Drosophila PLUTONIUM Protein Is a Specialized Cell Cycle Regulator Required at the Onset of Embryogenesis

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Unfertilized eggs and fertilized embryos from Drosophila mothers mutant for the pluto- nium (plu) gene contain giant polyploid nuclei resulting from unregulated S-phase. The PLU protein, a 19-kDa ankyrin repeat protein, is present in oocytes and early embryos but is not detectable after the completion of the initial rapid S-M cycles of the embryo. The persistence of the protein during the early embryonic divisions is consistent with a direct role in linking S-phase and M-phase. When ectopically expressed in the eye disc, PLU did not perturb the cell cycle, suggesting that PLU regulates S-phase only in early embryonic development. The pan gu (png) and giant nuclei (gnu) genes also affect the S-phase in the unfertilized egg and early embryo. We show that functional png is needed for the presence of PLU protein. By analyzing png mutations of differing severity, we find that the extent of the png mutant phenotype inversely reflects the level of PLU protein. Our data suggest that PLU protein is required at the time of egg activation and the completion of meiosis.

INTRODUCTION

During the development of multicellular organisms, it is critical that cell division be coordinated with differentiation. Not only must the number of cells contributing to a particular tissue be regulated, but the timing of mitotic divisions must be coordinated with gene expression and cell movement (Foe and Odell, 1989; Arora and Nüsslein-Volhard, 1992). The spatial orientation of mitosis also contributes to proper differentiation (Rhyu et al., 1994). Thus, extrinsic developmental controls must be integrated into the intrinsic control of the cell cycle.

The restart of the cell cycle at fertilization represents a critical point at which developmental events and cell cycle regulation intersect. Haploid gametes are produced by a specialized cell cycle, meiosis, in which two rounds of chromosome segregation follow a single S-phase. In most animal oocytes, meiosis is arrested to permit oocyte growth and differentiation. Although the particular arrest point of the meiotic cell cycle varies among organisms, all require developmental input to activate the completion of the meiotic cell cycle (Murray and Hunt, 1993; Sagata, 1996). In most cases, the completion of meiosis is triggered by fertilization. Sperm entry also serves to restart the mitotic cell cycle following fusion of the male and female pronucleus. The mechanism by which fertilization influences the meiotic and mitotic cell cycles is not understood at a molecular level, and the cell cycle regulators affected by fertilization remain to be identified.

Drosophila is an ideal organism in which to investigate the effect of developmental events on the cell cycle. The cell cycle is modified extensively during Drosophila embryogenesis, with at least four variant cell cycles utilized (Orr-Weaver, 1994). The ability to isolate mutants with defective cell division or altered cell cycles provides the means to recover key regula-

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tory genes. Mutants affecting the cell cycle early in embryogenesis exhibit a particular pattern of inheritance and are termed maternal-effect mutants. The early divisions in the embryo occur prior to transcription of the zygote's own genome, requiring that these divisions be controlled by maternal stores deposited during oogenesis. Consequently, mutations in genes needed for oogenesis or early embryogenesis exhibit a mutant phenotype only if the mother is a homozygous mutant.

*Drosophila* oocytes arrest first in prophase I to permit oocyte differentiation, but they arrest again at metaphase I as mature oocytes and can be held at this point for prolonged periods (King, 1970). The signals for the completion of meiosis and the restart of the cell cycle are separable in *Drosophila*: the oocyte is activated to complete meiosis as it moves into the uterus, regardless of whether it is fertilized, whereas fertilization triggers the resumption of the cell cycle (Mahowald et al., 1983). In these activated oocytes both meiotic divisions are completed, and the four meiotic products decondense their chromosomes in telophase II. If the egg is not fertilized, all four polar bodies condense their chromosomes and arrest in a metaphase-like state (Doane, 1960). The two meiotic divisions are nuclear divisions within the egg cytoplasm, and the polar bodies are not budded off as in vertebrate eggs. The condensed chromosomes of the meiotic products form a characteristic rosette structure on the dorsal side of the embryo.

Normally, oocyte activation is temporally linked to fertilization, and as the oocyte enters the uterus it is both activated and fertilized. In fertilized embryos, the four maternal meiotic products enter interphase after telophase II, and the male pronucleus also decondenses (Sonnenblick, 1950). While the three maternal polar bodies recondense their chromosomes on the dorsal surface, the male and female pronuclei undergo mitosis on a shared spindle in the interior of the embryo. The first 13 divisions consist of S-M cycles in which DNA replication directly oscillates with mitosis without intervening gap phases (Foe and Alberts, 1983). These are nuclear divisions in a shared cytoplasm; the zygotic nuclei divide fairly synchronously, whereas the polar bodies remain arrested with condensed chromosomes.

It is likely that specialized cell cycle controls are exerted at the onset of *Drosophila* development. Following telophase II, the meiotic products enter an interphase-like state in which the chromosomes are decondensed and may be vulnerable to the maternal cell cycle components packaged in the oocyte. Thus, the oocyte may require mechanisms to block the restart of the cell cycle after the completion of meiosis to coordinate it with fertilization. In addition, the early S-M cycles differ from archetypal G1-S-G2-M cycles because the regulation is exclusively posttranscrip-

tional (Edgar and Schubiger, 1986). The shared cytoplasmic signal implies there are special mechanisms to maintain polar body arrest while the zygotic nuclei replicate and divide.

The maternal-effect genes *plutonium* (plu),1 *pan gu* (png), and *giant nuclei* (gnu) act to control S-phase in the unfertilized egg and during the early divisions (Freeman et al., 1986; Freeman and Glover, 1987; Shamasaki and Orr-Weaver, 1991). Mothers mutant for any one of these genes produce unfertilized eggs that properly complete meiosis; however, DNA replication inappropriately occurs in all four meiotic products in these mutant eggs, leading to giant polyploid nuclei. In fertilized embryos from these mutant mothers, the polar bodies also undergo extensive DNA replication. In addition, the female and male pronuclei replicate their DNA but fail to divide, and they also become polyploid. Although the chromosomes do not go through mitosis, the centrosomes do divide and organize microtubule asters. Heterozygous mutations in plu or gnu worsen the phenotype of leaky png alleles. This dominant epistasis demonstrates that these three genes control the same biological process.

We previously cloned the *plu* gene and demonstrated that it encodes a 19-kDa protein with ankyrin-like repeats (Axton et al., 1994). Here, we analyze the developmental regulation of PLU protein. These experiments demonstrate a requirement for PLU protein after the completion of meiosis and emphasize the distinctions between the cell cycle capabilities of the polar bodies and the pronuclei. We investigate the regulatory relationship between PLU, png, and gnu and find that png function is required for the presence of PLU protein.

### MATERIALS AND METHODS

#### Drosophila Strains

The wild-type strain used, unless indicated otherwise, was Oregon R. The *plu* mutant strain used as a negative control for Western blots was *plu*1 (Shamasaki and Orr-Weaver, 1991). The previously described png13-1096, png13-1920, png7b-331a, and png2-2786 mutations were utilized (Shamasaki and Orr-Weaver, 1991), but for simplicity only the last four digits of the allele designations are used in this article. Other mutants examined were gnu, cort138, gray1970, and dh199 (Freeman et al., 1986; Freeman and Glover, 1987; Salz et al., 1994; Page and Orr-Weaver, 1996). The deficiency Df(1)A94 deletes png and was used as a control in some experiments. *Drosophila* strains were reared under standard conditions on cornmeal-molasses-agar medium (Ashburner, 1989).

#### Production of anti-PLU Antibodies

A full-length *Plu* cDNA was digested with *HindIII* and *NotI* and cloned into the pAE44 T7 expression vector (a gift from Paul Matsudaira and Michael Way, Whitehead Institute). The resulting

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1 Abbreviations used: cort, cortex; dh1, deadhead; gnu, giant nuclei; gnu, grauzone; plu, plutonium; png, pan gu; Dmcdc2, Drosophila melanogaster cdc2 protein.
plasmid was transformed into BL21 and BL21 LysS cells. Induction with isopropyl-β-D-thiogalactopyranoside resulted in the production of the full-length (19-kDa) PLU protein. This insoluble protein was purified by denaturing inclusion bodies in 6 M guanidine-HCl, 50 mM HEPES (pH 8.0), 0.1 mM EDTA followed by dialysis against 50 mM HEPES (pH 8.0), 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol to refold the protein.

Purified PLU protein was sent to HRP (Denver, Pennsylvania) for immunization of rabbits. Immune bleeds were assayed for reactivity against the bacterially expressed PLU protein on Western blots. Bleeds containing high immunoreactivity were affinity purified on renatured PLU protein coupled to Affigel columns (Bio-Rad, Hercules, CA) and eluted with glycine-HCl (pH 2.5). Alternatively, serum was affinity purified on Immobilon strips (Millipore, Bedford, MA) containing the bacterially expressed PLU protein and eluted with 3.5 M MgCl₂.

**Protein Extracts**

For ovary extracts, females were fattened on yeast for 3 to 5 days and ovaries were dissected in phosphate-buffered saline. Pooled ovaries were homogenized in 2× urea electrophoresis sample buffer (2% SDS, 10% glycerol, 80 mM Tris-HCl, pH 6.8, 2 mM EDTA, 100 mM dithiothreitol, 5 M urea) and heated at 95°C for 5 min. Samples were centrifuged for 5 to 10 min at 13,000 rpm. Supernatant was transferred to a fresh tube and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad).

For stage 14 oocytes, fattened females were disrupted using a modification of the protocol of Theurkauf (1994) as described by Page and Orr-Weaver (1997) and mixed populations of oocytes were methanol fixed. Mature stage 14 oocytes were hand-picked from the resulting mixture of oocytes, rehydrated, and homogenized as described above.

For unfertilized egg extracts, virgin females of the appropriate genotype were mated to sterile XO males. Zero- to 2-h collections of unfertilized eggs were collected on molasses-agar plates, dechorionated, washed extensively, and homogenized as described above. Similarly, fertilized embryo extracts were obtained by crossing females to wild-type males. Embryos were collected, dechorionated, and homogenized as described above.

For imaginal disc extracts, eye-antennal discs were dissected from third instar larvae and placed directly into urea sample buffer on ice. Pools of 20 pairs of discs were homogenized and were loaded in a single lane for electrophoresis.

**Western Blots**

Protein extracts were separated on 15% acrylamide (33.5% acrylamide:0.3% bis-acrylamide) gels. For most blots shown, 200 µg of total protein were loaded per lane. Gels were blotted onto Immobilon membranes (Millipore). Equal protein loading was verified by comparing the intensity of proteins on the Poncette 5-stained blots and, where shown, by incubating blots with anti-Dmed2 antibodies (Knoblich et al., 1994). Blots were blocked in 5% nonfat dry milk, 1% bovine serum albumin, and 0.1% Tween 20 in Tris-buffered saline and bound to affinity-purified anti-PLU antibody diluted in the same solution. Alkaline phosphatase-conjugated antirabbit secondary antibody (Promega, Madison, WI) and either 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (Promega) or chemiluminescent reagents (Tropix, Bedford, MA) were used to detect bound anti-PLU antibody.

**Northern Blots**

RNA was isolated from frozen ovaries as described previously (Ashburner, 1989). Polyadenylated RNA was isolated from total RNA and fractionated on 1% agarose/glyoxal gels. Roughly, 5 µg of poly(A)⁺ RNA were loaded per lane. Gels were blotted onto Hybond-N nylon membranes (Amersham, Arlington Heights, IL), UV cross-linked, prehybridized, and hybridized at 48°C in 0.25 M NaPO₄ (pH 6.5), 7% SDS, and 50% formamide. Single-stranded riboprobes were prepared by in vitro transcription of a Plu cDNA cloned into the Bluescript vector (Stratagene, La Jolla, CA).

**Staining of Embryos**

Embryos were collected, dechorionated, fixed, and prepared for microscopy as described by Shamanski and Orr-Weaver (1991).

**Scanning Electron Microscopy**

Adult flies were examined using an Electroscan environmental scanning electron microscope at the MIT Electron Microscopy Facility.

**Construction of Transgenes and P-Element Transformation**

For ectopic expression of PLU in the eye disc, two strategies were used: the pg38 plu transgene, which contains all of the sequences necessary for plu function (Axton et al., 1994), was digested with HindIII and SacII to generate a 0.7-kb fragment which contains only six nucleotides 5′ to the plu coding region. The ends of this fragment were end-filled using T4 DNA polymerase, and the fragment was cloned into the HpaI site of the pGMR1 vector (Hay et al., 1994). Alternatively, PCR mutagenesis was used to engineer a number of convenient restriction sites flanking a full-length Plu cDNA. The cDNA was digested with Smal and CiaI followed by end-filling with T4 DNA polymerase and cloning into the HpaI site of the pGMR1 vector.

The above subclones were introduced into the germline of y w+65-23 flies using standard techniques (Spradling, 1986). Insertions were mapped genetically, and strains which contained insertions of the Plu cDNA and genomic fragment under the control of the GMR enhancer on both the second and third chromosomes were constructed.

**RESULTS**

**Restricted Expression of PLU Protein**

Mature oocytes from plu mutants are arrested at metaphase I with normal morphology. However, in unfertilized mutant eggs, meiosis is completed, but the meiotic products overreplicate (Shamanski and Orr-Weaver, 1991). The overreplication observed in mutant unfertilized eggs indicates that PLU is needed to repress DNA replication prior to fertilization. Fertilized embryos laid by mutant mothers also exhibit a mutant phenotype in that mitosis does not occur and giant polyploid nuclei are produced. The polar bodies as well as the two pronuclei become polyploid, and frequently the five polyploid nuclei fuse together (Shamanski and Orr-Weaver, 1991). There are two interpretations for the defect observed in fertilized embryos. The first is that PLU is necessary only prior to fertilization and once the meiotic products inappropriately replicate it is no longer possible for them to undergo mitosis. This does not readily explain why the male pronucleus overreplicates. Another possibility is that PLU is also required after fertilization to
collections of fertilized embryos from wild-type females and Western blotted with the anti-PLU antibody (Figure 1B). The PLU protein derived from the different developmental stages migrated with the same mobility under several electrophoretic conditions, suggesting that there are not extensive modifications of PLU (Figure 1; Elfring and Orr-Weaver, unpublished results). The PLU protein was present in whole ovaries and unfertilized eggs and appeared to be slightly more abundant in stage 14 oocytes (Figure 1B). Levels of PLU were lowest in extracts from 0- to 3-h embryos. Thus, the PLU protein is present at detectable levels both before and after fertilization. The presence of PLU protein in fertilized embryos raised the possibility that PLU is actively required to regulate the cell cycle after fertilization.

Having observed that PLU persists after fertilization, we wanted to determine whether PLU regulates more than one of the several different cell cycles utilized during Drosophila embryogenesis. Zero to 2 h after fertilization, embryos undergo rapidly alternating S-M (cleavage) cycles; 2-4-h embryos complete the cleavage cycles and enter the first postblastoderm cycle in which a G2 phase is added; 4-6-h embryos undergo two additional postblastoderm divisions; and 6-22-h embryos undergo some mitotic divisions in the nervous system, while most tissues undergo endod cell cycles, which lack M phase (Orr-Weaver, 1994). To examine how long PLU protein persists in fertilized embryos, protein extracts were prepared from timed and appropriately aged collections of wild-type embryos, and Western blots were probed with the anti-PLU antibody (Figure 1C). PLU protein levels were maximal in 0–2-h embryos, detectable at low levels in 2–4-h embryos, and undetectable in later embryos. Previous experiments showed that the Plu transcript is undetectable in the larval or pupal stages or in adult males (Axton et al., 1994). The presence of PLU protein during the stages when rapid S-M cycles are occurring, and its absence in later stages, suggests that PLU protein specifically regulates the early division cycles.

Regulatory Relationship among plu, png, and gnu

Mutations in png and gnu are strict maternal-effect lethal. Females homozygous for these mutations produce unfertilized eggs which inappropriately replicate the four meiotic products. Fertilized embryos from gnu mutant mothers appear to be identical to those from plu mutants in that DNA replication occurs but there is no nuclear division (Freeman et al., 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). In contrast, the phenotype of fertilized png embryos reveals that there are two classes of png mutant alleles. Mothers mutant for three of five png alleles produce embryos like those from plu or gnu mutant mothers (Figure 2F). However, mothers mutant for the

couple S-phase to mitosis and to prevent replication in the polar bodies.

We wished to determine the relative levels of PLU protein before and after fertilization to distinguish between these two models. In addition, since wild-type embryos must possess a mechanism for inactivating PLU at fertilization in order for S-phase to occur in the zygotic nuclei, it seemed possible that PLU would be modified or degraded at fertilization. To analyze the developmental regulation of PLU, we prepared polyclonal antibodies against the full-length protein expressed and purified from Escherichia coli. The affinity-purified antibody recognizes a single protein of Mr 19,000 in protein extracts from wild-type ovaries (Figure 1A). This protein, which migrates at the predicted molecular weight for PLU, is not detected in extracts prepared from plu2 mutant ovaries. Thus, this is the Drosophila PLU protein.

Protein extracts were prepared from whole ovaries, mature stage 14 oocytes, unfertilized eggs, and 0–3-h
other two alleles lay fertilized eggs in which S-phase is transiently linked to mitosis to produce up to 16 nuclei (Figure 2D). In these embryos, the polar bodies begin to overreplicate while the zygotic nuclei still appear to be undergoing normal S-M cycles. Linkage between the S- and M-phases subsequently breaks down and the zygotic nuclei become polyploid (Figure 2E). In contrast, unfertilized eggs produced by these mutant females contain only single giant polyploid nuclei (Figure 2C). Our interpretation of this phenotype is that these two png alleles are leaky, and that residual png gene activity permits transient coupling of S- and M-phases for the zygotic nuclei, but not the polar bodies. This linkage breaks down after three or four cycles, resulting in multiple polyploid nuclei. The distinct phenotype of the two leaky png alleles was previously used for epistasis tests with plu and gnu. These studies found that plu and gnu are dominantly epistatic to png: even one mutant copy of plu or gnu enhanced the leaky png phenotype, eliminating the linkage between S- and M-phases and resulting in embryos indistinguishable from the one shown in Figure 2F (Shamanski and Orr-Weaver, 1991).

These genetic epistasis tests demonstrated that plu, png, and gnu control the same biological process, but did not distinguish whether the three genes acted in the same regulatory pathway or acted in parallel. To address this question, we examined the level of PLU protein in extracts prepared from png and gnu mutants. Whole ovaries, unfertilized eggs, and embryos were isolated from mothers homozygous for both classes of png alleles. Protein extracts from these col-

**Figure 3.** Expression of PLU protein in png and gnu mutants. Two hundred µg of each protein extract were Western blotted using anti-PLU antibodies. As a control for even loading, blots were subsequently probed with anti-Dmcdc2 antibodies. (A) Extracts were prepared from ovaries, unfertilized eggs, or 0–2-h fertilized embryos derived from wild-type, png<sup>105f</sup> (png<sup>trans</sup>), or png<sup>318</sup> (png<sup>ude</sup>) females. PLU protein was approximately equally abundant in these stages in wild-type females, but the protein was nearly undetectable in strong png mutants. In leaky png mutants, the protein was much more abundant in unfertilized eggs and fertilized embryos than in ovaries. This experiment has been repeated with at least four different sets of protein extracts, with identical results. Embryo extracts were produced by females mated to wild-type males, except in the last lane (*), where png mutant females were mated to sibling png mutant males. (B) Ovary and embryo extracts derived from gnu mutant females were probed with anti-PLU. Protein levels were lower in mutant ovaries than in mutant embryos, and levels in mutant embryos were slightly higher than levels in wild-type embryos.
The levels of PLU protein were dramatically affected by png mutations. PLU was nearly undetectable in ovaries from both leaky and strong png mutants (Figure 3A). Because oocytes from these mutants exhibit no sign of overreplication, PLU must not be required before activation and the completion of meiosis. In contrast, leaky and strong png mutations had differential effects on PLU levels at later stages. PLU levels were significantly higher in mature oocytes and unfertilized eggs from leaky png females than in strong png mutant extracts (Figure 3A; Elfring and Orr-Weaver, unpublished results). At these stages, however, leaky and strong png mutations have the same phenotype: mature oocytes do not overreplicate, whereas unfertilized eggs do. There are two possible reasons for why the PLU protein present in leaky png unfertilized eggs fails to block replication. First, the levels are lower in wild type, so PLU may not be present at sufficient levels. Alternatively, png may have additional, PLU-independent, functions before fertilization.

In fertilized embryos, PLU levels were also significantly higher in leaky png mutants than in strong png mutants (Figure 3A). In leaky png embryos, S- and M-phases are transiently coupled, whereas S-phase occurs in the absence of nuclear division in strong png mutants. Therefore, the PLU protein present in leaky png mutant embryos appears to function to link DNA replication to mitosis in the zygotic nuclei. Nevertheless, the polar bodies become polyplid in these mutants; therefore, as in unfertilized eggs, the residual PLU does not block inappropriate replication of the meiotic products.

To ensure that the altered PLU protein levels were due only to the png mutations and not to other recessive mutations on the png mutant chromosomes, PLU protein levels were also examined on Western blots containing extracts prepared from females carrying leaky and strong png alleles in trans to a deficiency that deletes png. Results similar to those in Figure 3A were obtained (Elfring and Orr-Weaver, unpublished results). Although it seemed unlikely, it was possible that the increased level of PLU in leaky png embryos was derived from their fathers. To address this possibility, we compared PLU levels in leaky png mutant embryos fathered by png mutant males to those of leaky png embryos fathered by wild-type males. Protein levels were equivalent and therefore were not dependent on the genotype of the fathers (Figure 3A).

The alteration in levels of PLU protein in png mutants could be due to differential transcription of the PLU mRNA or to effects on the PLU protein. We examined the level of the PLU transcript in ovaries from png mutant females to determine whether png mutations affected PLU message levels (Figure 4). Despite the dramatic effect of png mutations on PLU protein accumulation, PLU mRNA levels were relatively constant in png mutant females, indicating that png mutations affect a posttranscriptional aspect of PLU regulation. This could represent translational regulation, effects on PLU protein stability, or both.

To determine whether gnu mutations also affected the level of PLU protein, ovari and embryo extracts from gnu mutant mothers were probed with the anti-PLU antibody on Western blots (Figure 3B). As was the case for leaky png mutations, less PLU protein was present in gnu mutant ovaries than in embryos from gnu mutant mothers. However, the level of PLU protein in gnu embryos was slightly higher than that seen in wild-type embryos. Despite the fact that the phenotype of gnu mutations is more severe than that of leaky png mutations (Figure 3B), the levels of PLU protein in gnu mutants were higher than those in leaky png mutants. Therefore, it does not seem that the gnu phenotype is mediated solely through its effects on PLU protein levels.

Interaction with Other Genes that Block Early Cell Cycle Progression

Our experiments showed that PLU protein levels in wild-type extracts are highest in mature oocytes, which are arrested in meiosis I. We were interested in how PLU protein levels might be affected, and whether the overreplication seen in png mutants would still occur, if meiosis were not completed. To address this question, we investigated the effects of blocking development using two maternal-effect mutations, gran (grau) and cortex (cort), in which the second meiotic division is not completed (Page and Orr-Weaver, 1996). We prepared protein extracts of ovaries and arrested embryos produced by females mutant for cort and grau. Immunoblots probed with the anti-PLU antibody showed that PLU protein levels in ovaries and eggs collected from ho-
mozygous grau and cort females were indistinguishable from the wild type (Figure 5A).

Double mutants constructed with cort and a leaky png allele did not overreplicate their DNA; 63 eggs laid by double mutant mothers all showed the cort phenotype of metaphase II arrest. This suggests that activation and the completion of meiosis are required for the onset of the overreplication phenotype of leaky png mutants. In contrast, about half of 144 eggs produced by grau, leaky png double mutants did overreplicate their DNA. In these embryos from png\textsuperscript{33l8}, grau\textsuperscript{QE70} mutant mothers, however, the pronuclei did not undergo mitotic divisions. Thus, although overreplication can occur in this grau mutant, the transient S-M divisions normally seen in png\textsuperscript{33l8} mutants appear to require the completion of meiosis. The distinction observed between grau and cort when doubly mutant with png may reflect the fact that the onset of the grau defect is later than that of cort. Alternatively, the grau\textsuperscript{QE70} allele may be leaky.

It seemed possible that arresting development following the completion of meiosis, but before the onset of the cleavage cycles, would affect the PLU protein. The deadhead (dhd) mutation arrests development immediately following meiosis, but before the embryonic S-M cycles begin (Salz et al., 1994; Page and Orr-Weaver, 1996). Interestingly, the PLU protein in extracts prepared from dhd ovaries and unfertilized eggs migrated as a doublet; about half the PLU protein migrated as in wild-type extracts and the remainder ran as a slightly higher molecular weight band (Figure 5B). dhd encodes thioredoxin, a disulfide reductase with an extremely broad range of targets (Salz et al., 1994). The protein extracts were prepared under reducing conditions, thus it is unlikely that the mobility shift of PLU in thioredoxin mutants directly reflects the absence of the enzyme activity. Rather, the altered form of PLU may be an indirect effect of the stage at which the egg’s development is arrested.

Ectopically Expressed PLU Does Not Perturb the Cell Cycle during Eye Development

Our data suggest that PLU is required very early in embryonic development. The temporally restricted expression of PLU in wild-type embryos is consistent with a model in which PLU functions specifically from the completion of meiosis through early embryogenesis to regulate S-phase. To test the specificity of plu function, we tested whether PLU was capable of affecting a canonical G1-S-G2-M cell cycle when it was ectopically expressed.

To address this question, PLU was expressed in the eye imaginal disc during eye morphogenesis. The patterning of the ommatidia in this nonessential tissue requires precise regulation of the cell cycle (Wolff and Ready, 1993); mutations in cell cycle regulators or overexpression of cell cycle control genes disrupt this pattern, producing a readily observed rough-eye phenotype (Thomas et al., 1994; Richardson et al., 1995). Moreover, expression of the mammalian cyclin-dependent kinase inhibitor p21 during eye morphogenesis was shown to produce rough eyes (de Nooj and Harhara, 1995).

PLU was expressed in the developing eye disc under the control of the GLASS transcription factor using the pGMR vector (Ellis et al., 1993; Hay et al., 1994). We confirmed that PLU protein was induced in the eye discs of the pGMR-PLU transformants using Western blot analysis (Figure 6). The PLU protein from transformant eye discs showed an identical mobility to the protein from ovaries or embryos.

Although PLU protein was expressed in the developing eye disc, it did not affect patterning of the eye. The eyes appeared to be normal when examined by scanning electron microscopy (Figure 7). Additional copies of pGMR-PLU similarly had no effect on eye development (Elfring and Orr-Weaver, unpublished observation). Thus, when ectopically expressed in this developmental context, PLU protein does not appear to be capable of inhibiting S-phase as it does in early embryonic development.

Figure 5. Expression of PLU protein in cort, grau, and dhd mutants. cort and grau mutants arrest in the metaphase of meiosis II, whereas dhd mutants arrest following the completion of meiosis (Salz et al., 1994; Page and Orr-Weaver, 1996). Two hundred \(\mu\)g of each protein extract were immunoblotted and probed with the PLU antibody; Ponceau S staining of the blots was used to ensure even loading. (A) Extracts from ovaries and 0–2-h collections of embryos derived from wild-type, cort\textsuperscript{QE65} and grau\textsuperscript{QE70} females contained approximately equal levels of PLU protein. The ovary extract from grau females was slightly underloaded, as assayed by Ponceau S staining (Elfring and Orr-Weaver, unpublished result). (B) PLU protein in ovaries and embryos from dhd\textsuperscript{08} females migrated as a doublet, whereas wild-type extracts run on the same gel migrated as a single band of 19 kDa.
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**DISCUSSION**

We investigated the developmental regulation of the PLU protein and found that it controls S-phase specifically at the onset of *Drosophila* embryogenesis. Our analysis has molecularly defined the basis of the epistatic genetic interactions between *plu* and *png*. The *png* mutant phenotype inversely correlates with the levels of PLU protein, with strong alleles showing severely depressed protein levels and leaky alleles having depressed levels. In contrast, PLU protein levels are only slightly affected in *gnu* mutants.

The requirement for a specific cell cycle regulator like PLU in the egg and early embryo most likely arises as a consequence of the requirement for rapid development after fertilization. During oogenesis, maternal stockpiles of key cell cycle components are deposited into the developing oocyte. These reserves permit the rapid S-M cycles of early embryogenesis to proceed without zygotic gene expression (Edgar and Schubiger, 1986). The maternal cell cycle stockpiles, however, may make the oocyte particularly vulnerable to inappropriate DNA replication once the egg has been activated and the meiotic products have entered interphase. Regulators that can inhibit S-phase, such as PLU, *png*, and *gnu*, may be critical at this developmental transition.

In addition to blocking inappropriate DNA replication following meiosis, PLU is likely to control the early S-M divisions. The PLU protein is not immediately degraded after fertilization and is present during the rapid nuclear divisions. We found that in leaky *png* mutants, in which the zygotic nuclei go through several rounds of mitosis before becoming polyplloid, PLU protein is present, although at reduced levels relative to wild type. In contrast, in strong *png* mutants PLU protein levels are severely reduced, and S-phase is completely uncoupled from mitosis and nuclear division. These results imply that PLU serves to link S-phase with nuclear division in the zygotic nuclei of the fertilized embryo.

In fertilized embryos from leaky *png* mothers, the transient linkage of S- and M-phases correlates with the presence of some PLU protein. In contrast, unfertilized eggs from leaky *png* mutants overreplicate despite the presence of equivalent levels of PLU. One explanation for this discrepancy is that *png* could have additional targets that also function in keeping replication turned off before fertilization. A second explanation is that the reduced levels of PLU protein could simply be insufficient to inhibit replication in unfertilized eggs. In fertilized embryos from leaky *png* mutants, there may be initial proper S-M cycling because fertilization results in a localized signal (perhaps the centrosome) that enables the zygotic nuclei to respond to the reduced levels of PLU. If this is the case, it may be more accurate to think of PLU as a factor that...
promotes mitosis rather than an inhibitor of replication.

png mutants demonstrate the differential sensitivity of the polar bodies and pronuclei to PLU protein and emphasize the developmental distinctions between these nuclei. In fertilized embryos from plu, png, or gnu mutant mothers the polar bodies initially appear to overreplicate to a greater extent than the pronuclei, although the pronuclei do subsequently become polyploid (Carminati, 1995; Burney and Orr-Weaver, unpublished observation). This is most extreme in the leaky png mutants, in which the pronuclei actually go through several S-M cycles before becoming polyploid.

Given that the polar bodies and pronuclei share the same cytoplasm and presumably are exposed to the same levels of PLU in the leaky png mutants, why do the polar bodies undergo aberrant replication earlier or more rapidly? One possibility is that PLU is not uniformly distributed in the egg. In the wild-type embryo, the polar bodies remain arrested while the zygotic nuclei cycle through the S- and M-phases. Therefore, PLU might be predicted to be most concentrated around the polar bodies. To date, we have been unable to detect localized PLU with our antibodies, therefore this hypothesis cannot be excluded (Burney, Elfring, and Orr-Weaver, unpublished observations).

Another possibility, consistent with the model that PLU plays a role in promoting mitosis, is that the proximity of the centrosome to the zygotic nuclei influences the pronuclei and impedes inappropriate replication or promotes mitosis, provided sufficient PLU protein is present.

PLU protein diminishes in unfertilized eggs and is no longer present 4 h after fertilization. It appears that the degradation of PLU is a regulated process triggered by complete activation; the degradation of PLU also may be augmented by fertilization. PLU is stable in gnu and corf mutants, which do not complete all of the events of activation. However, in dhd mutants, which arrest after the completion of meiosis, modified forms of PLU appear. PLU protein with the same altered mobility can sometimes be detected in strong png mutants (Elfring and Orr-Weaver, unpublished observation). Recovery of modified PLU in png mutants is variable but always correlated with greatly reduced levels of PLU. Thus, we postulate that the modified protein represents an unstable form of PLU. There is a consensus cyclin destruction box in PLU, suggesting that the protein may be actively degraded, possibly by the cyclin destruction machinery (Glotzer et al., 1991).

Ectopic expression of PLU during the canonical G1-S-G2-M cycles in the developing eye disc did not affect the patterning of the ommatidia and bristles. Thus, PLU did not perturb the cell cycle or timing of these mitotic divisions. We confirmed that in these experiments PLU protein was expressed and exhibited an identical electrophoretic mobility to PLU from ovaries or early embryos. We cannot exclude the possibility that ectopically expressed PLU is not active because it requires additional cofactors or posttranslational modification, but we favor the interpretation that PLU is capable only of controlling the specialized S-phase of early embryos. Several unique aspects of DNA replication at this developmental time might necessitate specific regulation: the onset of S-phase is controlled posttranscriptionally (Edgar and Schubiger, 1986), S-phase is extremely rapid (Poe et al., 1993), and replication origins are about 10 times more frequent than in later mitotic cycles (Blumenthal et al., 1973).

In epistasis tests, plu, png, and gnu were shown to control the same process (Shamanski and Orr-Weaver, 1991). A functional png gene is necessary for the presence of PLU protein, and leaky png alleles retain some PLU protein. It is possible that the PNG protein controls the expression of PLU protein at a posttranscriptional level. Alternatively, PNG may be needed to stabilize PLU, possibly because they are in a complex. For example, when some components of the yeast SWI/SNF complex are mutated, other proteins in the complex become unstable (Peterson and Herskowitz, 1992).

Mothers mutant for gnu have phenotypes equivalent to strong png mutants or null plu mutants. However, PLU levels were slightly higher in fertilized embryos from gnu mutant mothers than in wild-type embryos and only slightly depressed in gnu mutant ovaries. Therefore, the gnu mutant phenotype cannot be the consequence of absence of PLU protein (although, like plu, gnu is epistatic to png). gnu could activate PLU protein independently of png, with only slight effects on the levels of PLU protein (Figure 8A).

Another possibility is that gnu is at the bottom of a regulatory hierarchy, below PLU (Figure 8B). The reduction of PLU in gnu mutant ovaries might then arise by a feedback loop.

Our results suggest that png acts through PLU and that PLU is specific in controlling S-phase only early in Drosophila development. It should be emphasized that these data do not restrict the role of png or gnu to embryogenesis. In addition to regulating PLU in the oocyte and early embryo, these genes could control other S-phase regulators later in development and regulate additional cell cycles as well as the S-M cycles. Although all five png alleles and the single gnu allele are strict maternal-effect mutations, these may not be null alleles; therefore, it is possible that these genes could function later in development.

A key to understanding how PLU controls S-phase at activation and during the early S-M cycles will be to identify the targets of PLU and to understand its potential influence on known cell cycle regulators. Since png controls PLU protein it will be interesting to iden-
The PNG protein. Identification of GNU may similarly facilitate its placement in the regulatory hierarchy of these three genes. It will be important to determine whether png and gnu show the same developmental specificity as plu or whether they are more promiscuous in the types of cell cycles they are capable of controlling.

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