 10 are each about 8 minutes long at 25°C, while during the next three cell cycles the time increases each cycle, until cycle 13 is about 18 minutes long (Foe, et al. 1993).

There are many genes maternally expressed during the early syncytial divisions, including conserved cell cycle regulators. It is often difficult to determine if a known regulator has a function during this time of development, because mutants might be lethal during late embryogenesis or during the larval and pupal stages, so viable females cannot be isolated to test for maternal effect. In addition, maternal effect mutations may also affect oogenesis, and these defects might precede and confound embryonic defects.
Homologs of conserved regulators which are highly expressed during the Drosophila syncytial divisions include cdc2, cdk2 (also known as cdc2c), cdc25 (string and twine), cyclins A, B, B3, and E, cdc5 (polo), wee1, and chk1 (grapes) (Campbell et al. 1995; Foe, et al. 1993; Fogarty et al. 1997; Jacobs et al. 1998; Llamazares et al. 1991; Sibon et al. 1997). Of these, genetic analysis has shown that cdc2 is required in vivo for these divisions (Stern, et al. 1993). Cdc25string is not required for the early divisions, but this is probably because of redundancy with Cdc25twine (Edgar and Datar 1996). Extensive analysis of Cdc2 phosphorylation and activity and Cyclin A and Cyclin B levels has shown no detectable fluctuations during cycles 2 through 7 and gradually increasing fluctuations during the later syncytial cell cycles, coincident with the start of zygotic transcription and lengthening of the cell cycle (Edgar, et al. 1994). One interpretation of these results is that undetectable degradation of localized Cyclins is occurring, perhaps of Cyclins associated with the spindle or nuclei. Another possibility is that the coordination of S phase and mitosis during the earliest divisions is controlled by other regulators.

Of the novel genes expressed at this time, fs(1)Ya is required during the first mitotic cell cycle (Lin and Wolfner 1989). Embryos mutant for strong alleles of fs(1)Ya arrest during the first mitotic division. The fs(1)Ya protein is localized to the nuclear envelope from interphase to metaphase, suggesting that it might be involved in nuclear membrane breakdown or formation.

**pan gu and Early Embryogenesis**

Three other maternal effect genes, pan gu (png), plutonium (plu), and gnu, play an important role in cell cycle regulation of the Drosophila early cleavage divisions (Freeman and Glover 1987; Freeman et al. 1986; Shamanski and Orr-Weaver 1991). Unfertilized eggs from mothers homozygous mutant for any of these three genes replicate their DNA even
though unfertilized. In addition, both unfertilized eggs and fertilized embryos produce giant polyploid nuclei that continue to replicate their DNA without undergoing nuclear division. In Drosophila embryos the sperm brings in the centrosome (Sonnenblick 1950). Centrosomes and spindles are absent in unfertilized *png* and *plu* mutant eggs (Shamanski and Orr-Weaver 1991). They are present in fertilized mutant embryos, but they are not tightly associated with the nucleus and their cycle is dissociated from the nuclear cycle: the centrosomes continue to double, unassociated with the nuclei.

These mutants offer an interesting opportunity for study because their phenotypes suggest a role for the wild-type genes in the regulation of DNA synthesis and the coordination of S phase and M phase. They could function to directly repress DNA replication at initiation of S phase until the egg is fertilized or mitosis is completed, to block rereplication of DNA during a single S phase, or to activate exit from S phase and entrance into mitosis.

The *png* gene is especially interesting because mutant alleles fall into two phenotypic classes indicating the gene has a direct role in the coordination of S phase and mitosis (Shamanski and Orr-Weaver 1991). Three of the five known alleles of *png* give phenotypes similar to the *plu* and *gnu* mutants: unfertilized eggs contain one to four giant nuclei representing the four maternal meiotic products, and fertilized embryos contain one to five giant nuclei representing the maternal meiotic products plus the paternal pronucleus. The paternal pronucleus also undergoes inappropriate DNA replication. Less than four or five giant nuclei are seen because they appear to fuse at later time points. In contrast, mothers homozygous for the other two *png* alleles produce embryos with six to 16 giant nuclei. Since these embryos contain more than the five nuclei found in newly fertilized embryos, some of the nuclei must undergo mitosis, suggesting that these *png* alleles are leaky. This multinuclear
phenotype is dependent on fertilization, since unfertilized eggs have only one to four nuclei.

The *plu* gene has been cloned, and encodes a small 19 kDa ankyrin repeat protein (Axton et al. 1994) Many lines of evidence suggest that *plu* is a specific regulator of the early embryonic divisions. First, alleles of *plu* that are null for *plu* transcript or PLU protein expression are homozygous viable and have no apparent zygotic defects (Axton, et al. 1994; Elfring et al. 1997). Secondly, PLU protein is only detectable until 4 hours of embryogenesis, and is not expressed during later embryonic development (Elfring et al. 1997). Finally, ectopic expression of PLU in the eye-antennal imaginal discs has no effect on eye development as would be predicted for disruption of the cell cycle, suggesting that PLU cannot regulate canonical G1-S-G2-M cell cycles (Elfring et al. 1997).

The *plu*, *png*, and *gnu* genes probably function in the same pathway, not only because of the similarity of their mutant phenotypes, but because they interact genetically. Mutations in *plu* or *gnu* dominantly enhance the phenotype of the weak *png*<sup>3318</sup> allele: if the dosage of the *plu* or *gnu* gene is decreased by half, *png*<sup>3318</sup> embryos are no longer capable of mitosis, so that the multinuclear phenotype becomes a single nucleus phenotype (Shamanski and Orr-Weaver 1991). In addition, PLU protein levels are slightly decreased in *png*<sup>3318</sup> embryos and greatly decreased in *png*<sup>1058</sup> embryos, so PLU stability or expression is dependent on *png* function (Elfring et al. 1997).

To understand how cell cycle regulation interacts with development, it is important to know if key regulators function at different stages during development, and whether their roles are the same or different during these different stages. It is unclear if wild-type *png* is required during later development when the zygotic genome is transcribed. If it were, a null allele of
*png* would cause a cell cycle defect and zygotic lethality. All five alleles of *png* are strict maternal effect: flies are homozygous viable even over a deficiency, and the embryonic phenotype is solely dependent on its mother's genotype (Shamanski and Orr-Weaver 1991). If one of these maternal effect alleles is a null, then the gene is not essential later in development. However, all five alleles were isolated in a screen on the X chromosome that would only isolate maternal effect mutations, because the mutant lines were established from hemizygous mutant males (Mohler 1977). No zygotic lethal alleles could have been isolated because these males would have died before adulthood. By genetic criteria, any of the alleles from both classes could be null mutations: the homozygous mutant phenotype is identical to the phenotype of the mutation in trans to a deficiency that uncovers it (Shamanski and Orr-Weaver 1991). They cannot all be null mutations, though, because the alleles represent two classes which give different phenotypes. The genetic test is never sufficient to define a null mutation; it can only rule out that a mutation is a null by showing that it is leaky. The question of whether *png* is required later in development cannot be answered until either a zygotic allele is isolated or the gene is cloned and one of the maternal effect alleles is shown to be a null.

It is unclear at what part of the cell cycle *png* acts. One possibility is that it functions as an activator of mitosis. This would be analogous to *cdc13* (cyclin B) in fission yeast, which is required for mitosis, but results in multiple rounds of DNA replication when deleted (Hayles 1994). In early Drosophila embryos, inhibition of the mitotic apparatus with microtubule depolymerizing drugs also prevents DNA replication, suggesting a checkpoint that inhibits S phase until completion of mitosis. If *png* functions as a mitotic activator, it must somehow bypass this checkpoint, or is itself required for the checkpoint. Another possibility is that *png* is required for inhibition of S phase before
fertilization and during mitosis. If this model is correct, it is unclear if it functions at entry into S phase or to prevent refiring of origins of replication within the same S phase.

The experiments in this thesis address many of the remaining questions about how *png* functions. Among those questions is whether *png* is a specific regulator of early embryogenesis, or if it globally regulates the cell cycle throughout development. We also wished to determine the nature of the two classes of *png* phenotypes, and if they can be explained in terms of the molecular changes caused by the mutations in the *png* gene product. We wanted to know if the mechanism of *png* function is a conserved mechanism found in other organisms, or if it functions in a novel way. Finally, we investigated the relationship of *png* to *plu* and other genes in the pathway regulating the coordination of fertilization and early development with the cell cycle.
References


Chapter Two

Materials and Methods
Mutations and Strains

Crosses were carried out using standard techniques (Greenspan 1997) and were done at 25°C unless otherwise noted. The original png alleles (png1058, png3318, png2786, png12-158, png1920) came from a maternal effect mutant collection of J. Dawson Mohler (Mohler and Carroll 1984; Mohler 1977) and are described in Shamanski and Orr-Weaver (Shamanski and Orr-Weaver 1991). The new png alleles (png50, png171a, png172, png246) have the genotype y png w P[FRT ry+ 19A] and were isolated by A. Swan and B. Suter from an EMS female sterile collection generated by A. Hilfiker and J. Lucchesi. The multi P[lacZ w+] and P[ry+ Δ2-3](99B) strains were gifts of F. Pelegri and described in Bier et al (Bier et al. 1989) and Robertson et al (Robertson et al. 1988), respectively. The duplication, deficiency, lozenge and FM1 strains were provided by the Bloomington Stock Center and are described in Lindsley and Zimm (Lindsley and Zimm 1992). The sterile males are missing a portion of the Y chromosome containing fertility factors necessary for sperm formation, and come from strain T(Y;2)#11 cn bwD mr2/b cn mr1 bs2/ SM6a, a gift of B. Reed. Other balancers and markers are described in Lindsley and Zimm (Lindsley and Zimm 1992).

yw recombinants of the original png alleles were generated from females of the genotype y png cv v f / w. Since png is closely linked to y, y w male progeny were selected and mated to png/ FM0 or Df(1)A94 / FM6 (Df(1)A94 uncovers png). Balanced progeny were stocked if their siblings failed to complement png or the deficiency uncovering png.

Unfertilized Eggs

To facilitate unfertilized egg collections, virgin females were mated to sterile males (see above) missing fertility factors on the Y chromosome. The
males do not produce sperm, but mating stimulates the females to lay their unfertilized eggs.

**Embryo Fixation and Staining**

Embryos (or unfertilized eggs) were dechorionated in 50% bleach and rinsed in 0.02% triton X-100. Embryos were devitellinized and fixed in a 1:1 mixture of heptane and methanol, and were left in methanol overnight at 4°C. They were then rehydrated through a PBS/methanol series to a final 1X PBS (136mM NaCl, 2.7mM KCl, 6.5mM NaHPO$_4$, 1.5 mM KH$_2$PO$_4$).

To visualize DNA, embryos were stained using DAPI (4', 6-diamidino-2-phenylindole) at 1µg/ml in 1X PBS for 20 minutes and then washed three times in 1X PBS before mounting in 70% glycerol in 1X PBS. Alternatively, embryos were stained with propidium iodide at 1µg/ml in 1X PBS, 0.005% triton X-100, 1 mg/ml RNaseA for 20 minutes and then washed three times in 1X PBS, 0.005% triton X-100 followed by three washes in methanol before mounting in 2:1 benzyl benzoate: benzyl alcohol, 50 mg/ml n-propyl gallate.

**Microscopy**

Zeiss Axiophot and Axioskop microscopes were used to examine fluorescence staining using Plan-neofluor 10X, 20X, and 40X objectives. Laser scanning microscopy was performed using an MRC 600 confocal scanning head (Bio-Rad Laboratories), mounted on a Zeiss Axioskop equipped with a Plan-neofluor 40X objective.

**Quantitative Southern Blots**

DNA preps were performed as described in Ashburner (Ashburner 1989), and DNA from 20 to 40 flies was loaded per lane (2-3 µg). Agarose gels
were run in 1X Loening buffer (40mM Tris, 36mM KH2PO4, 1 mM EDTA, pH 7.6) and DNA was transferred to nylon membranes (Nytran, Schleicher and Schuell) and UV cross-linked with a UV Stratalinker (Stratagene). Probes were labeled by random priming of linearized DNA fragments that had been isolated from low-melting-point agarose gels. Southern blots were prehybridized for 2 hours at 65°C in 6X SSC, 10X Denhardts, 1% SDS, and 100μg/ml denatured salmon sperm DNA. Blots were hybridized overnight with denatured probe at 65°C in 6X SSC, 5X Denhardts, 1% SDS, and 50 μg/ml denatured salmon sperm DNA. Blots were washed at 65°C with the following: twice for 15 minutes with 2X SSC, 1% SDS; twice for 30 minutes with 0.2X SSC, 0.1% SDS, and rinsed briefly in 0.2X SSC at room temperature. Quantitation of bands was done using a Fuji Bio-image analyzer 2000 (Fuji, Inc.). rosy genomic bands were used as DNA loading controls.

**Construction of Transgenes and P Element Transformation**

Inserts were cloned into the pCaSpeR4 vector (Pirrotta 1988) by standard techniques (Sambrook et al. 1989). Genomic fragments for transformation rescue experiments were cloned into pCaSpeR4 digested with the same restriction enzyme as the insert with the following exceptions: the 8 kb Sal I fragment was cloned into the compatible Xho I site; the 12.5 kb Sal I fragment was blunt end cloned into the Bam HI site; and for the 1.7 kb Bgl II/Hind III fragment, a 1.7 kb Sac I/Hind III fragment was first subcloned into pBluescriptSK+, then the insert was digested with Bgl II and Kpn I, and ligated into pCaSpeR4 digested with Bam HI and Kpn I. For the pGMR-png transgene, a 950 bp Fsp I/Nhe I genomic fragment subcloned into pGMR1 (Hay et al. 1994). For the hs-png transgene, a 950 bp Fsp I/Nhe I genomic fragment was subcloned into pCaSpeR-hs (Pirrotta 1988)
For the \textit{png-HA/His} transgene, the polymerase chain reaction (PCR) was used to create \textit{Xba} I sites surrounding the stop codon of \textit{png} in the 1.7 kb \textit{Bgl II/Hind III} rescuing fragment cloned into pBluescriptSK+. In addition, six histidine codons and an additional serine codon was added immediately prior to the stop codon. DNA encoding three tandem copies of the HA epitope (Tyers et al. 1993) was ligated into the \textit{Xba} I site in frame. A 238 bp \textit{Nae I/Nhe I} fragment containing the 3’ end of the open reading frame fused to the \textit{HA/6His} DNA was ligated into the 1.7 kb \textit{Bgl II/Hind III} rescuing fragment cloned into pCaSpeR4, replacing the genomic \textit{Nae I/Nhe I} fragment. The \textit{Nae I/Nhe I} fragment that had been PCR amplified was sequenced to verify there were no mutations.

The \textit{plu-Myc} transformant line was a gift of L. Elfring. It was generated as follows: PCR was used to create a \textit{BamHI} site in the C-terminus of a \textit{BglII/BglII} 3.8 kb genomic sublone (Axton et al. 1994). The site was created prior to the stop codon, and ablated the last arginine. Approximately 500 bp of DNA 3’ of the open reading frame was preserved. DNA encoding three repeats of the c-myc epitope (gift of S. Kron) was excised with \textit{BglII} and inserted into the \textit{BamHI} site. The entire was then cut with \textit{EcoRI} and \textit{XbaI} and cloned into the \textit{XbaI/EcoRI} sites of pCaSpeR4.

Injections were performed as described in Spradling (Spradling 1986). \textit{P} element constructs were co-injected with a helper plasmid containing a transposase gene (pIChs\(\Delta\)2-3), (gift of Ken Irvine). \textit{yw} embryos were injected and resulting flies were crossed with \textit{yw} flies to identify transformants. The \textit{P} element injection vector, pCaSpeR4, contained the mini \textit{white} gene as a selectable marker.
Test for Rescue of *png* Mutations

Transformation inserts were tested for the ability to rescue *png*\(^{1058}\) or *png*\(^{3318}\). Males carrying autosome-linked *w*+ transgenes were crossed to *y png w / FM0* females, and *w*+ non-FM0 male progeny were crossed to *y png w / FM0*. Progeny of the genotype *y png w / y png w ; P[w+] / +* were mated to sibling males, and their fertility was compared to *y png w / y png w ; + / +* sibling females not carrying the transgene.

Isolation of cDNAs

An ovary cDNA library (Stroumbakis et al. 1994) was screened with a 4.3 kb *Xho I* genomic fragment that overlaps the *png* open reading frame. A total of 40 positive cDNAs were isolated from 5 x 10\(^6\) clones screened. These clones were dot blotted onto filters and assigned to two transcription units based on patterns of hybridization to genomic probes. Clones that hybridized to the *png* open reading frame were amplified using primers against the phage vector, and PCR products of the 8 largest clones were sequenced (see below).

Sequencing

For sequencing of mutant alleles of *png*, genomic DNA was isolated from homozygous mutant adults, and the *png* ORF was amplified from the mutant genomic DNA. For mutant genomic DNA and cDNA clones, PCR products were directly sequenced. Wild-type genomic fragments were first subcloned into pBluescriptKS+. Sequencing was done by either Liuda Ziaugra and the Whitehead Institute Sequencing Facility or Research Genetics using fluorescence automated sequencing.
**Developmental Northern Blot**

The developmental Northern blot was a gift of M. Axton. Description of the blot and hybridization conditions is described in (Axton, et al. 1994). The probe contained the entire png ORF, and was PCR amplified, isolated on a low melting point agarose gel, and labeled by random priming.

**In Situ Hybridization to Ovaries**

Whole mount *in situ* hybridization to ovaries was carried out with digoxigenin-labeled RNA probes exactly as described in Royzman et al. (Royzman et al. 1999). The antisense png riboprobe was *in vitro* transcribed from a 950 bp *Fsp I/ Nhe I* genomic fragment subcloned into pBluescriptSK+.

**Polyclonal Antibodies Against PNG**

A fusion of GST to 167 amino acids from the C-terminus of PNG was used to generate antibodies in guinea pigs. The GST-PNG fusion protein was generated by cloning a 0.5 kb *Xho I/ Not I* png genomic fragment in frame with GST in the pGEX-4T-1 expression vector (Pharmacia Biotechnology, Inc.). The fusion protein was expressed in BL21(λDE3)pLysS cells by IPTG induction, and purified inclusion bodies containing GST-PNG were separated on standard 10% SDS polyacrylamide gels. The band corresponding to GST-PNG was excised, eluted, and injected into guinea pigs for antibody production (Covance).

**Immunoblots**

For the mutant allele immunoblot (figure 5-5), protein extracts were separated on a 15% 111:1 (acrylamide/bis-acrylamide), pH 9.08 gel. Otherwise, protein extracts were separated on 12% 150:1 (acrylamide/bis-
acrylamide), pH 8.8 gels. Gels were blotted onto Immobilon-P membranes (Millipore Corp.). 200 µg of total protein was loaded per lane. Bradford assays and Ponceau S staining were used to verify equivalent protein loading before immunoblotting. Blots were blocked in 5% nonfat dry milk and 2% BSA in TBST (0.01 M Tris, pH 7.5, 0.9% NaCl, and 0.1% Tween 20) for one hour at room temperature, and then incubated overnight at room temperature with diluted primary antibodies in the block solution. Guinea pig anti-PNG was used at 1:2000 dilution, affinity-purified rabbit anti-PLU (Elfring et al. 1997) was used at 1:200, and rat anti-Tubulin (YL1/2 and YOL1/34) was used at 1:200. Alkaline phosphatase-conjugated secondary antibodies diluted in block solution were used to detect bound primary antibodies. Anti-guinea pig antibody (Jackson ImmunoResearch Laboratories) was diluted 1:5000, anti-rabbit antibody (Promega) was diluted 1:7,500, and anti-rat antibody (Jackson ImmunoResearch Laboratories) was diluted 1:3,000. The PNG protein was visualized using the CDP-Star chemiluminescent substrate (Tropix). PNG protein migrates as a 33 kD band, consistent with its predicted molecular weight.

Protein Extracts

Embryonic extracts were made by dechorionating embryos in 50% Clorox bleach and homogenizing in urea sample buffer (USB: 8 M urea, 2% SDS, 5% β-mercaptoethanol, 100 mM Tris pH 7.6, and 5% Ficoll) at 3:1 USB/embryo (vol./vol.), and cleared by centrifugation. Imaginal discs, salivary glands, and brains were dissected from third instar larvae and placed directly into urea sample buffer on ice. Unactivated mature stage 14 oocyte extracts were a gift of T. Tang. All protein extracts were frozen in liquid nitrogen and stored at -80°C.
Immunoprecipitations

For immunoprecipitation experiments, embryos or unfertilized eggs were homogenized in 4 volumes IP buffer [100 mM NaCl, 25mM HEPES (pH 7.5), 1mM EGTA, 0.02% NaN₃, 1mM Na₃VO₄, 1mM NaF, 1μg/ml pepstatin A, 5μg/ml aprotinin, 100μg/ml chymostatin, 5μg/ml leupeptin, and 100μg/ml pefabloc]. Then, Triton X-100 was added to the extracts to a final concentration of 0.1% before extracts were cleared by centrifugation at 4°C for 10 minutes. IP extracts were precleared with protein A-sepharose (PAS) CL-4B beads (Pharmacia Biotechnologies) which had been previously coupled to a nonspecific purified antibody by DMP (Harlow and Lane 1988). Approximately one-third volume of a 50% slurry of beads in IP buffer was added to the extracts, and the mixture was incubated either at 4°C for 30 minutes with gentle agitation. To the precleared supernatant was added precoupled PAS beads with mouse 9E10 anti-Myc monoclonal antibodies (Santa Cruz Biotechnology) and allowed to incubate at 4°C for 40 minutes. A 1/10 volume of beads in a 50% slurry in IP-Triton buffer was used. The supernatant was removed from the beads and mixed with 4X SDS sample buffer. The beads were washed 4 times at 4°C with IP-Triton buffer [100 mM NaCl, 25mM HEPES (pH 7.5), 1mM EGTA, 0.02% NaN₃, 1mM Na₃VO₄, 1mM NaF, all protease inhibitors listed above, and 0.1% triton X-100]. Finally, 2X SDS sample buffer was added to the beads, and the samples were heated at 95°C for 5 minutes and centrifuged for 1 minute. The immunocomplexes were separated on SDS-polyacrylamide gels and analyzed by immunoblot.

Kinase Assays

For kinase assays, PLU-myc and PNG were first immunoprecipitated by the method described above, with the following exceptions: for the
experiment presented in figure 5-7, the extracts were cleared by ultracentrifugation at 65,000 RPM for one hour in a TLA100 rotor before incubation with protein-A sepharose. 190μl extract was precleared and then incubated with 7μl PAS beads coupled to mouse 9E10 anti-Myc monoclonal antibodies (Santa Cruz Biotechnology) and allowed to incubate at 4°C for 60 minutes. The beads were washed one time in IP buffer (see above), three times in IP buffer plus 0.5M NaCl, three times in IP buffer with no NaCl, one time in standard IP buffer, and one time in kinase buffer (75mM KCL, 10mM HEPES pH 7.5, 5mM MgCl₂, 1mM DTT, and 0.5mM EDTA). The pellets were then incubated in 10μl kinase buffer plus 1μCi 32P-γ-ATP (10 mCi/ml, 3,000 Ci/mMol, Dupont/NEN Research Products) at room temperature for 20 minutes. 4μl 6X SDS sample buffer was added, the samples were heated at 95°C for 5 minutes, and the the entire sample was separated on a 10-20% gradient SDS-PAGE gel (Owl Scientific) and analyzed by immunoblot. 32P-labeled proteins were detected using a Fuji Bio-image Analyzer 2000.

For the experiment presented in figure 5-8, the extracts were not ultracentrifuged or pre-cleared. 115μl extract was incubated with 20μl 9E10 coupled beads at 4°C for 50 minutes. The pellets were washed one time with IP buffer plus 0.5M NaCl, four times with standard IP buffer, and one time with kinase buffer. The pellets were then incubated with 50μl kinase buffer plus 10μCi 32P-γ-ATP (10 mCi/ml, 3,000 Ci/mMol, Dupont/NEN Research Products) at room temperature for 20 minutes. After addition of 20μl 4X SDS sample buffer, the pellets were heated at 95°C for 5 minutes and separated on a 4-20% gradient SDS-PAGE gel (Owl Scientific). 20μl sample was loaded per lane. The gel was fixed for 30 minutes in 10% isopropanol, 5% acetic acid, and 1% tetrasodium pyrophosphate, stained for 20 minutes in 0.1% coomassie brilliant blue, 25% isopropanol, 10% acetic acid, and destained for 20 minutes
in 10% isopropanol, 5% acetic acid. The gel was dried under vacuum and $^{32}$P was detected using a Fuji Bio-image Analyzer 2000.
References


Chapter Three

Screen for New Alleles of pan gu
Using P Element Mutagenesis

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Purpose of the Screen

In order to isolate new alleles of *png*, I completed a screen using *P* element mutagenesis. The screen had several purposes. First, new alleles would permit us to ascertain whether the gene was required only maternally, or if it regulated DNA replication throughout development. This is because the screen allowed isolation of zygotic lethal alleles as well as maternal-effect lethal (female-sterile) alleles. If zygotic lethal alleles were isolated, it would mean that *png* was required during later development. Weak alleles of genes required zygotically may give a maternal-effect phenotype because homozygotes may contain enough functional product to survive to adulthood. The five original alleles of *png* were isolated in a screen for female-sterile mutations on the X chromosome (Mohler 1977). Zygotic lethal alleles would not have been isolated in this screen because the mutations were recovered from sibling males hemizygous for the mutant X chromosome.

Secondly, new alleles might facilitate the molecular isolation of the *png* gene. Alleles caused by transposon insertions would provide homology tags for cloning the *png* gene. In addition to insertions, genetic lesions caused by chromosome rearrangements such as deletions and inversions would also provide clues to the molecular location of the gene.

Finally, isolation of additional weak alleles with the multinuclear phenotype would tell us that this phenotype results from loss of function of *png*. If the alleles with the multinuclear phenotype represent special mutations such as neomorphic gain-of-function mutations, isolation of additional alleles with this phenotype would be unlikely.

Genetic Design of the Screen

The strategy of the screen was to isolate mutations on the X chromosome (where the *png* locus is located) that fail to complement the
female-sterile phenotype of the previously isolated mutation \(png^{1058}\) (fig 3-1). The mutagen used in the screen was \(P\) element transposition. An “ammunition” \(X\) chromosome carrying four \(P[\text{lacZ } w^+]\) elements (Bier et al. 1989) was crossed to a strain carrying \(P[r_y^+ \Delta 2-3](99B)\), a stable third chromosome insertion which expresses high levels of active transposase (Robertson et al. 1988). Males carrying the mutagenized \(multi\ P\) chromosome and \(\Delta 2-3\) were then crossed en masse to females carrying the \(FM1\) balancer chromosome, generating female progeny carrying single mutagenized \(multi\ P\) chromosomes in the absence of transposase. Single females were then crossed to \(png^{1058}\) \(lz\) males. Female progeny from this cross could then be tested for fertility, because if the \(multi\ P\) chromosome carried a loss-of-function \(png\) mutation, these flies would have the \(png\) female-sterile phenotype. The mutant chromosome could then be recovered from sibling males.

Potential Isolation of Zygotic Alleles

To permit recovery of zygotic lethal alleles of \(png\), the mutations were rescued in males carrying a duplication containing wild-type \(png\) on the \(Y\) chromosome. Two duplications were used during the screen: \(Dp(1;Y)2E\) and \(Dp(1;Y)y^{2Y67g}\). These duplications both rescue the female sterility of \(png^{1058}\) in \(XXY\) females of the genotype \(png^{1058}/png^{1058}/Y, Dp(png^+).\) In addition, the duplications can rescue the lethality of \(Df(1)A94\) and \(Df(1)S39\), which both uncover \(png\). Therefore, these duplications are capable of rescuing a female-sterile allele of \(png\) as well as potentially lethal null alleles.

Use of lozenge Mutations to Reduce Labor

A \(lozenge(lz)\) mutation was used to reduce labor during the screen. The complementation tester chromosome carrying \(png^{1058}\) also carried \(lz^{34}\), a female-sterile allele of \(lz\). A female-sterile allele of \(lz\) is also present on the balancer \(FM1\). As a result, progeny from the last cross of the screen did not
Figure 3-1. Screen for new alleles of *png* using *P* element mutagenesis. A stable transposase gene, Δ2-3, is crossed into a *Drosophila* strain carrying four *P* elements. Progeny of these flies are tested for *P* element transpositions causing *png* mutations by their failure to complement the female sterile phenotype of the previously isolated *png*1058 mutation. Females not carrying a mutagenized chromosome are excluded from the complementation test because they are homozygous for *lozenge* (*lz*), a recessive female sterile mutation. Mutated chromosomes are rescued in males carrying a duplication containing wild-type *png* on the *Y* chromosome, permitting zygotic lethal alleles of *png* to be recovered.
\[ \text{yw multi } P \quad ; \quad + \quad \Phi \Phi \quad X \quad \pm ; \Delta 2-3, Sb \quad \sigma \sigma \]
\[ \text{yw multi } P \quad + \quad \sigma \sigma \]

\[ \text{FM1, lz } \quad ; \quad + \quad \Phi \Phi \quad X \quad \text{yw multi } P \quad ; \quad \Delta 2-3, Sb \quad \sigma \sigma \]
\[ \quad + \quad + \quad \]

\[ \text{yw multi } P^* \quad ; \quad + \quad \text{single } \Phi \quad X \quad \text{lz, png}^{1058} \quad ; \quad + \quad \sigma \sigma \]
\[ \text{FM1, lz} \quad + \quad \]

\[ \text{yw multi } P^* \quad \quad \text{yw multi } P^* \quad \left( \text{lz, png}^{1058} \right) \quad \text{FM1, lz} \quad \quad \text{yw multi } P^* \]
\[ \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 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have to be sorted, because females not carrying a mutagenized chromosome were sterile because they were homozygous for \( lz \).

**Test of Female Sterility**

Unsorted progeny from the the final cross were tossed into multi-walled chambers in which laid eggs can be collected and embryos from each line were collected on apple juice plates. After aging for at least 24 hours at 25°C, the collections were scored for the presence of hatched eggs, indicating that the embryos had completed embryogenesis. Males carrying the mutagenized chromosome were collected from lines that had no hatched eggs, and the males were crossed to \( png^{1058} lz^+ \) to retest the mutagenized chromosomes for failure to complement \( png \), as well as eliminate isolation of female-sterile mutations of \( lz \).

**Results of the Screen**

Approximately 10,000 lines carrying separate mutagenized \( X \) chromosomes were screened. There was no way to ensure isolation of a \( png \) allele, because \( P \) element mutagenesis is not random: there is some specificity for insertion site. It has been estimated that half of the Drosophila loci can be targets for insertion (Kidwell 1986). Consequently, if \( png \) lies in a “coldspot” it may be impossible to isolate a \( png \) insertion mutation. In addition, the size of the gene would affect its potential for being hit by a \( P \) element.

A single mutation was isolated from the screen. This mutation, \( png^{358H} \), failed to complement the female sterility of \( png^{1058} \) in multiple tests, and it was homozygous lethal. To characterize the phenotype of \( png^{358H} \), embryos and unfertilized eggs were collected from females heterozygous for this mutation and each of the five original \( png \) alleles. The embryos and eggs were fixed and stained with DAPI to visualize DNA. In all cases the eggs and embryos had a giant nuclei phenotype. Table 3-1 shows quantitation of the
Table 3-1. Phenotype of \( \text{png}^{358H} \) in trans to the original five \( \text{png} \) alleles. Unfertilized eggs were collected from virgin females mated to sterile males, while fertilized embryos were collected from females mated to sibling and/or \( y \text{ w} \) males. 0-2 hour collections were methanol/heptane fixed and stained with DAPI to visualize DNA. The strong single nuclei alleles are \( \text{png}^{1058} \), \( \text{png}^{12-158} \) and \( \text{png}^{1920} \). The weak multinuclear alleles are \( \text{png}^{2786} \) and \( \text{png}^{3318} \).
nuclear phenotype. Less than 5% of fertilized embryos from \textit{png}^{358H} in trans to the strong \textit{png} alleles have a multinuclear phenotype, while 16-27% of embryos from \textit{png}^{358H} in trans to the two weak alleles have a multinuclear phenotype. These phenotypes are similar to those of the \textit{png} alleles in trans to deficiencies which uncover the gene, consistent with \textit{png}^{358H} being a strong or null allele of \textit{png}.

\textbf{Southern Blot Analysis to Detect Rearrangements}

Former lab members Janet Carminati and Jessica Dines completed a chromosome walk across the region defined by duplications and deficiencies to contain the \textit{png} locus. Using germline-mediated transformation of Drosophila, they isolated a single 39kb cosmid, cosmid 3-3-1, that rescues \textit{png} mutations (Carminati 1995).

Quantitative Southern blot analysis was used to determine if \textit{png}^{358H} contained rearrangements in the 39kb rescuing region. A large rearrangement such as a \textit{P} element insertion, a deletion, or an inversion, would cause an alteration in the size of a restriction fragment containing the rearrangement. Quantitation was necessary because \textit{png}^{358H} is homozygous lethal: DNA was isolated from flies heterozygous for \textit{png}^{358H}, so only half the DNA would contain a rearrangement caused by the mutation.

The \textit{Eco} RI restriction map of cosmid 3-3-1 is shown in figure 3-2. Genomic Southern blots probed with parts of the rescuing cosmid show that the 14 kb and 3.3 kb \textit{Eco} RI fragments are deleted in \textit{png}^{358H} (figure 3-3). In addition, a new 1.6 kb fragment is seen in DNA from \textit{png}^{358H} and is not present in the \textit{multi} \textit{P} line which was mutagenized during the screen. This indicates that a large rearrangement has occurred in the region hybridized by cosmid 3-3-1. This rearrangement is most likely a deletion, because more than one fragment is altered. An insertion without any other rearrangements would
Figure 3-2. *Eco* RI restriction map of rescuing cosmid 3-3-1.

The sizes of the genomic restriction fragments which hybridize to the distal 4.5 kb fragment and the proximal 1.4 kb fragment is also shown.
Cosmid 3-3-1

distal

kb: 4.5 R 12.0 R 14.0 R R R
(7.8 genomic) 1.2 5.4 1.4

proximal

(3.3 genomic)

5 kb
Figure 3-3. Quantitative genomic Southern blot showing deleted fragments. Lane 1058: DNA from homozygous $png^{1058}$ females; lane multi P: DNA from homozygous multi $P$ females, which was the strain mutagenized in the screen; lane 358H/1058: DNA from females trans-heterozygous for $png^{358H}$ and $png^{1058}$. In all lanes the DNA was digested with $Eco$ RI. This blot was probed with different fragments from cosmid 3-3-1, including the 4.5 kb $Eco$ RI fragment, the 1.4 kb $Eco$ RI fragment, a 5.4 kb $Xho$ I fragment which overlaps the 12 kb and 14 kb $Eco$ RI fragments, and 4.5 kb $Xho$ I and 0.4 kb $Xho$ I fragments which are both within the 14 kb $Eco$ RI fragment. The blot was also probed with rosy DNA, which was used as a loading control standard for quantitation. Quantitation shows that the 14 kb and 3.3 kb fragments in $png^{358H}/png^{1058}$ are half the levels found in the multi $P$ or $png^{1058}$ lanes. The 7.8 kb fragment is not reduced in $png^{358H}/png^{1058}$, and the 12 kb fragment is too weak to quantitate accurately. This indicates that the $png^{358H}$ mutation is a deletion. An insertion would only affect one fragment (in which the insertion occurred). A novel 1.6 kb fragment can be seen in the $png^{358H}/png^{1058}$ lane, presumably from the fragment in which the deletion breaks.
multi 358H
1058   P   1058

14 kb
12 kb
8.3 kb rosy
7.8 kb
4.4 kb rosy
3.3 kb
1.6 kb
only affect the restriction map of one fragment. The entire 39 kb region is not deleted, however, because the 7.8 kb fragment hybridized by the distal end of the cosmid is not reduced in \textit{png}^{358H}.

The same blot was probed with the 12 kb RI fragment to determine if that fragment is affected by \textit{png}^{358H}. Quantitation of the phosphorimage shown figure 3-4 shows that the 12 kb fragment is not affected. The new 1.6 is also not detected by this probe. This result suggests that the breakpoint for the deletion is within the 14 kb \textit{Eco RI} fragment.

To determine where the deficiency breakpoint is within the 14 kb fragment, the same blot was probed with a 3.4 kb \textit{Xba I/Xho I} fragment. This fragment is at the distal end of the 14 kb \textit{Eco RI} fragment and slightly overlaps the 12 kb \textit{Eco RI} fragment. Figure 3-5 shows that this probe hybridizes to the 1.6 kb novel restriction fragment in \textit{png}^{358H}, proving that the deficiency breakpoint is within the 3.4 kb fragment.

The quantitative Southernss show that the proximal half of cosmid 3-3-1 is deleted in \textit{png}^{358H} (figure 3-6). The 3.4 \textit{Xba I/Xho I} fragment from the rescuing cosmid hybridizes to a new restriction fragment found in \textit{png}^{358H} but not wild-type, indicating that this fragment contains the distal breakpoint of the deficiency. It is unclear where the proximal breakpoint of the deficiency is located, but it must be beyond the proximal end of cosmid 3-3-1 because the 3.3kb \textit{Eco RI} fragment from the proximal end of the cosmid is deleted, and a probe of this fragment does not detect any new fragments in \textit{png}^{358H}.

Southern blots were also performed with the same DNA digested with \textit{Bam HI} and double digested with \textit{Xba I/Xho I}, and gave consistent results (data not shown).
Figure 3-4. Quantitative Southern blot probed with 12kb RI.

The same Southern blot from figure 3-3 was probed with the 12 kb Eco RI fragment and with rosy DNA. Quantitation relative to the two rosy bands shows that the 12 kb fragment is not reduced in \(^{358}\text{H}/^{1058}\text{png} \).
Figure 3-5. Southern blot probed with 3.4 kb Xba I/ Xho I.

The same Southern blot from figure 3-3 and figure 3-4 was probed with a 3.4 kb Xba I/ Xho I fragment which overlaps the 12 kb and 14 kb Eco RI fragments. The novel 1.6 kb band can be seen in lane $png^{358H}/png^{1058}$, indicating that this fragment contains the breakpoint for the deletion.
multi

358H

1058

P

1058

14 kb

12 kb

1.6 kb
Figure 3-6. Cosmid restriction map and location of the $png^{358H}$ deficiency. A restriction map for the 39 kb rescuing cosmid is shown. The DNA deleted by $png^{358H}$ is shown in blue. Abbreviations: Xb, Xba I; X, Xho I; R, Eco RI.
Cosmid 3-3-1

distal

Xb
R Xb X X

kb:
4.5 1.3 2.2 3.9 3.9 3.4 2.0 4.3

proximal

X
R Xb X X

kb:
4.0 1.2 1.0 4.4 1.4

Df(1)png^{358H}
Conclusion

These results indicate that the single mutation isolated from the screen is a deficiency removing at least 20kb from the region of png. Because the deficiency fails to complement $png^{1058}$, it must contain at least part of the gene. Since the deficiency does not delete all of cosmid 3-3-1, it delineates $png$ to a smaller region. Because the deficiency is large enough that it probably deletes more than one gene, the lethality could be due to loss of one of these other genes or loss of $png$. 
References


Chapter Four

Phenotypic Characterization of New Alleles and Molecular Characterization of pan gu

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*DDF did the phenotypic characterization of the new png alleles, subcloned the genomic fragments from cosmid 3-3-1, injected the 8 kb Sal I construct for Drosophila transformation, sequenced the wild-type genomic DNA and most of the mutant DNA, and did the phylogenetic analysis.
Phenotypic Characterization of New Alleles

We postulated that the multinuclear phenotype of some $png$ alleles is due to partial function (Shamanski and Orr-Weaver 1991). In order to confirm this, we thought it useful to characterize more alleles of $png$ and determine if other weak alleles have the same multinuclear phenotype or a different phenotype. The laboratory of Beat Suter kindly provided four new maternal effect lethal mutations with a giant nuclei phenotype. The mutations were originally isolated in an EMS screen on the $X$ chromosome carried out by A. Hilfiker and J. Lucchesi. A. Swan and B. Suter screened female-sterile and maternal-effect lethal mutations and isolated four mutations that have the giant nuclei phenotype. The four $X$-linked EMS mutations fail to complement the female sterile and giant nuclei phenotypes of $png^{1058}$ and $png^{3318}$, indicating that they are new alleles of $png$.

To examine the phenotype of the new $png$ alleles, we stained embryos from homozygous mutant mothers with propidium iodide to visualize the DNA (figure 4-1). All had giant polyploid nuclei, with phenotypes similar to the phenotypes of the previously isolated alleles. For the strong alleles, the nuclei are generally the same size, suggesting that they begin replicating together at a uniform rate. For multinuclear alleles, three of the nuclei are often larger than the others, probably because the polar bodies decondense after completion of meiosis and immediately begin over-replication without mitosis, while at the same time the pronuclei undergo a few divisions before they begin to over-replicate, resulting in smaller nuclei. Sometimes nuclei appear attached by DNA, probably due to incomplete anaphase. Previous characterization found mitotic defects during the first division for the strong allele $png^{1058}$ and the weak allele $png^{3318}$, resulting in spindle defects and anaphase bridges (Carminati 1995). The phenotypes of older mutant embryos
**Figure 4-1. Phenotype of new *png* alleles.**

Embryos from wild-type or mutant females mated to wild-type males were fixed and stained with propidium iodide to visualize DNA. (A) A fertilized wild-type embryo showing interphase nuclei. (B) *png^{172}* embryo showing a single giant nucleus. *png^{246}* has the same phenotype. (C) *png^{171a}* embryo showing the multinuclear phenotype. (D) *png^{50}* embryo. More than 20 nuclei is shown in this focal plane. More than 32 nuclei are observed in all focal planes of this embryo.
from long collections suggest that the nuclei eventually stop replicating and start degrading into less uniform spheres. This suggests that some replication factor is limiting and the nuclei can no longer replicate.

The new alleles fell into two classes: \textit{png}^{172} and \textit{png}^{246} showed no evidence of mitosis, while embryos from \textit{png}^{50} and \textit{png}^{171a} underwent a few mitoses before the nuclei overreplicated (table 4-1). One allele, \textit{png}^{50}, is different in that the embryos undergo more mitoses than had been seen with other \textit{png} alleles. Embryos from \textit{png}^{50} mothers have up to 64 nuclei, indicating that they can do one or two more divisions than \textit{png}^{171a} or \textit{png}^{3318}, the other multinuclear alleles. The two new multinuclear alleles confirm that this phenotype is due to loss of \textit{png} function, and if the strength of the allele is inversely proportional to the number of nuclei, then \textit{png}^{50} is the weakest of the eight alleles.

\textbf{Molecular Characterization of \textit{png}}

To determine the biochemical function of the \textit{png} gene product and if the nature of the mutations is consistent with the strength of the allele phenotypes, we cloned the \textit{png} gene. The \textit{png} locus maps between \textit{yellow} and \textit{white} on the X chromosome, and complementation tests between \textit{png} mutations and deficiencies and duplications delineated \textit{png} to a 130kb region (Carminati 1995). Former lab members Janet Carminati and Jessica Dines completed a chromosome walk across the region and isolated a single 39kb cosmid that can rescue \textit{png} mutations (Carminati 1995). A deficiency that breaks within the cosmid region suggested that at least part of the gene was in the proximal half of the 35 kb region (see chapter 3).

Using subcloned fragments from the rescuing cosmid in germline transformation rescue experiments, we isolated two overlapping fragments that rescued \textit{png} mutants, defining an 8 kb region containing the gene.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>1-5 nuclei</th>
<th>6-16 nuclei</th>
<th>17-32 nuclei</th>
<th>32-64 nuclei</th>
<th>number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>png^{50}</td>
<td>76.3%</td>
<td>19.8%</td>
<td>3.4%</td>
<td>0.4%</td>
<td>503</td>
</tr>
<tr>
<td>png^{50}</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>png^{171a}</td>
<td>88.0%</td>
<td>11.8%</td>
<td>0.2%</td>
<td>0</td>
<td>478</td>
</tr>
<tr>
<td>png^{171a}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>png^{172}</td>
<td>98.1%</td>
<td>1.9%</td>
<td>0</td>
<td>0</td>
<td>532</td>
</tr>
<tr>
<td>png^{172}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>png^{246}</td>
<td>98.6%</td>
<td>1.4%</td>
<td>0</td>
<td>0</td>
<td>143</td>
</tr>
<tr>
<td>png^{246}</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 4-1. Quantitation of nuclear number in new *png* alleles.

Homozygous mutant virgin females were mated to wild-type males to obtain fertilized embryos. 0 to 5.5 hour embryo collections were fixed and stained with propidium iodide and mounted in clearing solution, and the nuclei were scored using fluorescence microscopy. Nuclei that appeared to be fused were counted as one nucleus because they may have resulted from incomplete mitosis. Less than 5% of the embryos for each genotype were not scored because the nuclei were degraded or could not be seen because they were next to the slide.
Sequencing of the region revealed four large open reading frames (ORFs), including one with homology to protein kinases. Sequencing of DNA from the *png* mutants showed that all eight have mutations in this kinase ORF, indicating that this ORF is part of the *png* transcription unit. We then isolated a 1.7 kb *Bgl II/Hind III* restriction fragment capable of rescuing the maternal effect lethality of *png* homozygotes (figure 4-2). Genetic rescue indicates that the entire *png* transcription unit must be contained within the 1.7 kb, because this fragment contains all the DNA necessary for *png* function. Sequencing of the fragment revealed that the entire kinase ORF was contained within the 1.7 kb (figure 4-3). It seemed likely that this ORF was part of the *png* gene since it spanned most of the 1.7 kb and was entirely contained within it, and every *png* allele had a missense or nonsense mutation in this ORF.

To determine the *png* transcript, cDNA clones were isolated from the Tolias Drosophila ovary cDNA library (Stroumbakis et al. 1994). cDNAs were purified that hybridize to a 4.3 kb *Xho I* fragment that includes the 0.8 kb *Xho I/Hind III* fragment containing part of the ORF (see figure 4-3). Of the 40 cDNA clones isolated, 26 hybridize to a region distal to the 1.7 kb *Bgl II/Hind III* fragment, and 14 hybridize to the 1.7 kb fragment. The two groups do not overlap. Of the 14 which hybridize to the rescuing fragment, the 8 longest were sequenced. The longest cDNA is 1126 bp, not including the poly-A tail, and indicates that the 5' end of the transcript starts 80 bp upstream of the ATG (figure 4-3). The sequenced cDNAs show 3 alternate poly-A addition sites at 163, 170, and 191 bp 3' of the stop codon. None of the 8 cDNAs sequenced have introns, indicating that the *png* transcript is a single exon.

Conceptual translation of the longest cDNA reveals that the PNG protein has strong homology to Ser/Thr kinases (figure 4-4). The protein shows highest homology to members of the Snf1/AMP kinase family, with 27% amino
Figure 4-2. Smaller constructs delineate *png* to 1.7 kb.

The restriction map of cosmid 3-3-1 is shown along with the deficiency isolated in the screen (chapter 3) and the smaller fragments used in transformation rescue experiments. The following fragments complement *png*: 14 kb *Eco RI*, 13 kb *Bam HI*, and 1.7 kb *Bgl II/Hind III*. 
Cosmid 3-3-1

Deficiency isolated in screen:

Fragments tested for rescue:

- 14 kb Xba I
- 12.5 kb Sal I
- 8 kb Sal I
- 14 kb Eco RI*
- 13 kb Bam HI*
- 1.7 kb Bgl II Hind III*

*rescues png mutants
Figure 4-3. Restriction map of 1.7 kb *Bgl* II/ *Hind* III

The restriction map of the 1.7 kb fragment that contains *png* is shown. The *png* ORF is shown in blue, and the longest cDNA isolated from the Tolias ovary cDNA library is shown in red.
Figure 4-4. PNG encodes a new protein kinase.

Sequence of the PNG protein, based on conceptual translation of the genomic sequence of the longest cDNA. The mutations are shown above the sequence. Alleles shown in red are strong and those in blue are weak. The sequence was aligned with the kinase catalytic domain consensus shown below PNG by first aligning PNG to the Snfl kinase family using Clustal X (Thompson et al. 1997) and comparing to the alignment of Snfl to the kinase consensus in Hanks and Hunter (Hanks and Hunter 1995). In the consensus, uppercase letters are invariant, lowercase letters are nearly invariant, o postions are nonpolar, * postions are polar, and + positions are small residues with near neutral polarity.
acid identity to Snf1 over 265 amino acids (http://www.ncbi.nlm.gov/BLAST/). Phylogenetic analysis, however, shows that PNG is more distantly related to Snf1 family members than they are to each other, and probably represents a new family of Ser/Thr kinases (figure 4-5). PNG is small, encoding a 291 amino acid protein with a predicted molecular weight of 33 kD, and contains only a catalytic domain. This suggests there could be a regulatory subunit that associates with PNG.

We amplified the png ORF from homozygous mutants using the polymerase chain reaction (PCR), and sequenced the PCR products. The sequence revealed that all alleles cause changes in the kinase amino acid sequence (fig. 4-4). png

causes a missense mutation that causes a glycine to glutamate change in the DFG loop of the kinase domain. The DFG loop is required for primary Mg\(^{2+}\) chelation which helps orient the γ-phosphate for transfer, and the glycine residue is invariant in almost all protein kinases (Hanks and Hunter 1995).

\(png^{1920}\) causes a proline to serine change at residue 140 in the conserved catalytic loop. Proline-140 itself is not absolutely conserved in protein kinases, but it is surrounded by critical residues at the active site (Hanks and Hunter 1995). It is near aspartate-137, considered to be the catalytic base that accepts the proton from the attacking hydroxyl group of the substrate. It is also near asparagine-142, which stabilizes the catalytic loop by hydrogen bonding to the backbone carbonyl of aspartate-137, and chelates the secondary Mg\(^{2+}\) ion that bridges the α- and γ-phosphates of the ATP. Finally, it is next to lysine-139, which is invariant in all Ser/Thr kinases because it neutralizes the negative charge of the γ-phosphate during transfer (Taylor et al. 1992; Hanks and Hunter 1995).
Figure 4-5. Phylogenetic tree.

Phylogenetic tree showing that PNG is distantly related to the Snf1/AMP kinase family as defined by Hanks and Hunter (1995). The kinase catalytic domains were aligned using Clustal X (Thompson et al. 1997), and the phylogenies inferred by maximum parsimony using the PAUP software package version 4.0 (Swofford 1997). Minimum length trees were found using a "tree bisection-reconnection" heuristic algorithm. Two similar trees gave the same minimum length, of which one is shown. For both trees, the PNG branch is the longest, indicating that it is the most distantly related of the proteins shown. Branch length is relative to the number of amino acid substitutions required to reach hypothetical common ancestors at internal nodes.
The mutation in \textit{png}^{1058} predicts an arginine to cysteine change at residue 265 near the C-terminus. Arginine-265 is invariant in all kinases and is important for the kinase structure because it forms an ion pair with glutamate-181 (Taylor, et al. 1992). \textit{png}^{12-158} is a nonsense mutation that truncates the last 30 amino acids of the protein and also removes the invariant arginine-265.

The severity of the \textit{png}^{12-158} mutation and the alterations of conserved regions in \textit{png}^{172}, \textit{png}^{1920}, and \textit{png}^{1058} are consistent with these alleles having strong phenotypes. The weak \textit{png}^{3318} and \textit{png}^{2786} alleles have the same mutation, which alters an invariant residue in the glycine loop required for ATP binding. The glycine to serine change is a neutral change, so the kinase could still be partially functional. The strength of the other three alleles is not readily explained by the positions of the mutations.
Acknowledgements

We thank Dudley Wyman for technical assistance with cDNA isolation and Fran Lewitter for advice on phylogenetic analysis.
References


Chapter Five

Developmental Expression and Physical Interactions of the Pan Gu Protein Kinase

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*DDF examined png mRNA and PNG protein expression during development and effects of ectopic expression, purified the GST-PNG fusion protein, generated the anti-PNG antibodies, examined PNG and PLU expression in the png mutants, examined the kinase activity, and made the png-HA/His construct and analyzed its genetic interaction with the png alleles.
PNG Is Specific for Early Development

PNG is a cell cycle regulator specific for early embryogenesis. The early embryonic S-M cell cycle has a special regulation that allows DNA replication and mitosis to oscillate rapidly without gap phases. This regulation is controlled by maternal products, since zygotic transcription does not occur until cycle 10 of embryogenesis (Foe et al. 1993). If png functioned throughout development, then a null allele would be lethal. The png\textsuperscript{172} allele is likely to be null, however, because it alters a conserved amino acid of the kinase domain. This allele is fully homozygous viable, consistent with png being specific for the early, maternal-controlled, S-M cell cycles.

To test if expression of png is specific for early embryogenesis, we probed a Northern blot of polyA+ RNA isolated from different developmental stages with the png ORF (figure 5-1). Only a single transcript of 1.3 kb is detected, and it is expressed predominantly in adult females and early embryos until 3 hours of embryogenesis, which is consistent with its maternal effect inheritance. Barely detectable levels of the transcript are present in embryos between 3 and 6 hours, suggesting that the transcript is degraded or no longer expressed after the mid-blastula transition. png transcript is also present in the Schneider L2 embryonic cell line (Schneider 1972). To examine png mRNA expression during oogenesis, wild-type ovaries were probed with an anti-sense png riboprobe. Figure 5-2 shows that png transcript is highly expressed at stage 10B of oogenesis, and is present at lower levels in earlier stages. The png transcript shows no specific localization.

To examine PNG protein levels throughout development, we generated polyclonal antibodies against GST fused to the C-terminal 167 amino acids of PNG. Figure 5-3 shows an immunoblot of extracts from different developmental stages probed with anti-PNG antibodies. Extracts from
Figure 5-1. Expression of *png* transcript during development.

Developmental Northern of poly(A)+ RNA from embryos at various times after egg deposition, first, second, and third instar larvae, early and late pupae, female and male adults and the Schneider L2 cell line. The blot was probed with the *png* ORF.
Figure 5-2. *png* expression during oogenesis.

*png* transcripts were detected by whole mount *in situ* hybridization. *png* expression is seen in early egg chambers, but it is highly induced at stage 10B (large arrow). No expression is detected in the stage 14 oocyte (small arrow), because the chorion prevents the probe from entering the mature oocyte.
Figure 5-3. Expression of PNG protein during development.

Developmental immunoblot probed with anti-PNG. Protein extracts were made from 0 to 2 hour *png*<sup>12-158</sup> embryos, wild-type mature unactivated stage 14 oocytes, wild-type 0 to 1 hour unfertilized eggs, wild-type embryos of various ages shown, wild-type third instar larval brains, imaginal discs, and salivary glands. Three different immunoblots are shown: 0 to 2 hour wild-type embryo extract is shown on each as a reference. Wild-type oocytes and embryos were from strain *Oregon R*, wild-type unfertilized eggs and larval tissues are from *y w*.
$png^{12-158}$ embryos was used as a control for antibody specificity, because PNG$^{12-158}$ protein is truncated and has a faster mobility with SDS-PAGE. Wild-type PNG protein is highly expressed in unfertilized eggs and 0 to 2 hour embryos, which is consistent with the timing of the defect in $png$ mutants. PNG is present at low levels until 6 to 12 hours of embryogenesis. This could be due to purdurance of the high levels of maternal protein, or it could be from the presence of unfertilized eggs in the collections. PNG protein is not present in 12 to 24 hour embryos, nor is it expressed in the endoreplicating larval salivary glands, mitotic larval brains, or the imaginal discs which will form the adult tissues. Because mature oocytes from $png$- females have no apparent defects, it is likely that $png$ is not required until after metaphase I of meiosis (Shamanski and Orr-Weaver 1991). It was important to examine PNG protein levels in mature oocytes not yet activated by passage through the uterus, since $png$ might be regulated so that the protein is not present until after activation. This is because oocyte activation causes increased translation and the completion of meiosis, when $png$ is required by the embryo. Figure 5-3 shows that PNG protein is present in unactivated mature stage 14 oocytes, indicating that the protein is not translationally regulated at this time.

**Ectopic Expression of PNG**

To test if PNG can regulate an archetypical G1-S-G2-M cell cycle, we ectopically expressed $png$ in the eye imaginal disc during eye morphogenesis. PNG was expressed under the control of the Glass transcription factor using the pGMR vector (Hay et al. 1994). Figure 5-4 is a control showing that PNG protein is expressed in eye imaginal discs from larvae carrying the pGMR-png transgene. pGMR-png transformant flies had no apparent eye defects by examination with a dissecting light microscope (data not shown). To test if PNG requires PLU to affect eye development, we examined flies expressing
Figure 5-4. PNG protein is present in GMR-png eye imaginal discs

PNG protein was ectopically expressed in eye-antennal imaginal discs under the control of the GLASS transcription factor. Eye-antenneal discs were dissected from untransformed y w third instar larvae or from transformed larvae homozygous for an insertion of the png ORF in the GMR vector. The discs were homogenized, immunoblots prepared, and the blot probed with anti-PNG. The blot was also probed with anti-Tubulin (TUB) as a loading control.
both PLU and PNG in the eye imaginal discs. Animals carrying both pGMR-
png and a pGMR-plu transgene known to express PLU (Elfring et al. 1997) had completely wild-type eyes (data not shown). We also expressed PNG under the control of the inducible hsp70 promotor. Transformants carrying a hs-png transgene raised at 29°C had no apparent defects and were completely viable compared to wild-type sibling controls (data not shown).

**Effects of Mutations on PNG Protein**

It is likely that png172 is a null mutation, because it alters a conserved amino acid in a critical region of the kinase. To test if any of the alleles are protein nulls because the mutant protein is destabilized, we probed an immunoblot of mutant embryonic extracts with anti-PNG antibodies. Figure 5-5 shows an immunoblot of 0-1 hour embryo extracts from wild-type or homozygous mutant mothers. The png12-158 nonsense mutation which truncates PNG controls for antibody specificity because PNG protein from this mutant migrates faster. None of the alleles is a protein null, but png172 has greatly decreased PNG protein levels, as well as a slightly slower migration. PNG protein levels for the other seven alleles are slightly decreased compared to wild-type levels. This is likely to be an indirect effect of the png mutant phenotype rather than a structural instability caused by the mutations, because all seven alleles have a similar decrease.

Included on the immunoblot are embryonic extracts mutant for gnu and plu. Mutations in the maternal effect genes plu and gnu have giant nuclei phenotypes similar to the strong alleles of png and dominantly enhance png3318 (Freeman and Glover 1987; Shamanski and Orr-Weaver 1991). The plu gene encodes a 19 kD ankyrin repeat protein (Axton et al. 1994). We examined PNG protein levels in embryo extracts from plu3/plu6 mutant mothers. Both plu3 and plu6 are protein null alleles (Axton, et al. 1994;
Figure 5-5. Expression of PNG and PLU protein in different mutants.

Protein extracts were made from 0 to 1 hour embryo collections from wild-type (y w) or homozygous mutant females mated to sibling males. Also shown are wild-type (Oregon R) mature unactivated stage 14 oocytes and wild-type (y w) 0 to 1 hour unfertilized eggs. Each lane of the immunoblot contains two hundred μg of extract. The immunoblot was probed with anti-PNG antisera (top panel) or anti-PLU antibodies (middle panel). As a loading control, the immunoblot was also probed with anti-tubulin (bottom panel). plu embryos were from females trans-heterozygous for plu³ and plu⁶.
PNG and PLU Proteins Associate

Given the genetic interaction of the *plu* and *png* genes (Shamanski and Orr-Weaver 1991), we wished to test if the PLU and PNG protein interact
physically. We generated transformant lines carrying a Myc epitope-tagged PLU, which is functional because it can rescue the maternal effect lethality of \textit{plu}- homozygotes (Elfring, unpublished). We used anti-Myc antibodies to immunoprecipitate myc-PLU from embryo extracts and then immunoblotted the resulting pellet and supernatant. PNG protein coimmunoprecipitates with Myc-PLU using anti-Myc antibodies, while PNG is not present in control immunoprecipitates using embryos which do not carry the \textit{plu-myc} transgene (figure 5-6). PNG does not associate with the Myc tag, because it does not coimmunoprecipitate with Myc-tagged MEI-S332 under the same conditions (data not shown). PNG and PLU may associate because PLU is a substrate for the PNG kinase, or PLU may be a regulatory subunit.

**PNG and PLU Associated Kinase Activity**

To test if a kinase activity is associated with PNG and PLU, \textit{in vitro} kinase assays were performed on immunoprecipitates. Figure 5-7 shows that myc epitope-tagged PLU co-migrates with a protein that can be phosphorylated after immunoprecipitation from \textit{plu-myc} unfertilized egg extracts using anti-myc antibodies. The phosphorylated protein is not present in an immunoprecipitate that does not contain PLU-myc (wt lane). The kinase activity may be PNG dependent, because the phosphorylation is slightly reduced in \textit{png}^1058; \textit{plu-myc} extracts, though the reduction is difficult to quantitate because of high background and reduced levels of PLU protein in the \textit{png}^1058; \textit{plu-myc} immunoprecipitate. This result suggests that PNG may phosphorylate PLU.

In addition to possible phosphorylation of PLU-myc, a 55 kD endogenous protein is also phosphorylated in immunoprecipitates containing PNG and PLU-myc from embryonic extract (figure 5-8). This kinase activity is also PLU and PNG dependent, because it is not detected in an immunoprecipitate
Figure 5-6. PNG coimmunoprecipitates with PLU-myc from embryo extracts. Mouse monoclonal anti-myc antibodies (9E10) were used to immunoprecipitate myc epitope-tagged PLU from embryo extracts and the pellet (P) and supernatant (S) were loaded on an SDS-PAGE gel for immunoblot analysis. All of the pellet and one sixth of the supernatant was loaded for each immunoprecipitate. The top panel was probed with anti-PNG and the bottom panel was probed with anti-PLU. PNG protein coimmunoprecipitates with PLU-myc in extracts from embryos carrying a plu-myc transgene. Neither PLU-myc nor PNG is present in the pellet from control extracts, prepared from embryos with a plu-GFP transgene instead of a plu-myc transgene.
Figure 5-7. PLU protein is phosphorylated in immunoprecipitates containing PNG. Mouse monoclonal anti-myc (9E10) antibodies were used to immunoprecipitate myc epitope-tagged PLU and endogenous PNG from 0-4 hour unfertilized egg extracts. The resulting pellets were labeled in an in vitro kinase reaction containing $^{32}$P-$\gamma$-ATP and loaded on a 10-20% gradient SDS-PAGE gel for immunoblot analysis. The left panel is a phosphorimage of the immunoblot showing $^{32}$P-labeled proteins, while the right panel was probed with anti-PLU antibodies. The arrows indicate the migration of myc epitope-tagged PLU. Genotypes of the mothers are shown above each lane. The “wt” lane contains extracts from $y\,\text{w}$. 
Figure 5-8. PLU and PNG dependent kinase activity is detected in embryonic extracts. Protein kinase activity from 0-2 hour embryonic extracts was assayed in immunoprecipitates prepared with anti-myc (9E10) antibodies, and the proteins were separated on a 4-20% gradient SDS-PAGE gel. A phosphorimage of $^{32}$P-labeled proteins is shown. The arrow indicates a protein of 55 kD that is phosphorylated in immunoprecipitates containing myc epitope-tagged PLU and endogenous wild-type PNG (plu-myc lane), but is not labeled in immunoprecipitates that contain mutant PNG ($png^{1058}$; plu-myc, $png^{12-158}$; plu-myc, and $png^{3318}$; plu-myc) or do not contain PLU-myc (wt lane). The genotype of the “wt” embryos is $y w$. 
using wild-type extract that does not contain PLU-myc, and it is not present in extracts mutant for png\textsuperscript{1058}, png\textsuperscript{12-158}, or png\textsuperscript{3318}. It is not known what this 55 kD protein is. Autophosphorylation of PNG has not been detected in \textit{in vitro} kinase assays.

**PNG Inhibition Mediated Through the C Terminus**

A \textit{P} element construct containing the 1.7kb rescuing fragment with an HA/His insertion at the C-terminus of the \textit{png} ORF showed allele-specific rescue when transformed into Drosophila (table 5-1). Surprisingly, the construct shows the highest degree of rescue for png\textsuperscript{12-158}, the strong allele which truncates the last 30 amino acids of the PNG protein. 12.5\% of the embryos from png\textsuperscript{12-158} homozygotes carrying the \textit{png-HA/His} transgene complete embryogenesis, while survival is less than 2\% for the other alleles. To control for other mutations on the \textit{png} chromosome, png\textsuperscript{3318}, png\textsuperscript{172} and png\textsuperscript{1058} were tested in trans to each other. For all three trans-heterozygotes the degree of rescue was still less than 1\%. To ensure that the location of the inserted transgene is not causing the effect, three independent \textit{png-HA/His} insertions were tested with png\textsuperscript{12-158}, png\textsuperscript{1058}, and png\textsuperscript{3318}. The amount of rescue was different for each insert, presumably because of different levels of expression by the transgene, but for each insert png\textsuperscript{12-158} showed the highest level of viability, and png\textsuperscript{3318} showed the lowest (data not shown). This result suggests that endogenous mutant PNG protein inhibits the function of a PNG-HA/His fusion protein, probably by competing for regulatory subunit or substrate binding, and the C-terminus of the endogenous mutant protein is required for this inhibition.
Table 5-1. Rescue of png- maternal effect lethality with a png-HA/His transgene. Viability shown is the percent of embryos which complete embryogenesis, and represents the mean of at least 9 independent collections. More than 2000 total embryos were scored for each genotype. The variability was high (see standard deviation and range), but by the ANOVA test the difference between png^{12-158} P[png-Ha/His] and the other genotypes was significant (p < .0001).
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References


Chapter Six

Discussion and Future Considerations
This chapter reviews the results presented in this thesis and discusses them in terms of the questions answered about \textit{png}, and what questions still remain to be answered. The topics are presented in approximately the same order as in the previous three chapters. Also included within relevant sections and in the second half of the chapter are ideas for future experiments which may answer the remaining questions about \textit{png}'s function and mechanism of action.

\textbf{P Element Screen for New \textit{png} Alleles}

We completed a screen for new alleles of \textit{png} using \textit{P} element mutagenesis (chapter 3). A single mutation, \textit{png}\textsuperscript{358H}, was isolated during the screen, and quantitative Southern blot analysis showed that this mutation was a deficiency which deleted at least 20 kb. This deficiency probably resulted from imprecise excision of a \textit{P} element which had inserted in the region. Complementation tests of \textit{Df(1)png}\textsuperscript{358H} with other \textit{png} alleles showed that this deficiency behaves similarly to strong \textit{png} alleles or other deficiencies that uncover \textit{png}. \textit{Df(1)png}\textsuperscript{358H} is homozygous lethal, but this could result from deletion of other genes. The deficiency aided in the molecular isolation of \textit{png} because it delineated the region containing at least part of the \textit{png} gene, and it was eventually shown that \textit{Df(1)png}\textsuperscript{358H} deleted the entire \textit{png} transcription unit (chapter 4).

The main purpose of the \textit{P} element screen for new alleles of \textit{png} was to isolate \textit{P} insertions in \textit{png}, which would both aid in genetic analysis of \textit{png}'s function and in cloning the \textit{png} gene. In this respect the screen was not successful because no \textit{P} alleles of \textit{png} were isolated. There are several possible reasons for this. First of all, \textit{P} element transposition is non-random: some regions are "hotspots" for \textit{P} element insertion (Kidwell 1986). Therefore some other regions or genes such as \textit{png} may have a very low probability for \textit{P}
element insertion. Secondly, the size of \textit{png} probably affected its mutagenesis rate: the entire region necessary for \textit{png} function is less than 1.7 kb, which on average is smaller than most Drosophila transcription units, and represents a smaller target for mutagenesis. Finally, it is possible that the multi \textit{P} line mobilized during the screen carried full length \textit{P} elements expressing wild-type transposase, because transposition was observed in non-mutagenized lines (data not shown). This would have decreased the probability of isolating stable \textit{P} element insertions.

The screen may have been improved by the use of “local hopping”. It has been shown that \textit{P} elements are more likely to transpose to regions near the original site (Tower et al. 1993). Using an ammunition chromosome carrying a \textit{P} element in the region of \textit{png} may have improved the mutagenesis rate.

It is interesting to consider what was not isolated in the screen. It was of interest to attempt to isolate zygotic lethal alleles of \textit{png}, since none of the alleles were isolated in screens which would allow such a mutation. While the low mutagenesis rate may have precluded this, other results suggest that loss of function mutations with a zygotic defect could not exist because \textit{png} is specific for early development (see below). Another type of mutation which could have been isolated is a second site non-complementer. Since none were isolated, it suggests a low probability for finding this type of \textit{png} interactor on the \textit{X} chromosome, although such interactors are rare for most genes.

\textbf{Characterization of New EMS Alleles}

We were interested in characterizing new alleles of \textit{png} to determine if the multinuclear phenotype of some \textit{png} alleles is due to partial loss-of-function. If additional alleles with this multinuclear phenotype could be isolated, it would mean that these alleles did not result from unusual gain-of-
function mutations. The laboratory of Beat Suter gave us four new female-
stereile EMS alleles that fail to complement "png" (chapter 4). These alleles also
have the giant nuclei phenotype. Two of the new alleles, "png172" and "png246", have phenotypes similar to "strong" "png" alleles, in which mitosis never occurs; the new allele "png171a" is similar to the weak allele "png3318", in which a few mitoses occur in the early embryo; and one new allele, "png50", is different because the embryos have up to 64 nuclei, more than had been seen with any other "png" allele. Of the original alleles, there were two with a multinuclear phenotype ("png3318" and "png2786"), but when "png" was cloned it was shown that these two alleles have the same mutation (chapter 4). Isolation of two more alleles which have the multinuclear phenotype confirms that this phenotype is due to loss of function, and not some type of special mutation. The ability of "png50" embryos to complete up to 6 mitotic divisions increases the evidence that "png" not only functions to inhibit DNA replication before the first division, but plays a direct role coordinating the first several divisions. However, it is still formally possible that the phenotype of "png50" results from a defect during the first cell cycle.

The giant nuclei phenotype is rare in that in screens of maternal effect mutants, we and others have identified only three genes with this degree of inappropriate DNA replication (Shamanski and Orr-Weaver 1991). This suggests that these genes play an important and specific role in the regulation of S phase.

The phenotype of "png50" embryos was a surprise because it was different from the phenotype of the previously isolated alleles. It is possible that there are additional aspects of the "png" phenotype that have not yet been observed, and examining these may suggest new ideas about "png"'s function. It is also possible that different aspects of the phenotype, such as "png"'s function
before fertilization and its function during the early divisions, are actually independent functions that can be separated genetically. Since \textit{png} encodes a protein kinase, it may have different substrates involved in different processes, so mutations that affect substrate specificity may affect one aspect of \textit{png}'s functions and not others. It would be worth examining the tubulin and centrosome phenotype of the new alleles, to determine if the centrosome cycle also continues in these alleles as it does in the original four alleles. It would also be worth examining unfertilized eggs from the new alleles, because this phenotype could also be different. \textit{png}^{50} may give different phenotypes because it is the weakest allele, so it may be more similar to wild-type. The other alleles may have different phenotypes if they specifically effect interactions with other proteins.

We would also like to know if \textit{png} functions only during the first cell cycle, or throughout early embryogenesis. The phenotype of the weak alleles may result from defects which occur during the first cell cycle that do not prevent mitosis until later cell cycles. If some of the weak alleles are able to partially complement other alleles, it might be possible to observe even weaker phenotypes, and allow observation of several normal divisions before the mutant defect manifests. This would confirm that \textit{png} does function to coordinate \textit{S} phase and mitosis after the first cell cycle. Another way to answer this question is to observe development of the multinuclear phenotype using live analysis with \textit{histone-GFP} or \textit{tubulin-GFP} transgenes. Yolk auto-fluorescence can obscure nuclei in early embryos, but Histone-GFP can still be observed because it fluoresces at a different wavelength (H. LeBlanc, personal communication). Observation of the nuclei would also be improved using confocal microscopy.
We believe that png, plu, and gnu interact in the same pathway because mutations in both plu and gnu can dominantly enhance the phenotype of png$^{3318}$. Since this genetic interaction has only been observed with one png allele, it is possible it results from background mutations. To confirm this result, it is important to examine the genetic interactions of the new weak png alleles with plu and gnu. If the interaction is also true of png$^{50}$ and png$^{171a}$, it would confirm that these genes function in the same pathway. In addition, since other data suggests that PLU and PNG proteins physically interact, allele-specific genetic interactions between plu and png would suggest that specific regions of PNG are important for the interaction.

In wild-type embryos, treatment with microtubule depolymerizing drugs arrests DNA synthesis, suggesting a checkpoint that coordinates replication with the mitotic machinery (Foe et al. 1993). It is possible that png mutations affect this checkpoint, since mutant embryos continue the cycling of centrosome duplication and mitotic spindle formation, physically decoupled from DNA replication of the nuclei (Shamanski and Orr-Weaver 1991). To test this, the giant nuclei phenotype can be analyzed after treatment with microtubule depolymerizing drugs such as colchicine. If colchicine inhibits DNA replication in the mutant embryos, it means that the checkpoint is independent of plu and png. If the phenotype is unaffected, it means that the microtubule checkpoint is defective in these mutants, or it could mean that attachment of the spindles to the nuclei is important for the checkpoint. Treatment with taxol, which stabilizes microtubules, may distinguish these possibilities if it maintains the spindle attachment to the nuclei and prevents replication in the mutants.

Finally, it is unclear if the mutant phenotypes result from repeated firings of replication origins within the same S phase or if repeated rounds of
complete genomic replication are occurring without intervening nuclear divisions, though preliminary evidence suggests that replication is cyclic in png mutants (Shamanski and Orr-Weaver 1991). Attempts have been made to answer this question by electron microscopic analysis of mutant DNA: repeated firings of origins within the same S phase would give rise to replication bubbles within replication bubbles, while bubbles within bubbles would not be observed if the entire genome were replicated before the next round of replication. Too few intermediates were observed, suggesting the experiment may be technically too difficult to give conclusive results (T. Orr-Weaver, personal communication).

**png Encodes a New Protein Kinase**

Molecular isolation of png showed that it encodes a protein kinase (chapter 4). We are confident that we have molecularly isolated png because all eight alleles have missense or nonsense mutations in the kinase open reading frame, and because a 1.7 kb fragment containing only this transcription unit is capable of rescuing png mutations. Isolation of cDNAs showed that the png transcript contains no introns. Eight cDNAs that span the entire open reading frame were sequenced and none have introns (chapter 4). In addition, Northern blot analysis shows that a single 1.3 kb transcript hybridizes to the png ORF, suggesting that there are no alternatively spliced transcripts (figure 5-1). Intronless genes are rare in Drosophila, but a general search of the literature revealed a few examples, including forkhead (Weigel et al. 1989) and several of the hsp genes (Corces et al. 1980).

PNG is most similar to Ser/Thr kinases. Phylogenetic analysis suggests that PNG represents a new family of Ser/Thr kinases (figure 4-5). It would be interesting to find homologs in other species, both to learn about conserved domains in PNG and to learn if PNG's function is conserved in evolution. It
would be especially interesting to find a homolog in Xenopus, which also has maternally-driven early embryonic S-M cell cycles.

Whether or not specific homologs of PNG exist in other species, PNG may function in a pathway with conserved regulators. At present, it is unknown if $\textit{png}$ functions mainly to inhibit S phase before fertilization and during mitosis, or to activate mitosis, coordinating S phase and M phase during the early divisions. Determination of the substrates of the PNG protein kinase and how it influences known cell cycle regulators will help answer this question. If its main function is to inhibit S phase, it may phosphorylate and inhibit Cdk4/6, Cdk2, or cyclin E. If it is most important as an activator of mitosis, it may provide the activating phosphorylation of Cdc2 or a mitotic cyclin. Since global regulation of Cdc2 activity is not observed during the early divisions, this regulation may occur in local compartments of the embryo, such as the nucleus. In addition, since kinases are often regulated by phosphorylation, PNG may be controlled by other kinases such as CaMK II or Mos, although a Drosophila Mos homolog has not yet been found.

**Molecular Analysis of the $\textit{png}$ Mutations**

Analysis of the amino acid changes in the $\textit{png}$ mutants confirms its role as a protein kinase and suggests an explanation for the phenotype of the weak $\textit{png}$ alleles. Three of the five strong alleles alter conserved amino acids in the kinase domain, and an additional strong allele, $\textit{png}^{1920}$, alters an amino acid in a very conserved region. This mutation is a proline to serine change in the catalytic loop. While this proline is not absolutely conserved, this exact same mutation disrupts the function of yeast $\text{p34}^{\text{Cdc28}}$ (Carr et al. 1989). Of the three weak alleles, only $\textit{png}^{3318}$ affects a conserved amino acid, and this is a neutral glycine to serine change, which may mean that the kinase is still partially active. The other weak alleles could also still have activity because
they cause changes in less conserved parts of the kinase. It is unclear what effect the strong mutation $\text{png}^{246}$ has on PNG activity because this amino acid is not conserved.

We believe $\text{png}^{172}$ could be a null because the mutation probably affects $\text{Mg}^{2+}$ chelation and thus abolishes kinase activity. We also examined PNG protein levels in embryos mutant for the eight $\text{png}$ alleles (figure 5-5). None of the alleles is null for protein expression, but $\text{PNG}^{172}$ does show greatly reduced levels, suggesting that this allele is unstable or poorly expressed. One problem with this interpretation is that the wild-type form of PLU is present in $\text{png}^{172}$ embryos, suggesting that this allele is still functional (see PLU and PNG interactions, below). The decrease in $\text{PNG}^{172}$ may not be biologically relevant, because maternal genes are highly expressed in the early embryo, so decreased levels may have no effect on function. $\text{PNG}^{172}$ also has a slightly slower mobility, consistent with the mutation changing a glycine to a glutamate, altering the charge of the protein. The other alleles show only modest reduction in PNG protein levels, suggesting an indirect affect of the embryonic phenotype rather than a direct reduction of PNG stability caused by the mutations.

**PNG Is Specific for Early Development**

The phenotypes of all the alleles, including the putative null $\text{png}^{172}$, suggest that $\text{png}$ is only required during early embryogenesis, because homozygous mutants are fully viable and show strict maternal effect inheritance. Consistent with this idea, the developmental Northern and immunoblot show that $\text{png}$ expression is limited to early embryogenesis (chapter 5). Low levels of PNG protein can be detected in extracts from 6 to 12 hour embryos, well beyond the early embryonic divisions, but it is unclear if this is new expression, purdurance of maternal product, or the presence of unfertilized eggs in the collections. There is an experiment which could be done
to test if PNG is zygotically expressed. We have generated transgenic lines carrying *png-GFP* and *png-myc* transgenes which are functional because they can completely rescue *png* mutations (data not shown). The fusion proteins have a slower mobility with SDS-PAGE, while PNG\textsuperscript{12-158} protein has a faster mobility because it is truncated. Females homozygous for *png*\textsuperscript{12-158} and one of the transgenes can be mated to *png*\textsuperscript{+} males, obtaining viable embryos which could only express PNG of wild-type mobility if *png*\textsuperscript{+} was zygotically transcribed.

Interestingly, *png* transcript is detected in the Schneider L2 cell line, which is derived from embryos (figure 5-1). It is unclear if PNG protein is expressed in these cells, though it might be worth examining. *plu* transcript is not expressed in L2 cells (Axton et al. 1994), suggesting that if PNG protein is present, it may not be functional. Studies of PNG kinase activity and localization in this cell line may tell us about PNG's requirement for the presence of PLU.

Ectopic expression studies also suggest that *png* functions specifically during early development. Expression of PNG separately or PLU and PNG together in the eye-antennal imaginal discs has no effect on adult eye morphology (chapter 5). Eye development is sensitive to cell cycle perturbations: for example, weak alleles of *cyclin E* which have no effect on viability can have a rough eye phenotype (Secombe et al. 1998). These results suggest that PLU and PNG are incapable of regulating cell cycles other than the early S-M cell cycles. Furthermore, ectopic expression of PNG throughout development under the control of the *hsp70* promotor has no effect on viability. It is possible that ectopic expression of these genes did have subtle cell cycle defects which did not affect viability or adult visible phenotypes. It might be worth repeating these experiments and directly examining cell cycle
progression by techniques such as BrdU incorporation. It is interesting to consider why PLU and PNG might not be functional during later development. It suggests that they require another protein to function, and this other protein is also only expressed in early embryos.

It is interesting to think about why a special cell cycle regulator would be required during the early embryonic divisions. Obviously, the first division is critical because the rest of embryogenesis depends on its success. In addition, because there is no zygotic expression, the early embryo is filled with high levels of maternal proteins and RNAs necessary for the first several divisions. It is quite possible that a special regulator is needed at this time to regulate and coordinate DNA replication and keep the massive quantities of cell cycle regulators under control. This is especially true immediately after meiosis, because the meiotic products immediately enter interphase and may be particularly vulnerable to inappropriate DNA replication. Moreover, because there is no transcription, additional post-transcriptional regulation may be required at this time. This is consistent with png encoding a protein kinase.

**PLU and PNG Interactions**

The *plu* and *png* genes have similar phenotypes and interact genetically, suggesting that they function in a common pathway to control the early embryonic cell cycle. We have shown that PLU mobility on SDS-PAGE is altered in *png* mutant embryonic extracts (figure 5-5). A faster mobility form of PLU is present in wild-type embryos, and the same form can be detected in unfertilized eggs and unactivated mature oocytes. In contrast, some of the *png* mutants have a slower mobility form of PLU not seen in wild-type extracts. This form is seen in four out of five strong *png* alleles, the exception being *png*172. The difference between *png*172, the putative null, and the other strong alleles is surprising, and suggests that either the mobility of PLU is not
dependent on PNG kinase activity, or that png\textsuperscript{172} is not a null. (See next paragraph for more discussion of this.) The three weak png alleles all have the wild-type form of PLU, although png\textsuperscript{171a} extracts also have the slower mobility form, which predominates. These results suggest that png controls covalent modification of PLU, directly or indirectly. We have also shown that the interaction is probably direct, because PNG co-immunoprecipitates with epitope-tagged PLU (figure 5-6). PLU might be a substrate of the PNG protein kinase, or it might form a complex with PLU, and as a result prevent or allow covalent modification of PLU to occur. The fact that the weak alleles have the wild-type form while most of the strong alleles have the slower mobility form suggest that the new form directly results from loss of PNG function because PLU is a substrate for PNG. Treatment of embryo extracts with lambda-phosphatase (New England Biolabs) results in slower mobility of PLU protein on SDS-PAGE, though it is unclear if the mobility correlates with the slower PLU form observed in some png mutants (D. Burney, unpublished).

The only confounding mutant is png\textsuperscript{172}, which has the wild-type form of PLU, yet has a mutation which certainly affects kinase activity. An alternative model is that PLU is a regulatory subunit of PNG, most likely an activator because of the phenotypes and genetic interaction, and that PLU is covalently modified by other regulators depending on whether it is bound to PNG or not. This would be analogous to cyclin-dependent kinase complexes, in which the cyclin, which is required for kinase activity, is phosphorylated, ubiquitinated, and degraded after activation of the kinase (Deshaies et al. 1995). Suggestively, the PLU protein contains a putative cyclin destruction box like those which are required by cyclins for ubiquitination and degradation. In addition, PLU is a generally unstable protein (D. Burney, personal communication) and is greatly reduced in longer collections of png\textsuperscript{1058} embryos.
(Elfring et al. 1997). By analogy, a good interpretation of the results is that mobility of PLU reflects the ability of the two proteins to complex. The functions of the two proteins may still depend on each other, if the physical interaction were required for kinase function. This is consistent with all the weak *png* alleles having the wild-type form of PLU: this form is dependent on PLU's physical interaction with PNG, and PNG's kinase activity is dependent on PNG's physical interaction with PLU. In this case, PNG	extsuperscript{172} may still be kinase inactive, yet still capable of the physical interaction with PLU which is required for wild-type PLU mobility.

Many experiments need to be completed to address these issues. More *in vitro* kinase assays should be performed to confirm if PLU is a substrate for PNG, and also to test if the presence of PLU is required for PNG kinase activity. We know that immunoprecipitated PLU from embryonic extracts can be phosphorylated *in vitro* (figure 5-7 and D. Burney, personal communication). However, these experiments suggest only a modest decrease in phosphorylation of PLU from *png* mutant extracts, so it is unclear if PLU phosphorylation is dependent on PNG *in vivo*. These experiments should be repeated with highly purified PLU and PNG. Furthermore, an endogenous 55 kD protein may be phosphorylated by PNG in embryos (figure 5-8). It would be interesting to identify this protein, since it may tell us more about the PNG pathway. Surprisingly, we have not detected PNG autophosphorylation. Autophosphorylation activity has been detected for members of the Snf1 kinase family (Celenza and Carlson 1989; Mitchelhill et al. 1994). PNG, however, is distantly related to the Snf1 family and may not phosphorylate itself. It is also possible the autophosphorylation activity requires different reaction conditions.
In addition, site directed mutagenesis followed by Drosophila transformation can be used to test if PLU’s destruction box, phosphorylation sites, and nuclear localization signal is required for its function. Some of these mutations may have dominant effects on embryo viability, but even this could be tested because PLU is only expressed maternally: transformants could be isolated and maintained as males.

The partial rescue of png mutants by a png-HA/His fusion also suggests protein interactions with PNG. Since full-length mutant forms of PNG can inhibit a partially functional PNG-HA/His fusion, while truncated PNG\textsuperscript{12-158} cannot inhibit (Table 5-1), it is likely that the C-terminus of PNG needs to interact with other proteins to function. Our interpretation of these results is that full-length mutant PNG is competing with PNG-HA/His for binding to substrates or regulators. The C-terminus is required for this interaction because PNG\textsuperscript{12-158} is missing the last 30 amino acids of PNG. It is possible that this interaction involves PLU. Immunoprecipitation experiments or other binding assays may show a difference in PLU’s ability to associate with the different mutant forms of PNG.

**Interactor Screens**

It is also likely that other proteins interact with PNG, and it is important to find other proteins functioning in the PNG pathway. Immunoprecipitation experiments can be carried out to test if known cell cycle regulators co-immunoprecipitate with PNG. A number of antibodies against Drosophila Cyclins and CDKs are available. This type of approach is limited, though, to known regulators. Other approaches would allow isolation of both known and novel Drosophila cell cycle regulators. Biochemical purification of the PLU and PNG complex might co-purify substrates or regulatory subunits. The yeast two-hybrid approach could also be used to isolate proteins that
interact with PLU, PNG, or both. The yeast two-hybrid would also allow examination of the interaction of PLU and PNG: if the PLU/PNG physical interaction can be established and monitored in yeast, it would be easy to use site directed mutagenesis to examine the protein domains required for the physical interaction. Finally, if purified, active PNG is available, it would be possible to screen an expression library for proteins which can be phosphorylated by PNG.

The screens mentioned would isolate direct physical interactors, while a genetic approach would allow isolation of all genes functioning in the png pathway. The lab has carried out a screen for deficiencies which dominantly affect the phenotype of png$^{3318}$ and several enhancers and suppressors have been isolated (L. Elfring and G. Bosco, personal communication). Isolation of point mutants and $P$ alleles in these intervals which are causing the interaction and cloning of these genes will give us more information about the pathway. A more thorough approach could also be carried out using $P$ element or EMS mutagenesis, allowing screening of the entire genome and reducing the background effects of large deficiencies. Some of the genes isolated might encode substrates of the PNG protein kinase. Since the png$^{3318}$ mutation alters the glycine loop at the N-terminus of the kinase domain, it probably affects binding to ATP, but might not effect binding to substrate. This makes it more likely that genetic interactors with png$^{3318}$ could be substrates. It is common for kinases to be regulated by phosphorylation; if png is part of a kinase pathway, genes encoding kinases and phosphatases both downstream and upstream could be isolated in the screen. General cell cycle regulators, such as genes required for mitosis, would probably also be isolated, but these genetic interactions may be indirect because mutations in anything required for mitosis might enhance the png phenotype.
Localization

Since PLU contains a putative nuclear localization signal, localization of one or both proteins might be important for function. If they are localized to the nucleus, they might function specifically to inactivate DNA replication factors or affect chromosome condensation. If they localize to centrosomes or mitotic spindles, it would suggest that they function to activate mitosis or maintain the connection between the mitotic machinery and the nuclei. Localization at specific times of the cell cycle may also be important for their regulation. To answer these questions, several approaches have been taken to localize both PLU and PNG in early embryos, but none have detected any specific localization. Transformant lines carrying plu-GFP and png-GFP transgenes have been established, but no GFP signal was detected (L. Elfring, personal communication, and data not shown). Similar approaches with anti-PLU antibodies have been attempted, with no success. Anti-PNG antibodies did give punctate staining in embryos relative to controls, but there was no apparent pattern to the localization (data not shown), and since none of the alleles is a protein null, it is difficult to rule out antibody cross-reactivity. It is worth expressing GFP fused PLU and PNG in Drosophila embryonic Schneider cells to see if they are localized, and if localization of one depends on the presence of the other. These experiments could also be attempted with affinity purified antibodies or with different antibodies, which may have a higher specificity.

Structural Studies

Structural studies of protein kinases and their interactors have provided much basic information about how kinases function. It is not clear what important structural differences there are, if any, between different families of Ser/Thr kinases. Since PNG represents a new type of kinase, solving its
structure using X-ray crystallography or NMR techniques may provide more information about the highly conserved kinase superfamily and the differences between kinase families. In addition, recent structural studies have shown how ankyrin repeat proteins can inhibit the function of protein kinases (Baumgartner et al. 1998; Venkataramani et al. 1998). The physical relationship between PLU and PNG has to be different, because PLU is either an activator of PNG or a substrate. The structure of the PLU/PNG complex might explain this difference, as well as provide general information about the interaction of ankyrin repeat proteins with kinases.

**Fertilization and Development**

It would be interesting to understand more about the signals coordinating fertilization in the embryo and determining the maternal pronucleus and polar bodies. In fertilized embryos and unfertilized eggs the polar bodies, which are the three maternal meiotic products which do not contribute to the embryo, remain arrested in a condensed state and eventually degrade. The polar bodies of *plu* or *png* mutant embryos over-replicate along with the pronuclei. This suggests that the *png/plu* pathway also involves determination of the maternal nuclei. It would be interesting to know how this signal is established. Genetic interactors may affect pronucleus determination. In addition, paternal effect mutations which affect embryonic development have been discovered (Fitch et al. 1998), and it would be interesting to see the effects of mating these mutants to *png* or *plu* mutant females. Since one function of *plu* and *png* is to inhibit replication in unfertilized eggs, it is possible that these paternal effect genes encode factors which interact with the *plu/png* pathway. Finally, fertilization in other organisms is associated with changes in Ca$^{2+}$ levels (Sagata 1996). Studies could be carried out in Drosophila to establish if fertilization in this organism is...
also associated with changes in Ca^{2+} levels, which may regulate the png/plu pathway.

**What Does gnu Do?**

No one knows for sure how *gnu* fits into the *png* pathway, and this information will probably wait until the *gnu* gene is cloned. Since only one allele of *gnu* exists, the wild-type gene may not normally function in the pathway if the mutation is neomorphic. If it does function in the pathway, it will be interesting to see if it encodes a homolog of a known cell cycle regulator, or if it is novel, and if it could be a substrate or activator of PNG.

**Final Thoughts**

We have learned much in recent years about the mechanism and function of PNG. The results presented in this thesis have answered many questions about when and how PNG controls the embryonic cell cycle, but each answer has also raised many more questions. Continued studies will not only tell us about the PNG pathway, but also about fertilization, embryogenesis, protein kinases, and cell cycle regulation in general.
Acknowledgment

I thank D. Burney for the many discussions we have had about \textit{plu} and \textit{png}. This chapter contains many of those ideas.
References


126


