The inhibitor of DNA replication encoded by the *Drosophila* gene *plutonium* is a small, ankyrin repeat protein

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The *plutonium* (*plu*) gene product controls DNA replication early in *Drosophila* development. *plu* mutant females lay unfertilized eggs that have undergone extensive DNA synthesis. In fertilized embryos from *plu* mutant mothers, S-phase is uncoupled from mitosis. The gene is expressed only in ovaries and embryos, null alleles are strict maternal effect mutations, and the phenotype of inappropriate DNA replication is the consequence of loss-of-gene function. *plu* therefore negatively regulates S-phase at a time in early development when commitment to S-phase does not depend on cyclic transcription. *plu* encodes a protein with two ankyrin-like repeats, a domain for protein–protein interaction. *plu* is immediately adjacent to, but distinct from, the *PCNA* gene.

Key words: ankyrin/cell cycle/DNA replication/*Drosophila*/mitosis

Introduction

DNA replication in eukaryotes is regulated in three ways: (i) all of the genome is replicated once, and only once, per cell cycle; (ii) S-phase is coupled with cell growth and division; and (iii) during the development of multicellular organisms there are changes in the coordination of S-phase with other cell cycle events in response to developmental signals. The control that ensures the genome is replicated only once is most likely exerted at DNA replication origins. This can be accomplished by an adequate distribution of origins such that replication of the genome can be completed within one S-phase, and by activating each origin only once during a given S-phase. The mechanism that blocks multiple initiations at an origin during DNA replication is not understood. It is assumed that reinitiation of replication while synthesis is ongoing is blocked because a triggering mechanism must be reset by a mitotic event. One attractive hypothesis proposes that a 'licensing factor' essential for the initiation of replication at origins can only gain access to the nucleus during nuclear envelope breakdown at mitosis, and that the factor is inactivated after initiation (Blow and Laskey, 1988). This model arose from experiments conducted with *Xenopus* extracts showing that only one

round of replication took place in added nuclei, but that permeabilization of the nuclear envelope would permit another round of DNA synthesis (Blow and Laskey, 1988). Licensing factor is at present hypothetical, and remains to be identified. However, several yeast proteins required for DNA replication do show the cell cycle fluctuations in their cellular location predicted for a licensing factor (Hennessy *et al.*, 1990; Nasymth *et al.*, 1990; Chen *et al.*, 1992).

In the G₁-phase of the cell cycle there is a point at which the cell becomes committed to undergo DNA replication. This is known as START in the yeast *Saccharomyces cerevisiae* and the restriction point in mammalian cells. This regulatory point serves to couple S-phase with growth, nutrient conditions and response to mating pheromone in yeast. It is the response point for growth factors, cell density and cell size in mammalian cells. Considerable effort has been devoted to elucidating the control of the G₁/S transition (Roberts, 1993). Key regulators in *S.cerevisiae* are the kinase encoded by the *CDC28* gene and the G₁/S *CLN* cyclins. In mammalian cells the cyclin dependent kinases (CDKs) are active at the G₁/S transition.

Passage through START or the restriction point marks the commitment to S-phase, but DNA replication does not immediately follow. The intervening controls that actually trigger the initiation of DNA synthesis remain to be isolated, although candidates have been identified. In *S.cerevisiae* several genes have been shown to act between START and S-phase (Hartwell, 1976). Some substrates of the CDKs could be critical for the initiation of replication. Two replication proteins, SV40 T antigen and RPA are CDK substrates, and the polymerase- δ accessory factor PCNA is found in a complex with cyclin D (McVey *et al.*, 1989; D'Urso *et al.*, 1990; Dutta and Stillman, 1992). The initiation of S-phase does not have to be positively regulated, it could be tripped by inactivation of an inhibitor (Roberts, 1993). In the Chinese hamster ts41 mutant cell line at the non-permissive temperature S-phase repeatedly occurs without the intervening G₂-, M- and G₁-phases (Handeli and Weintraub, 1992). This suggests that the gene inhibits entry into S-phase. In *Schizosaccharomyces pombe* certain temperature sensitive *cdc2* mutants can be induced to inappropriately re-enter S-phase by temperature shifts (Broek *et al.*, 1991).

DNA replication is regulated in response to developmental events in multicellular organisms. Fertilization is necessary for restarting the cell cycle. The signals inducing the first S-phase are not understood. In *Xenopus* and *Drosophila*, S-phase is regulated differently in the early divisions in the embryo than in later development. The early divisions have a modified cell cycle in which S-phase immediately follows mitosis without an intervening gap phase or a G₁/S decision point (Foe and Alberts, 1983). Since abundant maternal transcripts are stockpiled in these embryos and expression of the embryo's own genome is not required for the early divisions, gap phases are not necessary to permit

transcription of replication components. S-phase is very rapid during these early cycles, partly due to closer spacing between replication initiation sites (Blumenthal *et al.*, 1973). Despite the rapidity of the cycles, the block to reinitiation of replication is present (Blumenthal *et al.*, 1973; Harland and Laskey, 1980).

Understanding the regulation of DNA replication in multicellular organisms will require not only the identification of regulators for the entry into S-phase and for replication within an S-phase, but also elucidation of the interface between developmental signals and phases of the cell cycle. Three interacting maternal effect genes, *gnu*, *pan gu* (*png*) and *plutonium* (*plu*) regulate DNA replication in early *Drosophila* development (Freeman *et al.*, 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). Our analysis of *plu* indicates that the gene product inhibits S-phase and is necessary for nuclear division. Since few repressors of DNA replication have been identified in eukaryotes, it is important to understand the mechanism by which *plu* controls DNA replication. Here we describe the cloning of the *plu* gene and demonstrate that it encodes a protein with two ankyrin-like repeats. The gene only controls DNA replication early in development, since null alleles are strict maternal effect mutations, and the transcript is detectable only in ovaries and early in embryogenesis.

Results

Drosophila females that have mutations in the *plu* gene produce unfertilized eggs and embryos that have giant, polyploid nuclei (Figure 1). In these fertilized embryos DNA replication occurs, centrosomes divide and organize asters, but nuclear division does not take place (Shamanski and Orr-

Fig.1. Phenotype of *plu*. The embryo derived from an homozygous *plu*³ mutant mother (A) has undergone extensive DNA replication without nuclear division to form fused, giant, polyploid nuclei from the male and female pronuclei and from the polar body nuclei. The polar bodies (arrow) remain condensed throughout embryogenesis in the wild-type (B). The wild-type embryo in (B) is oriented with anterior to the left and dorsal down. Embryos were stained with the DNA-binding fluorochrome DAPI. Bar is 50 μ m.

Weaver, 1991). Thus, inappropriate DNA replication occurs in the unfertilized egg, and in the fertilized embryo S-phase is not coupled to mitosis. This replication does not initiate prior to egg activation, because stage 14 oocytes from mutant mothers have an apparent diploid set of chromosomes that are arrested at metaphase I of meiosis (data not shown). Meiosis can be completed normally; in early unfertilized eggs or embryos the four meiotic products can be seen as separate polyploid nuclei. As their ploidy increases, these nuclei often fuse into a single, giant nucleus.

Cloning of the plutonium gene

We used a transposon inserted within *plu* as a tag to isolate the gene. The initial allele of *plu* was recovered from a hybrid dysgenic screen and thus was likely to contain a P-element insertion within or near the gene. The *plu*¹ stock contained a P-element insertion at 56F, the cytological location of *plu*, as well as at least five other P-element inserts. To show that the P-element at 56F was responsible for the *plu* mutant phenotype, we reverted the *plu*¹ mutation by placing it in the presence of a strong source of transposase (Robertson *et al.*, 1988), and found that restoration of fertility was accompanied by excision of the P-element from 56F.

The genomic interval containing the *plu* gene was isolated from a lambda library constructed from the *plu*¹ strain by hybridization with a P-element probe. One of these phage, which hybridized *in situ* to 56F, was used to isolate homologous cosmids from a wild-type library (Tamkun *et al.*, 1992). The P-element insertion site was localized by

Fig. 2. Map of the *plu* region. The restriction map for enzymes *Bam*HI (B), *Bgl*II (G), *Eco*RI (E) and *Sal*I (S) is shown at the top. The *plu*¹ P-element is inserted between the internal *Eco*RI site and the polymorphic *Bam*HI site (at nucleotides +283 and +486, respectively, in Figure 4). For clarity, *Sac*I (C) sites in the genomic DNA have been omitted. In the center are *plu*, *PCNA* and two other distinct transcription units (A and B) with their respective directions of transcription arrowed. Intron/exon structure and exact limits of A and B are not shown. Below are four restriction fragments that, when reintroduced into the germline by P-mediated transformation, do (+) or do not (–) rescue *plu* mutants. G3.8RKO is a derivative of G3.8 where the *plu* ORF was frameshifted after codon 62 by elimination of the internal *Eco*RI site (X).

restriction mapping and Southern analysis. The genomic DNA sequences adjacent to the P-element were determined by sequencing across the junctions of genomic and P-element sequence (see Figure 2 and Materials and methods). The P-element appears to be full length, although an internal deletion of 100 bp or less would not have been detected by our analysis.

Identification of the plutonium transcript

cDNAs for *plu* were isolated from a *Drosophila* ovary library and from a 0–4 h embryonic library (see Materials and methods), and they were localized on the genomic map (Figure 2). This interval is densely packed with transcription units, the adjacent transcripts being separated by only several hundred base pairs. Several lines of evidence indicate that the 0.7 kb transcript spanning the P-element insertion site is the *plu* transcript. The insertion of the P-element in the middle of the transcript unit would be expected to affect the transcript, either quantitatively or qualitatively, an expectation confirmed below. Moreover, an inversion that mutates the *plu* gene has a breakpoint that also mapped within the ~200 bp *EcoRI*–*Bam*HI fragment (Figure 2, and data not shown). The other breakpoint of *In(2R)plu* maps to 49C–D. Interestingly, the transcript on the 3' side of the *plu* transcript has been previously identified as the replication factor PCNA (Yamaguchi *et al.*, 1990). The *plu* cDNAs are considerably less abundant than those of the flanking genes: 364 clones of transcript B, 158 of *PCNA*, 106 of transcript A and 11 of *plu* were detected in the 0–4 h embryonic library.

To confirm the identity of the *plu* transcript, we performed a set of transformation rescue experiments. In transformed stocks, the 6.5 kb *Bam*HI fragment complemented the female sterility of the *plu*² and *plu*⁶ mutations, but strains containing

the 5.2kb *Eco*RI fragment which breaks within the putative *plu* transcription unit still exhibited the mutant phenotype (B6.5 and E5.2 in Figure 2). Both of these constructs were shown to complement the lethality of *mus209* stocks that are mutated in the *PCNA* gene (D.S.Henderson, S.S.Banga, T.A.Grigliatti and J.B.Boyd, submitted). Transformants were generated containing a 3.8 kb *Bgl*III fragment that lacks most of the *PCNA* gene, and this fragment restored *plu* function to the *plu*², *plu*³ and *plu*⁶ mutant strains (G3.8 in Figure 2). Finally, we filled in an *Eco*RI restriction site to create a frameshift in the putative *plu* open reading frame (ORF) and transformed flies with this derivative of the *Bgl*III fragment. Transformants containing this construct failed to rescue any of the three mutations in the *plu* gene tested (G3.8RKO in Figure 2). These experiments not only identify the *plu* transcript, but they also show that *plu* is a distinct gene from *PCNA*.

RNA blots showed that three of the mutations affect the *plu* transcript and that at least two of the *plu* mutations are null alleles. A specific *plu* RNA probe was used to detect the *plu* transcript in polyadenylated RNA isolated from ovaries of wild-type or *plu* mutant females (Figure 3A). There was a single transcript of 0.7 kb present in RNA from wild-type, *plu*² and *plu*³ females. No transcript was detected in ovary RNA from *plu*¹ or *plu*⁶ females, and a transcript of increased size was observed in RNA isolated from *In(2R)plu/Df(2R)M173* heterozygous females. None of the other transcripts in the *plu* region (illustrated in Figure 2) are affected by *plu* mutations (data not shown).

plutonium is only expressed early in development

The *plu* transcript is only present in ovaries and during early embryogenesis. A developmental Northern blot with

Fig. 3. Expression of the *plu* gene. (A) *plu* mutations disrupt the maternal *plu* transcript. Poly(A)⁺ RNA from ovaries of wild-type, homozygous *plu* mutant and *In(2R)plu/Df(2R)M173* female flies was blotted and sequentially probed with *plu* and then with *PP1 87B* (Dombrádi *et al.*, 1989) to control for loading. (B) Developmental expression of *plu*. This RNA blot comprises poly(A)⁺ RNA from embryos at various times after egg deposition, first, second and third larval instars, two pupal stages, female and male adults and the Schneider L2 cell line. This blot was hybridized as described above.

poly(A)⁺ RNA from several stages of embryogenesis, first, second and third instar larvae, early and late pupae and adult males and females was hybridized with an RNA probe for the *plu* transcript (Figure 3B). Only a single transcript of 0.7 kb was present in adult females, and this was found until between 6 and 12 h of embryogenesis. The transcript in adult females most likely derives from transcription in the ovary. Thus the gene is expressed early in development, and multiple forms of the transcript are not present. Additionally, *plu* transcripts were not detected in the Schneider L2 cell line, originally derived from embryonic cells (Schneider, 1972).

plutonium encodes a protein with ankyrin-like repeats

The DNA sequence of cDNAs and the genomic DNA for the *plu* gene was determined (Figure 4). We found that five cDNAs extend from nucleotide +2 to +739 and one shorter variant, cDNA11, extends from nucleotide +35 to +749. In primer extension experiments we observed three transcriptional start sites, all in close proximity to the 5' ends of the longer cDNAs (Figure 4). This, together with the observation that the size of the transcript on Northern blots is the same size as the cDNAs, indicates that most of the cDNAs are full length. Comparison of the cDNA sequences with the genomic sequence shows that the gene contains two introns of 58 and 54 bp located as shown in Figure 4. Splice donor and acceptor sites are in good agreement with the consensus sequences for short *Drosophila* introns (Mount *et al.*, 1992). At nucleotide +724 there is a canonical polyadenylation signal (Wickens, 1990). The poly(A) tails of the two classes of cDNA begin after 10 and 20 nucleotides 3' to this signal, respectively.

The *plu* gene is predicted to encode a protein of 174 amino acids with a molecular weight of 19 328 Daltons and a pI of 9.7 (Figure 4). The cDNAs contain no other large ORFs. The four nucleotides preceding the initial ATG codon at +45 fit well with the consensus for *Drosophila* initiation codons (Cavener, 1987). Given the primer extension data, it is unlikely that any of the *plu* coding region lies upstream of this site. This conclusion is further reinforced by the observation that a stop codon lies in frame, five codons 5' to the N-terminal methionine.

The BLAST search program (Altschul *et al.*, 1990) shows Plu to be most similar to ankyrin proteins. The region of homology is a 33 amino acid domain that is repeated 22 times in the human ankyrin protein (Lux *et al.*, 1990). This ankyrin repeat has been found in a wide range of eukaryotic proteins that fulfill diverse biological functions (Figure 5A). The Plu protein has two full ankyrin repeats and weaker homology to a third (Figure 5B).

The presence within the Plu sequence of several clusters of basic amino acids raises the possibility that the protein might be capable of nuclear localization (for review see Garcia-Bustos *et al.*, 1991). Robbins *et al.* (1991) proposed that the bipartite nuclear localization signal found in nucleoplasm is generally present in many proteins that enter through nuclear pores. Such a signal would minimally comprise a structure (B)₂(X)₁₀(B)₃₋₄, where B represents arginine or lysine and X other residues. The 16 C-terminal residues of the Plu sequence are 159KR(X)₁₀NRLR, one basic residue short of the consensus.

We also scanned the sequence (Fuchs, 1991) for clues as to how Plu inhibition of DNA replication might be modulated by the signals of fertilization and mitosis via calcium or

Fig. 4. Sequence of *plu*. Genomic sequence was derived from the clone G3.8 that rescues *plu* mutants. cDNA sequence is that of cDNA 1. The extent of cDNAs 1 and 11 is indicated with '+' over the sequence. Nucleotide +1 is the 5' end of the shorter of two equally abundant primer extension products, marked '+'. The first nucleotide shown (-13) is the 5' end of a third extension product which is much less abundant (not shown). Coding DNA is capitalized to show the two small introns. The region similar to ankyrin is underlined, with slash marks to delimit the repeats shown in Figure 6. Strain-specific nucleotide polymorphisms are overlined and amino acid residues are in parentheses where these polymorphisms would result in altered protein sequence. A candidate polyadenylation signal is underlined. The vertical line after nucleotide +400 marks the insertion site of the P transposable element causing the *plu*¹ mutation. The asterisks indicate stop codons. The nucleotide sequence number is shown on the right and the amino acid number on the left.

protein phosphorylation. There is nothing apparent in the Plu sequence to suggest binding of calcium or calmodulin. Protein kinases have been shown in many cases to exhibit sequence dependent substrate specificity (Pearson and Kemp, 1991). According to this model, 94RRRS is a theoretical substrate for cAMP dependent protein kinase, and the C-terminal 16 amino acids include the sequence 164SP, which we are investigating as a candidate for phosphorylation by the p34^{cdc2} mitotic histone kinase. There are also three possible sites for phosphorylation by casein kinase II and three for protein kinase C.

Sequence of *plutonium* mutations

To characterize the mutations in the *plu* gene we used the polymerase chain reaction (PCR) to amplify genomic DNA from flies homozygous for *plu*³, the fertile *plu*¹ revertant J4, *plu*², its unmutagenized progenitor chromosome *cn bw sp* 'iso1' and the wild-type Canton-S strain. The amplified DNA was then directly sequenced. The standard genomic

Fig. 5. Plu protein contains repeated ankyrin motifs. (A) Identical amino acids from various protein sequences aligned to Plu by the BLAST search program are displayed. Matching proteins are listed in order of decreasing similarity to Plu. Occasionally several regions of one protein were found to match. The putative Plu protein shares significant regions of amino acid identity with human erythrocyte ankyrin ($P = 2.6 \times 10^{-9}$) (Lux *et al.*, 1990), the *C.elegans* sex determination protein fem-1 ($P = 3.9 \times 10^{-4}$) (Spence *et al.*, 1990), Shope rabbit fibroma virus ORF H3R ($P = 1.1 \times 10^{-3}$) (Massung *et al.*, 1992), *S.pombe* cell cycle regulator res1/sct1 ($P = 1.5 \times 10^{-3}$) (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993), *Xenopus laevis* EGF repeat protein Xotch ($P = 1.9 \times 10^{-3}$) (Coffman *et al.*, 1990), Human mediator of interferon action 2-5A dependent RNase ($P = 3.6 \times 10^{-3}$) (Zhou *et al.*, 1993), B-cell lymphoma gene BCL3 ($P = 1.4 \times 10^{-2}$) (Ohno *et al.*, 1990) and the *Drosophila melanogaster* regulator of ventral cell fate Cactus ($P = 1.7 \times 10^{-2}$) (Geisler *et al.*, 1992; Kidd, 1992). The consensus represents agreement in 50% or more of the aligned sequences. (B) The regions of ankyrin homology within Plu comprise two contiguous ankyrin-like motifs of 33 amino acid residues from residues 33 to 65 and 66 to 98. A third contiguous region, from 99 to 131 shows a similar structure, but with fewer matching residues. Plu sequence was optimally aligned with itself using the Dotplot Align program (DNA Star). Identities are represented by vertical lines, conservative substitutions (Dayhoff *et al.*, 1983) are indicated by asterisks. Identity of two out of three aligned residues was taken as the Plu consensus sequence shown below. The ankyrin consensus is taken from Lux *et al.* (1990).

sequence of clone G3.8 was also from the lethal-free wild-type line *y; cn bw sp 'isol'* (Tamkun *et al.*, 1992). cDNA 1 was derived from an isogenic *dp cn bw* stock (Brown and Kafatos, 1988).

Figure 6 shows the mutations that disrupt *plu* function. *plu*¹ is a null mutation (Figure 3) but *plu*² and *plu*³, if translated normally, should produce truncated proteins missing C-terminal sequences. The observation that the phenotype of these alleles is indistinguishable from the null suggests strongly that the C-terminus, and not just the ankyrin domains, is essential for protein stability or function *in vivo*.

A number of polymorphisms are present in these stocks (Figure 4). All strains tested contained the sequence 392CC. Yamaguchi *et al.* (1990) reported part of what we have identified as the *plu* sequence in a *PCNA* clone from the wild-type Oregon-R strain, and in that sequence these two nucleotides are 392GG. This difference would result in a 97S→W change in the *plu* ORF of Oregon-R. This substitution suggests that the serine residue in the sequence discussed above (94RRRS) is dispensable for *plu* function. This change would also alter a conserved position within the second ankyrin repeat (Figure 5B). A set of three silent substitutions in the third position of three adjacent codons yields 411C, 414T, 417T in Oregon-R, *plu*¹, *plu*¹ revertant J4, *plu*³ and *dp cn bw*, whereas Canton-S, *cn bw sp 'isol'* and *plu*² have 411G, 414C and 417G. The polymorphic *Bam*HI site shown in Figure 2 is absent from *cn bw sp 'isol'* and *plu*². However, the change 490T→C is present in

Canton-S, Oregon-R, *dp cn bw*, *plu*¹, *plu*¹ rev J4 and *plu*³, creating the *Bam*HI site as well as causing a predicted coding change of 130S→P. The second intron contains several polymorphisms: 561A→T is present only in Canton-S; 568TT in *cn bw sp 'isol'* and *plu*² is replaced with 568TGTAATC in Oregon-R, *plu*¹ rev J4 and *plu*³, while in Canton-S the sequence is 568TGTAATT. Thus, the second intron may be either 54 or 59 nucleotides long without affecting *plu* function. Overall, *plu*¹ and its derivatives appear, on the basis of these polymorphisms, to be more closely related to Oregon-R than to Canton-S.

Discussion

We have isolated the *plutonium* gene and have shown that this essential gene inhibits DNA replication early in *Drosophila* development. The *plu* gene encodes a small protein that has two ankyrin repeats. The *plu* gene was identified by the presence of a P-element within the coding sequence in one allele, and by alterations of the coding sequence in two other *plu* mutations. Transformed flies bearing a DNA fragment containing this gene rescued the *plu* mutant phenotype, while transformants with the same fragment, but in which the ORF had been mutagenized, failed to complement *plu* mutations.

The genomic region containing plutonium

It is striking that the *plutonium* gene is separated from the gene encoding an essential replication protein, PCNA, by

Fig. 6. Sequence of *plu* mutations. In the *plu*¹ sequence, insertion of a P-element disrupts the *plu* coding region. A block of 8 bp of genomic DNA duplicated at the P-insertion site (O'Hare and Rubin, 1983) is indicated with the number 1. *plu*³ is a derivative of *plu*¹ resulting from imprecise excision of the P-element leaving 20 bp of P-element terminal inverted repeat sequence (overline arrows). *plu*³ also includes an additional duplication of genomic sequence (1 and 2) to give a total insertion of 48 bp of extraneous DNA. A TGA termination codon (*) within the P sequence is predicted to terminate the *plu* ORF after one missense residue 100D→A. Comparison of the unmutagenized *cn bw sp* chromosome and EMS-induced *plu*² allele revealed only the 577G→A mutation. If spliced as shown, this would result in a +1 frameshift predicted to terminate the Plu2 protein after 11 missense codons.

<150 bp. PCNA is an accessory factor for DNA polymerase- δ that increases the processivity of the enzyme (Bravo *et al.*, 1987; Prelich *et al.*, 1987). Mutations in the *mus209* (*mutagen sensitive*) gene have been shown to lie in the *Drosophila* PCNA gene (D.S.Henderson, S.S.Banga, T.A.Grigliatti and J.B.Boyd, submitted). These mutations are zygotic lethal, demonstrating that the functional gene must be expressed in the zygotic genome during development for viability (D.S.Henderson, S.S.Banga, T.A.Grigliatti and J.B.Boyd, submitted). Conditional lethal alleles at the permissive temperature are mutagen sensitive, and they are female sterile due to defects in oogenesis.

Several results establish that, despite their close linkage, *plu* and *mus209* are distinct genes. The *plu*¹, *plu*², *plu*³ and *plu*⁶ alleles fully complement the two lethal alleles of *mus209*: D292 and 775 (data not shown). The *plu* and PCNA transcripts are separate, and despite extensive screening no cDNAs were found to overlap both genes. A genomic fragment that contains the PCNA gene, but includes only the 3' two-thirds of the *plu* gene, rescues *mus209* but fails to rescue *plu* mutants, while a fragment with the *plu* transcription unit but only 15 amino acid residues of PCNA complements only *plu*. Most importantly, none of the three nearby transcripts were affected by the mutations that abolished *plu* expression.

Genes regulating the same biological process are in some cases clustered to ensure their coordinate expression. However, the PCNA gene is expressed at highest levels in early development but at a low constitutive level throughout development (Yamaguchi *et al.*, 1990). This is in contrast

with the *plu* gene which is expressed only in ovaries and until 6 h of embryogenesis. Moreover, another gene (B in Figure 2) that is divergently transcribed from *plu* and whose 5' end is separated from the 5' end of the *plu* gene by ~300 bp is abundantly expressed throughout development (data not shown). These differences may result from differential message stability. Alternatively, transcriptional control signals for each gene may be small or embedded in the flanking genes. Indeed, Yamaguchi *et al.* (1990) proposed that PCNA expression is regulated by binding of homeodomain transcription factors within what we have shown to be the second intron and 3' exon of *plu*.

plutonium encodes an ankyrin repeat protein

The 33 amino acid ankyrin repeat sequence has been identified in a variety of eukaryotic proteins, such as transcription factors, cytoplasmic regulators of transcription factors, integral membrane receptors and proteins linking integral membrane proteins to the cytoskeleton (Blank *et al.*, 1992; Nolan and Baltimore, 1992). The proteins containing ankyrin-like repeats share the common property that they bind to other proteins, and thus it is likely that the ankyrin motif may mediate protein-protein interactions. The ankyrin repeats have been shown to be required for the interaction of the GA binding protein β subunit with the α subunit, and in the I κ B family the ankyrin repeats of the Cactus and pp40 proteins are necessary for their binding to dorsal and the NF κ B p50-p65 heterodimer, respectively (Thompson *et al.*, 1991; Inoue *et al.*, 1992; Kidd, 1992). The ankyrin repeats of Cactus have been shown to be sufficient for its

interaction with Dorsal (Kidd, 1992). The Swi4 and Swi6 proteins of *S.cerevisiae* interact to synchronize gene transcription with the cell cycle, but the ankyrin repeats of these proteins are dispensable for their binding to each other (Andrews *et al.*, 1992). Nevertheless, these motifs may permit the binding of Swi4 and Swi6 to other regulatory proteins.

Plu represents a new class in the family of ankyrin repeat proteins. The other family members have four or more tandemly repeated copies of the motif or two separated copies, as in Swi4 and Swi6 (Blank *et al.*, 1992; Nolan and Baltimore, 1992). The two copies in the predicted Plu protein are adjacent. Multiple ankyrin repeats are needed for the binding of pp40 and Cactus to their Rel family cognates (Inoue *et al.*, 1992; Kidd, 1992). Mutations in four of the five repeats of pp40 tested blocked binding, and a polypeptide containing three of the Cactus repeats was unable to bind to Dorsal. Four temperature sensitive point mutations in *Caenorhabditis elegans* glp-1 and one in lin-12 map to the ankyrin repeats, demonstrating that mutations in individual repeats can affect protein function (Greenwald *et al.*, 1990; Kodoyianni *et al.*, 1992). It will be of interest to determine the significance of the copy number and spacing of the repeats within the members of this protein family. For instance, these features might dictate the stoichiometry of interacting proteins.

It is likely that, like other members of this family, Plu interacts with other proteins. We speculate that it may inhibit DNA replication by binding to and blocking the action of an essential replication factor. If Plu is nuclear it could bind to and block the action of an initiation factor. However, if the protein is cytoplasmic, it could, by analogy with I κ B, sequester a replication factor. Binding of I κ B family members to Rel proteins blocks the nuclear localization signal, preventing translocation of the Rel proteins to the nucleus (Blank *et al.*, 1992; Nolan and Baltimore, 1992). A test of this hypothesis awaits the determination of the cellular location of Plu.

plutonium only inhibits DNA replication early in development

Our genetic analysis of the *plu* locus suggested that the action of the gene was restricted to early development, since all of the alleles we obtained were strict maternal effect mutations both as homozygotes and *in trans* to a deficiency. If the gene were essential to regulate the cell cycle throughout development then null alleles would be zygotic lethal mutations. Here we have shown that at least two of the maternal alleles are null mutations that fail to produce detectable transcripts. Furthermore, transcripts are only present in ovaries and during the first third of embryogenesis. While it is formally possible that the protein is extremely stable, persisting through the 12 days of development, it is most likely that *plu* does not regulate DNA replication during the cell cycle in the larval and pupal stages.

plu controls DNA replication in the unfertilized egg, and in the absence of the gene product inappropriate DNA synthesis occurs. In fertilized embryos, giant, polyploid nuclei are also found. The Plu protein is likely to be required after fertilization to ensure that DNA replication and nuclear division are linked. The *plu* transcript persists after fertilization of the egg, and in mutant embryos lacking the transcript, the incoming male pronucleus is also subject to

loss of replicative control. Following fertilization, the action of *plu* is limited to the syncytial nuclear divisions, or possibly continuing through the three post-blastoderm divisions, since the transcript is no longer present after their completion.

Why would a negative regulator of DNA replication exist only early in development and not function in later cell cycles? The developmental period in which *plu* acts is remarkable in that DNA replication is not transcriptionally regulated. Transcription of most zygotic genes does not begin until cellularization, so that the early syncytial divisions are controlled by maternal components. Zygotic transcription does occur during the three post-blastoderm divisions, but these divisions are unusual in that there is a G₂-phase but no G₁-phase to the cell cycle (Edgar and O'Farrell, 1990). S-phase immediately follows mitosis, precluding transcriptional regulation as a means for controlling entry into S-phase. Therefore, during the first third of embryogenesis when the syncytial and post-blastoderm divisions take place, entry into S-phase must be regulated post-transcriptionally. The model in which Plu binds to and inhibits an initiation factor provides a mechanism for the post-transcriptional triggering of S-phase required at this point in development.

The role of plutonium in inhibiting DNA replication

The Plu protein could inhibit DNA replication by blocking entry of nuclei into S-phase, by inhibiting reinitiation at origins within one S-phase, or by triggering exit from S-phase, possibly by permitting chromosome condensation. The first model predicts that DNA synthesis in the mutant embryos would be cyclic, while in the other two models it would be expected to be continuous. Experiments in which *plu* mutant embryos were pulse-labeled with BrdU suggested that DNA replication was cyclic (F.Shamanski and T.Orr-Weaver, unpublished results). Thus we favor the hypothesis that the Plu protein blocks entry into S-phase and when mutated leads to multiple S-phases, analogous to the mammalian ts41 mutation (Handeli and Weintraub, 1992).

Although the Plu protein inhibits DNA synthesis in the unfertilized egg, the exact point at which this repression is exerted remains to be determined. In the *Drosophila* unfertilized egg both meiotic divisions are completed, but it is not known whether the first S-phase can take place prior to fertilization or if it is dependent on fertilization. Based on the cytological appearance and intensity of DAPI staining of polar body chromosomes it is unlikely that more than one round of S-phase occurs in the unfertilized egg. Therefore, the Plu protein either blocks entry into the first S-phase in the unfertilized egg, making it dependent on fertilization, or it limits DNA synthesis to a single round of S-phase.

In unfertilized eggs and fertilized embryos from *plu* mutant mothers, all four meiotic products undergo extensive DNA replication and become polyploid. Since the polar body chromosomes in wild-type embryos remain arrested in a condensed state, it is likely that the Plu protein continues to repress DNA replication in the polar bodies after its action on the female pronucleus has been relieved. The specificity for eliminating Plu action at the pronucleus may be provided by the centrosome, because the key difference between the pronucleus and the polar bodies is the proximity of the male pronucleus and its centrosome (Sonnenblick, 1950). The centrosome could alleviate the repressive function of Plu by its effect on the nuclear envelope, since during the early embryonic divisions the nuclear envelope breaks down only

in the vicinity of the centrosome (Stafstrom and Staehelin, 1984). Alternatively, spindle assembly may modify the function of Plu. A centrosomal ability to regulate Plu function could explain how Plu would couple the S- and M-phases in fertilized embryos.

The isolation of the *plu* gene and identification of its product suggest a mechanism for how this protein, one of the few identified regulators of eukaryotic DNA replication, exerts its repressing action. It will be of interest to identify proteins that interact with Plu.

Materials and methods

plutonium mutant strains

The initial allele of *plu* was isolated from a collection of maternal effect mutations generated by PM hybrid dysgenesis in Trudi Schüpbach's laboratory. We conducted a non-complementation screen to isolate additional mutations in the gene (Shamanski and Orr-Weaver, 1991). Although this screen employed ethylmethanesulfonate (EMS) mutagenesis, the cross to test for non-complementation of the *plu*¹ mutation was a dysgenic cross, as was the cross to recover the mutagenized *cn bw sp* chromosome. Consequently, after we had cloned the gene and were able to analyze the alleles molecularly it was found that the *plu*¹, *plu*³, *plu*⁴, *plu*⁵, *plu*⁶ and *ln(2R)plu* alleles all carried a polymorphic *Bam*HI site not present on the isogenized chromosome mutagenized in the screen. This makes it likely that these alleles are derivatives of the *plu*¹ allele induced by the dysgenic conditions in the screen. The *plu*³, *plu*⁶ and *ln(2R)plu* mutations have different molecular structures to *plu*¹. *plu*⁶ is either an inversion or insertion, and the *ln(2R)plu* inversion is cytologically visible. The *plu*⁴ and *plu*⁵ mutations are indistinguishable in their molecular restriction maps from *plu*¹. These latter two alleles may be re-isolates of *plu*¹ that arose by recombination in the males used to recover the mutagenized chromosome (Shamanski and Orr-Weaver, 1991). The *plu*² allele is an EMS-induced point mutation. *Df(2R)M173* is a cytologically invisible deletion that uncovers the *plu* locus and an adjacent *Minute* (Shamanski and Orr-Weaver, 1991). This deletion removes ~10 kb of DNA covering the *plu* gene, *PCNA* and the transcription unit B at the 5' end of the *plu* gene (D.S.Henderson, S.S.Banga, T.A.Grigliatti and J.B.Boyd, submitted).

Library construction and cloning of the genomic region containing plutonium

High molecular weight genomic DNA was prepared from *plu*¹ flies as described (Levis *et al.*, 1982), except that the nuclear extract was repeatedly phenol extracted and then dialyzed against 50 mM Tris pH 8.0, 10 mM EDTA and 10 mM NaCl. A partial *Sau*3A digest of this DNA was partially end-filled and cloned into a partially end-filled *Xho*I digest of Lambda Fix II (Stratagene). The library was screened with a full length P-element probe (O'Hare and Rubin, 1983), and positives were combined in pools. These pools were biotin-labeled by nick translation and hybridized to polytene salivary glands from a Canton-S stock lacking P-elements (Engels *et al.*, 1986). A single pool was identified that hybridized to 56F, the cytological location of the *plu* gene. A single phage isolate from this pool hybridized to 56F, λ35.

Cosmids homologous to the phage λ35 were isolated from a cosmid library made from the same isogenous *y;cn bw sp* 'iso1' stock used in our EMS mutagenesis (Tamkun *et al.*, 1992). The phage and cosmids were mapped with restriction enzymes, and Southern blots were done to localize the position of the P-element. Seven restriction enzyme sites within the P-element were checked, shown to be present, and produced fragments of the expected size for a full length P-element. Comparison of the restriction map of λ35 with the cosmids revealed a polymorphic *Bam*HI site present in the phage (Figure 2). The *plu* alleles were restriction mapped by Southern blots using the genomic clones and the P-element as probes.

To determine the exact position of the P-element insertion within λ35, a 1.25 kb left junction fragment was cloned into pBluescriptKS⁺ (Stratagene), extending from the *Eco*RI site within the *plu* transcription unit to the *Sac*I site inside the P-element (Figure 2). The right junction fragment was also cloned into the pBluescriptKS⁺ plasmid as a 1.7 kb *Sac*II fragment. The ends of the P-element and the flanking genomic DNA were then sequenced using the KS oligonucleotide primer and a synthetic oligonucleotide corresponding to nucleotides +528 to +514. This analysis revealed that the P-element was inserted 489 nucleotides 5' to the *PCNA* transcriptional start site (Yamaguchi *et al.*, 1990). Several restriction fragments from the cosmids were also subcloned: *Bam*HI 6.5 kb, *Eco*RI 5.2 kb, *Bam*HI–*Eco*RI 3.5 and 3 kb and *Bgl*III 3.8 kb.

Germline transformation and rescue

The restriction fragments shown in Figure 2 were ligated into the w⁺ P-element vector CasPer (Pirrotta, 1988) and introduced into the germline of y w flies by standard techniques (Spradling, 1986). The end-filling and religation of the frameshifted G3.8RKO clone were confirmed by sequencing across the former *Eco*RI site (+283). G₀ flies were crossed to y w and G₁ and G₂ to y w; *SM1/Sco* in order to identify insertions on the X and chromosome 3, and to replace the second chromosomes with dominantly marked ones. Transformant-bearing stocks of the genotype w; *Cyo/Df(2R)M173*; P[w+]/+ were crossed to w; *plu/Cyo*. The fertility of hemizygous *plu/Df* females bearing the transposon was compared with the invariable sterility of their w; *plu/Df* sisters. For both of the constructs B6.5 and E5.2, one insertion on the X and one on chromosome 3 were tested. For G3.8, two on the X and three on chromosome 3 were tested, and one on the X and three on chromosome 3 for G3.8RKO. The integrity of the restriction fragments in the inserted transposons was checked by Southern blot.

Isolation of cDNA clones

cDNAs corresponding to *PCNA* and transcripts A and B were obtained by hybridization of the 6.5 kb *Bam*HI fragment to an ovary cDNA library in the λZAP II vector (provided by T.Hazlerigg). 671 of ~5 × 10⁵ 0–4 h embryo cDNA clones in the plasmid vector pNB40 hybridized with the same fragment (Brown and Kafatos, 1988). These were then probed with cDNAs corresponding to each of the identified transcription units. Of the remaining positives, 11 were confirmed as *plu* transcripts by hybridization with a 187 bp PCR product spanning the *plu*¹ P-element insertion site.

RNA blots and primer extension

RNA preparation and blotting was performed as previously described (Dombrádi *et al.*, 1989), except that RNA probes transcribed *in vitro* were employed to determine the orientation of the transcription units (Figure 2). The *plu* probe was transcribed *in vitro* from an exonuclease III deletion endpoint at nucleotide +487 to a *Hind*III site at +33 (Figure 4). Primer extension (Sambrook *et al.*, 1989) made use of a ³²P-phosphorylated oligodeoxynucleotide primer matching nucleotides +45 to +22 (Figure 4) which was allowed to anneal to polyadenylated RNA from adult females and 0–1 h embryos at 25°C in 80% formamide, then extended using MMuLV reverse transcriptase.

DNA sequencing

The ends of the cDNAs were sequenced from oligonucleotides complementary to the pNB40 vector (Brown and Kafatos, 1988). The insert of cDNA 1 was transferred as a *Bgl*III–*Not*I fragment to *Bam*HI–*Not*I cut pBluescriptSK⁺ to generate clone AE1B1. Exonuclease III deletions were then created from the 3' end (SN series) and 5' end (KH series). Double-stranded deletion clones were then sequenced from the M13 and reverse primers homologous to the vector (Henikoff, 1987). The 3.5 kb genomic clone was sequenced in the same way. The *plu* region of the genomic 3 kb and 3.8 kb clones was sequenced using the M13 and reverse primers or specially synthesized primers listed below. Sequence compressions were eliminated using inosine triphosphate in place of guanosine triphosphate. Sequence was assembled using MacVector and DNASTar software. Sequence searches were run using BLAST (Altschul *et al.*, 1990).

Sequence of plu mutations

Genomic DNA was amplified using PCR (Saiki *et al.*, 1988) and primers located at +6 to +23 and +681 to +664 (this latter has a 12 nucleotide 5' extension). The amplified DNA was directly sequenced by cycles of extension and chain termination (Promega) from ³²P-phosphorylated PCR primers and from oligonucleotides +342 to +361 and +528 to +509. PCR and sequencing was repeated at least three times for each, with consistent results. As a further control for fidelity, the sequence of amplified *cn bw sp* genomic DNA was identical to that of DNA amplified from the G3.8 genomic clone and to the sequence of the clone itself sequenced by conventional [³⁵S]dATP incorporation.

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