THE REGULATION OF DNA REPLICATION DURING DROSOPHILA DEVELOPMENT

by

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ABSTRACT

DNA replication is developmentally regulated throughout the life cycle of *Drosophila melanogaster*. DNA replication is regulated at several levels and thus can be modified in a variety of ways in response to different developmental cues. The amplification of the chorion genes provides a model system to study a developmentally controlled replicon. During oogenesis, amplification of the chorion genes occurs in the follicle cells and enables the chorion proteins to be produced in a short developmental time window. We show that a 440 bp cis-acting control element, termed ACE3, (amplification control element from the third chromosome), is sufficient to regulate amplification. Constructs containing either a single 440 bp ACE3 element or multiple copies of ACE3 are able to undergo amplification with the proper developmental specificity. When controlled solely by ACE3, amplification initiates either at ACE3 or within closely linked flanking DNA.

DNA replication is also developmentally regulated in the early embryo to ensure that the restart of the cell cycle is dependent on fertilization. The maternal-effect gene, *pan gu*, is involved in this regulation of DNA replication and may also regulate S phase in the early cell cycles following fertilization. *pan gu* unfertilized eggs and fertilized embryos undergo inappropriate DNA replication resulting in giant, polyploid nuclei. The phenotypic characterization of early cell cycle defects in *pan gu* embryos is described. We show that DNA replication is transiently coupled to mitosis in *pan gu* embryos, and that defects occur as early as the first mitotic division. The analysis of germ-line clones shows that *pan gu* is required in the germ line to regulate replication. To understand the mechanism by which *pan gu* regulates S phase, experiments are described aimed at the molecular identification of the *pan gu* gene. We show that *pan gu* is localized to a 39 kb region by transformation rescue experiments, and that several DNA mutations are present in this region in *pan gu* alleles. The analysis of transcription units throughout the region is described and several candidate *pan gu* transcripts are identified.

Thesis Supervisor: Dr. Terry L. Orr-Weaver
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CHAPTER I.

Introduction:
Changes in DNA Replication During Animal Development

I. INTRODUCTION

Throughout animal growth and development, controls are exerted upon DNA replication and S phase to coordinate cell cycle events with ongoing developmental processes. DNA replication is subject to developmental controls at several levels, and replication can therefore be modified in a variety of ways in response to different developmental cues. For an overview of the developmental changes and subsequent alterations in DNA replication discussed throughout the review, refer to Table I. Before discussing the developmental effects on replication, it is first necessary to give a brief overview of the normal controls that govern the onset of S phase and the replication process itself. DNA replication is normally controlled both by cell cycle regulators that control the onset and timing of S phase, as well as by regulators affecting the specific properties of DNA replication. These parameters include origin usage, activation, and the rate of replication fork movement.

S phase entry is controlled by cell cycle regulators that coordinate DNA replication with other cell cycle events. Cyclin kinases (cyclin-cdk complexes) including cyclins E, D, and A complexed with cdk2, 4, and 6 are all known to play a role in S phase regulation of higher eukaryotes (reviewed in Sherr, 1993; Sherr, 1994). G1 cyclin kinases are thought to phosphorylate the retinoblastoma gene product, pRb, releasing it from the transcription factor E2F, thus allowing the S phase transcriptional program (Sherr, 1994). Other S phase kinases, perhaps cyclin A kinase, might then inactivate E2F once its transcriptional program is complete (Dynlacht et al., 1994; Krek et al., 1994). In the yeast *S. cerevisiae*, three functionally redundant cyclins, *CLN 1, 2,* and *3,* control the G1/S transition and two cyclin B homologs, *CLB5* and *6* also act to

Table I. Alterations in regulation of DNA replication during development

Developmental Change	S phase control	Replication Modifications
Meiosis	Distinct entry into S phase	Slower S phase S. cerevisiae: same origins used, asynchronous activation Mouse: slower fork rate Newt: fewer origins activated
Oocyte Activation	Xenopus: Mos inhibition of S phase during meiosis	Sea Urchin: Inactive initiation factors in the unfertilized egg
Restart of S phase at fertilization	Drosophila: Inhibition of S phase prior to fertilization by *plu*, *png*, and *gnu*	
Early Embryonic Cycles	S/M cycle Post-transcriptional control of S phase genes Drosophila: Genes that couple S & M phases	Xenopus & Drosophila: Periodic spacing of origins; sequence independent & synchronous activation
Onset of transcription & addition of G1 phase (MBT)	G1 phase added; transcriptional control of S phase genes	Longer S phase; asynchronous origin activation & late replicating heterochromatin
Polyploidy/Polyteny	Drosophila: Endo cell cycle (S/G); spatial & temporal pattern	Drosophila: Removal of block to rereplication Late replication & underrepresentation of heterochromatin
Amplification		Tissue & temporal control; removal of block to rereplication

control S phase (Richardson et al., 1989; Epstein and Cross, 1992; Schwob and Nasmyth, 1993).

In the normal cell cycle, inhibitors of cyclin kinases exist that are able to inhibit progression of the cell cycle in response to various environmental cues. The cdk inhibitors, $p21^{CIP1}$, $p27^{KIP1}$ and $p16^{INK4}$ all act during the G1/S transition and cause a G1 arrest in response to certain conditions (reviewed in Sherr, 1994). In yeast, an inhibitor $p40^{SIC1}$ controls the entry into S phase by inhibiting the *CLB5* and *6* genes (Schwob et al., 1994).

As well as the above cell cycle regulators, an added level of control includes checkpoints and feedback controls that act to ensure the proper execution and order of cell cycle events, in relation to growth conditions and environmental cues. Checkpoint and feedback mechanisms also act to couple M phase events with S phase events as well as ensuring that replication occurs only once per cell cycle. One postulated mechanism that limits DNA replication to once per cell cycle is the existence of licensing factor. This factor is proposed to permit one round of DNA replication and to then become inactivated following replication (Blow and Laskey, 1988). Active licensing factor is then excluded from the nucleus, and reentry into the nucleus is only permitted following nuclear envelope breakdown in mitosis. Three genes from *S. cerevisiae*, *CDC46*, *MCM2*, and *MCM3*, are required for DNA replication, and all show nuclear localization patterns characteristic of the predicted licensing factor (Hennessy et al., 1990; Yan et al., 1993).

As opposed to the cell cycle regulation described above, parameters of DNA replication such as origin usage and temporal activation are also controlled. In eukaryotes, many origins must be regulated in concert, such that DNA replication occurs only once along the chromosome. Origin studies in the yeast *S. cerevisiae* have identified specific sequences, or *ARS* elements,

that act as chromosomal origins of replication (Brewer and Fangman, 1987; Huberman et al., 1987). Recently, a complex that binds to the essential region of *ARS* elements has been identified and termed the origin recognition complex (ORC) (Bell and Stillman, 1992). ORC is comprised of six proteins which remain bound to the origin throughout the cell cycle (Bell et al., 1993; Diffley et al., 1994). Presumably the interaction between ORC with other factors during G1 is necessary for replication initiation, and one possible interactor includes Dbf4 which was recently found to interact with yeast origins (Dowell et al., 1994).

In higher eukaryotes the identification of specific origins of replication has been less successful; replication origins may consist of regions more complex than the *ARS* elements identified in yeast. One well characterized origin in higher eukaryotes is located in the amplified sequences surrounding the DHFR gene in a methotrexate-resistant Chinese hamster ovary cell line. Initially, two specific initiation regions within 28 kb were identified downstream of the DHFR gene, based on several origin mapping techniques (Heintz and Hamlin, 1982; Anachkova and Hamlin, 1989; Leu and Hamlin, 1989). By mapping the position of Okazaki fragments, a predominant origin of bidirectional replication was delineated to a 0.45 kb sequence (Burhans et al., 1990). However upon analysis of the region using two-dimensional gel techniques, it was found that replication initiation events can be detected within a larger 50 kb region (Vaughn et al., 1990). One hypothesis to explain this apparent paradox is that within this larger zone of initiation many origins can fire, yet initiations from specific origins are favored which lead to mature replicons (Linskens and Huberman, 1990).

Other mammalian origins have been identified as well using techniques to isolate Okazaki fragments or nascent strands generated at

replication forks. Both the human β-globin and *c-myc* genes were found to contain bidirectional replication origins located in their 5' upstream regions (Vassilev and Johnson, 1990; Kitsberg et al., 1993). The *c-myc* origin was localized to an 8 kb region located 1.5 kb upstream of the first exon. Similarly, a bidirectional origin was recently mapped by nascent strand analysis, near the promoter of the human heat shock protein 70 (*hsp70*) gene (Taira et al., 1994). One replication origin was recently localized to a 2 kb region in the middle of the Chinese Hamster ribosomal protein S14 (*RPS14*) locus, in contrast to most origins previously identified in either 5' or 3' regions (Tasheva and Roufa, 1994). The characterization of initiation events throughout these origin-containing regions has not yet been determined by two-dimensional gel analysis.

In Drosophila, replication origins have not been extensively characterized at a molecular level. One of the first origins identified however was the origin used during chorion gene amplification (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Two-dimensional gel analysis identified a predominant origin within a larger 12 kb zone of initiation. Recently, Drosophila tissue culture cells have been used to identify replication origins within the histone gene repeats, as well as downstream of a single copy locus, the DNA polymerase α gene (Shinomiya and Ina, 1993; Shinomiya and Ina, 1994). Both regions have been characterized using both nascent strand and two-dimensional gel analysis. Multiple initiations occur throughout the histone repeats, and similarly, multiple initiations are found within a 10 kb zone located downstream of the DNA polymerase α gene. These results again suggest that higher eukaryotes contain complex origins consisting of larger zones of initiation.

In addition to the observed spatial control of eukaryotic origins, a temporal control of origins also exists, such that some origins are activated early in S phase while others are activated late in S phase. A second factor determining the timing of replication is the relative spacing of origins. Regions within a large replicon might replicate late due to the time needed for the replication fork to travel through the region. Indeed, in yeast examples of both types of late replicating regions can be found (Reynolds et al., 1989; Ferguson et al., 1991; Ferguson and Fangman, 1992). There are also examples in higher eukaryotes of temporal control of replication. Replicon asynchrony is seen in cycle 14 of the early Drosophila embryo, with origins in euchromatin being activated throughout the first 20 minutes of S phase, followed by the initiation of replication of centromeric heterochromatin (McKnight and Miller, 1977; Edgar and O'Farrell, 1990).

Finally, origins are controlled within S phase such that rereplication of DNA does not occur. This control might be separable from those that activate origins once during the cell cycle. However, these two controls might be linked by a common regulator, and the model for licensing factor proposes that it both allows only one initiation per cycle, and is then inactivated thus acting as a block to rereplication. Examples that override this block to rereplication occur during development, such as polyteny and amplification, and will be discussed later.

Other parameters of eukaryotic DNA replication include polymerases and factors found at origins and forks of replication. The known replication proteins found in Drosophila are listed in Table II, and include DNA polymerase α primase and DNA polymerase γ. Recently a DNA polymerase δ has been purified from embryos and consists of a single 120 kd polypeptide which is not responsive to PCNA as in other organisms (Chiang et al., 1993).

However, PCNA has been identified, as well as replication protein A (RP-A), two ribonucleotide reductase genes and DNA topoisomerases I and II. The analysis of replication in Drosophila awaits the further characterization of these known proteins as well as the identification of other factors involved in DNA replication. The use of in vitro replication systems may also aid in its characterization, and Drosophila extracts from 0-2 hour embryos have been successfully used to assay replication of added Xenopus sperm nuclei (Crevel and Cotterill, 1991). However, this system was found to be less efficient that the Xenopus extract system, perhaps due the fact that extracts are not made from synchronous populations of embryos.

Table II. Drosophila Replication Proteins

DNA Polymerase α/primase	(Cotterill et al., 1987) (Hirose et al., 1991) (Cotterill et al., 1992) (Peck et al., 1993)
DNA Polymerase γ	(Wernette and Kaguni, 1986)
DNA Polymerase δ	(Peck et al., 1992) (Chiang et al., 1993)
PCNA	(Henderson et al., 1994)
Replication protein A (RP-A)	(Marton et al., 1994)
Ribonucleotide reductase 1, 2 (*RNR1, 2*)	(Duronio and O'Farrell, 1994)
DNA Topoisomerase I	(Hsieh et al., 1992) (Lee et al., 1993)
DNA Topoisomerase II	(Hsieh et al., 1987) (Wyckoff and Hsieh, 1988) (Wyckoff et al., 1989)

II. MEIOTIC VS. MITOTIC S PHASE

A. Meiotic S phase

1. Regulation of Entry Into S phase

Studies addressing the regulation of premeiotic S phase have been investigated mainly in the yeast, *S. cerevisiae*. Many genes that govern S phase during mitosis have been characterized, yet several of these are found not to be required for the control of premeiotic S phase. Interestingly, some of these genes that are involved in mitotic S phase do have a role in meiosis, yet are required after replication and before meiosis I. One example is the mutation *cdc7*, in which mitotic cells are blocked prior to replication, yet meiotic cells are arrested following premeiotic DNA synthesis (Schild and Byers, 1978; Buck et al., 1991). Recently, Cdc7 has been linked to replication origins in that a protein that binds to and activates the Cdc7 kinase, Dbf4, also binds to *ARS* elements (Jackson et al., 1993; Dowell et al., 1994). If Cdc7 does prove to have a role at the replication origin, this might then suggest that premeiotic replication is differentially regulated. Other similar examples include the *S. cerevisiae* mutations, *cdc28*, and *cdc4*, in which mitotic DNA replication is blocked yet premeiotic synthesis occurs (Simchen and Hirschberg, 1977; Piggott et al., 1982; Shuster and Byers, 1989; Reed and Wittenberg, 1990). *cdc28* is a component of maturation promoting factor (MPF) and is required both at the G1/S and G2/M transitions during the mitotic cell cycle, yet does not appear to be required for the G1/S transition of premeiotic S phase.

2. Spatial Control of Meiotic Replication Origins

Comparisons of meiotic and mitotic S phase using fiber autoradiography indicated that a similar spacing of origins is present and that replication forks travel at a similar rate (Johnston et al., 1982; Newlon, 1988). However the resolution of fiber autoradiography was not precise enough to determine whether the specific origins used were the same origins used during mitosis. Given the fact that not all *ARS* elements identified by the plasmid assay are active chromosomal origins during the mitotic cell cycle, it was possible that the other *ARS* elements were specifically used during meiosis. In one study designed to examine *ARS1* function in meiosis, plasmid loss occurred in both mitosis and meiosis upon induction of transcription through the *ARS1* element (Hollingsworth and Sclafani, 1993). Although this study uses an indirect assay for *ARS* function, it suggests that the same origin can function in both meiosis and mitosis. In another study to identify replication origins active during meiosis, two-dimensional gel analysis was used to examine origins on chromosome III (Collins and Newlon, 1994). The five meiotic origins map to the same *ARS* elements as the mitotic origins. One possible exception was a weak mitotic origin located at *CEN3* in which there did not appear to be any meiotic activity. No additional meiotic origins were found that did not correspond to mitotic origins. Further analysis will be needed to determine if this conclusion is true for all chromosomes, yet these studies strongly suggest that meiotic origins are indeed the same as mitotic origins.

In higher eukaryotes, perhaps this will not be the case. Using fiber autoradiography in the newt Triturus, the spacing between origins used in meiosis is larger than that seen during mitosis, suggesting that either

different origins are used or a subset of mitotic origins are activated (Callan, 1974).

3. Temporal Control of Meiotic Origins

S phase in eukaryotes can vary widely in length, from extremely short S phases during embryonic development (several minutes) to longer S phases in somatic cells (hours) and often very prolonged S phases during premeiotic S (at least 24 hours in the Drosophila ovary) (Chandley, 1966; Grell, 1973). S phase seems to lengthen as development places increasingly complex controls on the cell cycle. In *S. cerevisiae* premeiotic S phase is at least two times as long as mitotic S phase (65 min. vs. 30 min.) (Williamson et al., 1983). Despite this lengthening of S phase, origin usage and the rate of fork movement are the same as during mitotic S phase (Johnston et al., 1982). Two possibilities for the longer meiotic S phase are either that origins within a chromosome are fired asynchronously or that different chromosomes are replicated at different times. In the study of meiotic origins on chromosome III mentioned above, the efficiency of usage of specific *ARS* elements and characteristic termination patterns were similar between meiosis and mitosis (Collins and Newlon, 1994). This implies that within a single chromosome the kinetics of initiation and termination are roughly the same in meiosis and mitosis. Further studies are needed to determine if this is true for all chromosomes. The authors therefore suggest that the longer meiotic S phase is due to different initiation times for different chromosomes.

In the premeiotic S phase of mouse spermatogonia, the lengthening of S phase was studied by fiber autoradiography (Jagiello et al., 1983). Similar to yeast, origin spacing during meiosis and mitosis is similar. However, the rate

of fork movement is slower during meiosis and might account for the longer S phase.

III. S PHASE IN EARLY EMBRYOGENESIS

A. Inhibition of DNA Replication in the Developing Oocyte and Unfertilized Egg

Many animals undergo rapid cell divisions following fertilization, including Xenopus, Drosophila, sea urchin, starfish and clam. DNA replication factors are stockpiled in the egg to prepare for the high demand during the rapid embryonic divisions. However, DNA replication must be repressed in the oocyte so as not to occur during meiosis or prior to fertilization. This repression could be at the level of S phase control such that entry into S phase is blocked during meiosis, or alternatively at the level of replication parameters, such as initiation factors that might be kept in an inactive state. Two well characterized mechanisms of replication control in the developing oocyte and unfertilized egg have been shown in Xenopus and sea urchin.

The inability of Xenopus oocytes to replicate DNA has been found recently to be due to Mos activity blocking the entry into S phase during meiosis (Furuno et al., 1994). Mos is a serine/threonine kinase that is expressed at high levels in germ cells of vertebrates as well as maternally in

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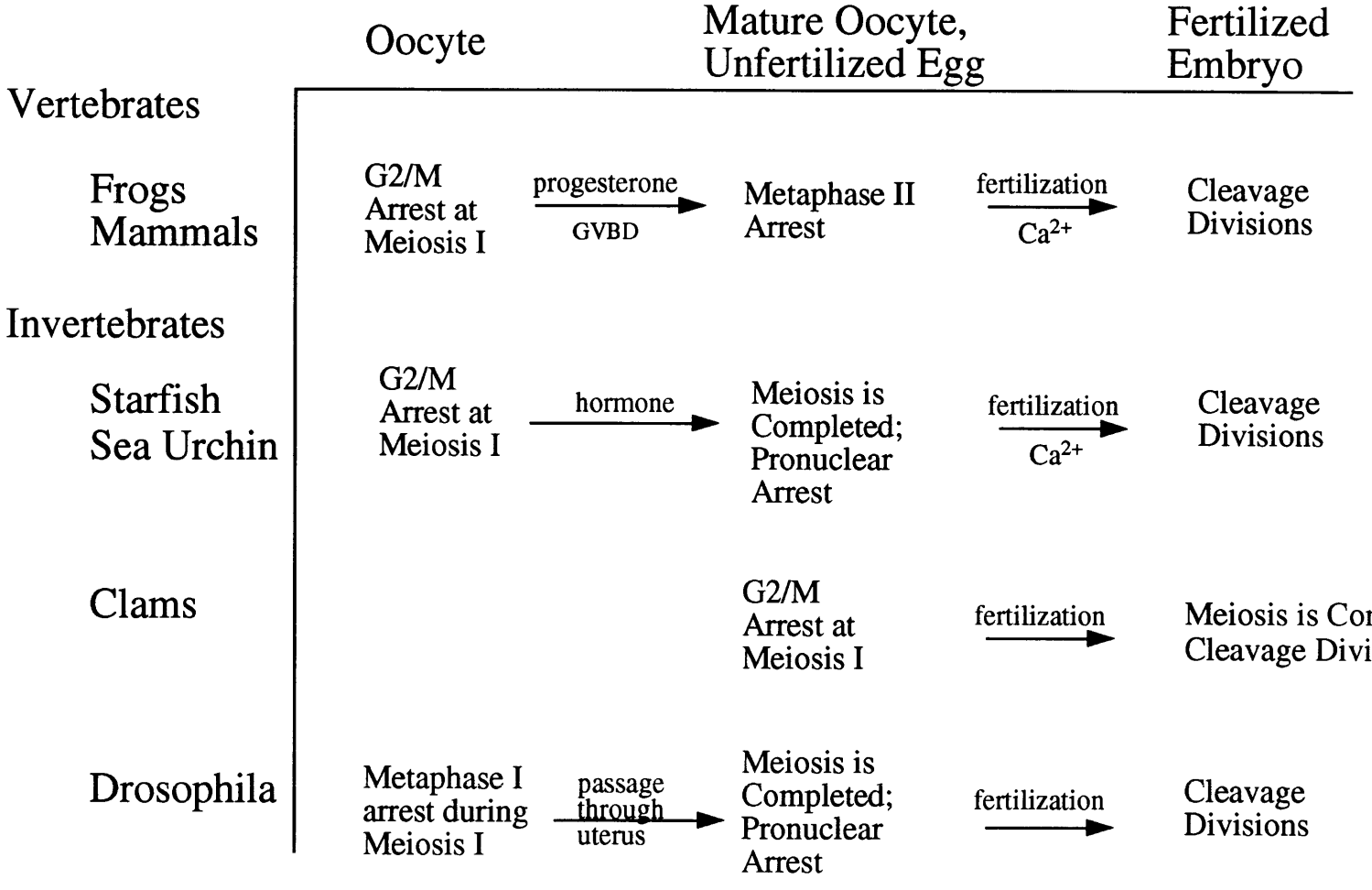
Figure 1. The progression from oocyte maturation to fertilization is shown for several organisms, including both vertebrates and invertebrates. Arrest points during oogenesis as well as the relationship between fertilization and meiosis are unique to each organism.


Oocyte
Mature Oocyte, Unfertilized Egg
Fertilized Embryo
Vertebrates
Frogs
Mammals
G2/M Arrest at Meiosis I
progesterone
GVBD
Metaphase II Arrest
fertilization
Ca2+
Cleavage Divisions
Invertebrates
Starfish
Sea Urchin
G2/M Arrest at Meiosis I
hormone
Meiosis is Completed; Pronuclear Arrest
fertilization
Ca2+
Cleavage Divisions
Clams
G2/M Arrest at Meiosis I
fertilization
Meiosis is Completed Cleavage Divisions
Drosophila
Metaphase I arrest during Meiosis I
passage through uterus
Meiosis is Completed; Pronuclear Arrest
fertilization
Cleavage Divisions

the Xenopus oocyte (Sagata et al., 1988). Xenopus immature oocytes (stage VI) are arrested at G2/M of meiosis I (Fig. 1). Following hormone stimulation germinal vesicle breakdown (GVBD) occurs, and oocytes progress to metaphase II of meiosis. This maturation is dependent on the translation of Mos following hormone stimulation, after which mature oocytes remain arrested at meiosis II until fertilization.

By examining a precise time course of MPF activity throughout meiosis, it was found that MPF is inactivated early in meiosis I (early metaphase I) and is then reactivated during late metaphase I, well before meiosis II (Furuno et al., 1994; Ohsumi et al., 1994). Mos mediates this reactivation of MPF, which then suppresses DNA replication between the two meiotic divisions. Ablation studies using *c-mos* antisense RNA or Mos antibodies showed that upon inactivation during GVBD, mature oocytes enter S phase inappropriately following meiosis I (Furuno et al., 1994). The role of MPF in suppressing an intervening S phase was also confirmed by studies that block MPF activation at meiosis II, resulting in DNA replication (Furuno et al., 1994; Ohsumi et al., 1994). Once MPF is reactivated, it is stabilized by the action of cytostatic factor (CSF) at the metaphase II arrest (reviewed in Minshull, 1993). Interestingly, CSF is composed of both Mos and an unknown factor. Whether Mos acts alone in reactivating MPF after meiosis I is not known.

Mos homologs have not been identified in invertebrates, suggesting that different mechanisms might act to control replication during oocyte maturation. In contrast to the control of S phase entry seen in Xenopus, the mechanism that inhibits DNA replication in the unfertilized sea urchin egg is the absence of active initiation factors responsible for DNA synthesis. Following oocyte maturation, sea urchin eggs complete meiosis and arrest at

G1 of the first mitotic cycle (Fig. 1). Fertilization then releases this arrest and replication factors are post-translationally activated within a short 3 minutes following fertilization (Zhang and Ruderman, 1993). The first S phase occurs 20 minutes following fertilization, even in the absence of protein synthesis.

The unfertilized egg of sea urchin is not permissive for DNA replication as assayed by the inability of egg extracts to replicate added sperm nuclei or double-stranded DNA templates (Zhang and Ruderman, 1993). Conversely, embryonic interphase extracts are capable of supporting replication of added templates. Mixing experiments of egg and embryonic extracts showed no evidence of negative factors in the egg capable of repressing replication; the egg extract did not inhibit the ability of the embryonic extract to replicate DNA. Thus, the unfertilized egg does not contain inhibitors of replication, and instead, the inhibition of replication seen is due to inactive initiation factors present at this stage of development.

B. Restart of S phase at Fertilization

In many organisms the unfertilized egg is arrested during or following meiosis, and fertilization then releases the egg from this arrest (Fig. 1). This coupling of fertilization with the resumption of the cell cycle ensures that the female and male pronuclei can then enter the first S phase and subsequent cell cycles with the proper timing. In Drosophila as well as starfish and sea urchin, the completion of meiosis is not coupled to fertilization. In Drosophila, the mature oocyte is arrested at metaphase I in the ovary. Upon passage of the egg through the uterus, the egg becomes activated, and meiosis is completed regardless of whether fertilization occurs. Fertilization is necessary to restart the cell cycle in the embryo following the completion of meiosis, and unique regulators may be needed at this developmental step.

In Drosophila three genes that act at this point to couple fertilization with DNA replication are the maternal-effect genes *pan gu* (*png*), *plutonium* (*plu*), and *giant nuclei* (*gnu*). Unfertilized mutant eggs complete meiosis resulting in four meiotic products, yet then undergo improper DNA replication, resulting in large polyploid nuclei (Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). These genes normally act as negative regulators of DNA replication to make the restart of S phase dependent on fertilization. Fertilization must overcome the action of these genes in order to resume the cell cycle.

In many organisms, including starfish, Xenopus, sea urchin and mouse, a transient increase in intracellular calcium (Ca^{2+}) occurs at fertilization and is associated with the onset of development including meiotic maturation, pronuclear migration, DNA replication, and nuclear envelope breakdown. The levels of inositol triphosphate (IP_3) also fluctuate during fertilization and might act to trigger the calcium transients seen during sea urchin development (Ciapa et al., 1994). However, the cause and effect relationship between calcium and the second messengers in other systems remains less clear (reviewed in Whitaker and Swann, 1993).

Recently it was determined that following the calcium burst in Xenopus mature oocytes, the calmodulin-dependent kinase, CaM K_{II}, is responsible for the inactivation of both MPF and CSF, thus releasing the oocyte from a metaphase II arrest (Lorca et al., 1993). How this then regulates the resumption of DNA replication following meiosis is less clear. The role of calcium bursts in controlling DNA replication can be seen in starfish, where the calcium ionophore, A 23187, acts as a parthenogenic agent capable of inducing several rounds of replication in mature oocytes (Picard et al., 1987). The calcium ionophore also triggers the onset of the cell cycle in

unfertilized sea urchin eggs (reviewed in Whitaker and Patel, 1990). Whether calcium transients play a role in Drosophila fertilization and the restart of S phase is unclear.

C. Rapid Early Cycles in Xenopus and Flies

Many organisms undergo rapid embryonic cycles following fertilization. The early embryonic cycles of Xenopus and Drosophila have been well characterized and consist of rapid cycles of alternating S phase and M phase that are controlled by maternally supplied products present in the egg. DNA replication is differentially regulated during this time in development. Unique regulation occurs at both the entry into S phase and in the replication parameters used. S phase entry is regulated post-transcriptionally due to the absence of zygotic transcription. DNA replication parameters are also developmentally controlled such that many synchronous origins are activated to ensure complete replication within the very rapid 3-10 minute S phases.

1. Post-Transcriptional Control of S phase

The rapid early cycles of Xenopus and Drosophila are controlled by post-transcriptional modifications of regulators during S phase and M phase. In Drosophila, the first 13 divisions in the fertilized embryo are rapid, synchronous nuclear divisions within a common shared cytoplasm, where S phase occurs in an extremely short 3-4 minute period. The early cycles in Xenopus are similar in that the first 12 divisions consist of rapid, synchronous cycles with S phase occurring in a brief 10 minutes. During these early embryonic cycles of both Xenopus and Drosophila, transcription of the zygotic nucleus does not occur. In Drosophila maximal zygotic

transcription occurs following cellularization at cycle 14, whereas in Xenopus, zygotic transcription occurs at the midblastula transition (Newport and Kirschner, 1982b; Edgar and Schubiger, 1986).

Known regulators of S phase are present at high levels in Drosophila and Xenopus embryos. In Drosophila, the S phase cyclin E-cdk2 kinase is present throughout early embryonic development (Richardson et al., 1993; Knoblich et al., 1994). Cyclin E protein cycles between a nuclear localization in S phase and a cytoplasmic localization during most of mitosis (H. Richardson and R. Saint, personal communication). The nuclear localization of cyclin E during the rapid S phases must be controlled by either post-transcriptional or post-translational modifications. Similarly, a large pool of maternal cyclin E is present during the early cleavage divisions of Xenopus (T. Hunt, P. Jackson, and M. Kirschner, personal communication).

In addition to the post-transcriptional control of known S phase regulators, unique regulators might also be used early in development for the rapid cell cycle. In Drosophila, three maternal-effect genes *pan gu* (*png*), *plutonium* (*plu*) and *giant nuclei* (*gnu*) appear to be novel cell cycle regulators. As discussed above, these genes are needed to couple S phase with fertilization such that mutant unfertilized eggs undergo inappropriate DNA replication. When fertilized, these embryos also give rise to giant polyploid nuclei, suggesting that these genes also control S phase during the early cycles. Although fertilized, these embryos fail to properly couple S phase and M phase, and some aspects of mitosis such as centrosome duplication continue to cycle independently from nuclear division (Freeman et al., 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). *png* is unique in that several presumably leaky alleles transiently couple S phase and M phase, resulting in embryos containing many more giant nuclei. However, defects

in mitotic figures and DNA condensation can be seen as early as the first division, and the uncoupling of replication and mitosis progresses as development proceeds. The unfertilized and fertilized phenotypes can be explained by the proposal that these genes inhibit DNA replication. In unfertilized eggs they function to make S phase dependent on fertilization, whereas in fertilized embryos these gene products make S phase dependent on the proper completion of mitosis. However, at what level these genes act is unclear. They might control either the entry into S phase, the block to rereplication, or other aspects of the cell cycle such as chromosome condensation that link mitosis to replication.

Molecular data confirms that *plu* is a unique regulator that acts solely during the early Drosophila divisions and not in later canonical cell cycles. RNA null alleles are maternal-effect alleles and expression of the *plu* transcript is not present during later stages of development (Axton et al., 1994). Plu encodes a 19 kD protein consisting of 3 ankyrin repeats. Interestingly, another small ankyrin repeat protein is the cdk4 inhibitor, $p16^{INK4}$, which acts to inhibit cdk4, thus disrupting its association with the S phase cyclin D protein (Serrano et al., 1993). Plu might act analogously in its role in repressing DNA replication, by possibly inhibiting S phase cyclin kinases until their proper time of action.

2. Origins?

Studies in both Xenopus and Drosophila suggest that in the extremely rapid cycles of the early embryo, origins are controlled by a unique mechanism perhaps involving chromosome folding or attachment to the nuclear envelope. This could ensure the complete replication of the genome during the brief S phase. This mechanism acts temporally and spatially, such

that periodically spaced origins are activated synchronously at the beginning of each S phase.

Experiments using Xenopus eggs showed that plasmid replication upon injection into the egg is under cell cycle control. Replication is not dependent on specific sequences, but instead depends on the size of the plasmid injected (Harland and Laskey, 1980; Mechali and Kearsey, 1984). Two-dimensional gel analysis of replication intermediates revealed that in both Xenopus eggs and extracts, plasmids containing either rDNA repeats or single copy sequences initiated and terminated replication at random sites throughout the plasmid (Hyrien and Mechali, 1992; Mahbubani et al., 1992). It was also determined that although initiation could occur at random sites on the plasmid, a single initiation event gave rise to complete replication of each plasmid molecule.

Similar conclusions were reached upon examination of the replication of chromosomal rDNA repeats in early embryos prior to the midblastula transition (Hyrien and Mechali, 1993). Initiation occurred at random positions, and the estimated replicon size was 9-12 kb. In order to complete replication in the rapid S phase, all origins were presumed to be activated synchronously at the beginning of S phase. Whereas replication initiation is random with respect to sequence, the periodic spacing of replicons suggests that initiation is not random with respect to higher order chromatin folding. If initiation were entirely random there could be instances where some replicons were too far apart to finish replication in the short S phase. Thus the authors suggest that chromosomal folding might specify a periodic spacing of origins, guaranteeing complete replication within the 10 minute S phase.

A similar periodic spacing of replicons was seen in the Drosophila early cleavage nuclei in which replication occurs in a 3-4 minute S phase. By EM studies the average replicon size was 7.9 kb with a preferred periodicity of 3.4 kb, as well as a maximum size of 19 kb (Blumenthal et al., 1973). An estimated 20,000 bidirectional origins must be activated nearly synchronously to finish S phase in the extremely short time. The maximum replicon size correlates with the amount of DNA that can be replicated in 3-4 minutes given the rate of fork movement observed. However, these studies did not resolve whether initiation occurs at defined or random sequences. Using two-dimensional gel analysis of replication intermediates from early embryos, it was found that random initiation occurs both within the histone repeats and within a 40 kb single copy sequence (Shinomiya and Ina, 1991). Therefore, the periodicity of replicon spacing and the sequence independent nature of replication is similar to that found in early Xenopus embryos. Thus, there appears to be a specific control of origin usage in the early rapid cycles.

3. S phase Slow Down

Following the rapid embryonic cell cycles, the cell cycle lengthens to allow certain developmental processes to occur such as the onset of zygotic transcription and gastrulation. In both Xenopus and Drosophila, the increase of the nuclear to cytoplasmic ratio results in the slowing of the cell cycle (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b; Edgar et al., 1986). During this time in development, both the control of replication origins as well as the entry into S phase are altered. In Xenopus this is a one step process that occurs at the midblastula transition (MBT), whereas in Drosophila these events occur in two distinct steps. First, following

cellularization at cycle 13, alterations in the temporal control of origins occur. At this time, a G2 phase is added to the cell cycle, and zygotic transcription commences. Following three more divisions a G1 phase is then added, permitting transcriptional control of S phase. This transcriptional control in Drosophila and the MBT in Xenopus are discussed below in section IV.

In Drosophila, replication proceeds from the fast synchronous S phase of the early cycles to a prolonged S phase in which origin activation becomes asynchronous. A change in condensation of both euchromatin and heterochromatin occurs during this lengthening of S phase (Foe et al., 1993). During the first 13 cycles of the Drosophila embryo, the euchromatin remains decondensed throughout interphase. The heterochromatic regions decondense for a short time in S phase, decondensing progressively later in interphase as the cycle slows during cycles 11 to 13. By cycle 13, S phase has lengthened from 3-4 minutes to 13 minutes. During the three post-blastoderm cycles 14 to 16, S phase is 8 times as long as the early S phases, taking 35-45 minutes.

In contrast to the early cycles, at least 200 particles of highly condensed euchromatin can be seen during interphase of cycles 14, 15, and 16 (Foe et al., 1993). EM studies show that new forks appear throughout the first 20 minutes of S phase during cycle 14, indicating that origin activation becomes asynchronous during interphase of these later cycles (McKnight and Miller, 1977). Heterochromatin remains condensed throughout most of interphase and is not replicated until euchromatic replication has finished (Edgar and O'Farrell, 1990; Foe et al., 1993). This altered regulation of origins and concurrent lengthening of S phase correlates with the onset of zygotic transcription and tissue specific expression of certain genes. Replication must be coordinated with other processes now occurring during the cell cycle. In

the post-blastoderm cycles, replication is followed by a G2 phase of varying length while mitosis occurs in distinct temporal and spatial domains.

D. Mouse

1. Injection Experiments

Similar to Xenopus eggs, mouse embryos are also able to support the replication of injected double-stranded DNA templates, whereas injected plasmids are not replicated in oocytes (Wirak et al., 1985). Replication in embryos is dependent on specific cis-acting origin sequences of polyomavirus (PyV) or simian virus 40 (SV40) present in cis to plasmid sequences. This is in contrast to results in Xenopus eggs in which replication is independent of cis-acting sites and initiates at random sequences.

Using mouse embryos, an added layer of control is seen when comparing arrested one-cell embryos containing the unfused male and female pronuclei with two-cell embryos containing zygotic nuclei (Martinez-Salas et al., 1988; Martinez-Salas et al., 1989). One-cell embryos are able to replicate DNA containing a minimal origin core sequence from PyV, whereas the two-cell embryo requires enhancer sequences present in cis to the PyV origin. Enhancers are also required for gene expression in the zygote as opposed to the one-cell embryo. Enhancers are postulated to prevent the repression of origins and promoters by altered chromatin structure that is thought to occur upon zygote formation.

Replication in the mouse embryo is distinct from that occurring in the rapid early embryonic cycles of Xenopus and Drosophila, possibly due to the difference seen in zygotic transcription in these organisms. Xenopus and Drosophila have an early period lacking zygotic transcription, so are able to support fast cycles of alternating S and M phase. In contrast, transcription

occurs in the early mouse embryo following zygotic formation, and thus replication must be coordinated with transcription. Enhancers might be associated with origins to overcome chromatin effects due to a transcriptionally active genome.

IV. DEVELOPMENTAL SHIFT TO TRANSCRIPTIONAL REGULATION OF S PHASE

Following the rapid cycles of Xenopus and Drosophila that are controlled by post-transcriptional modifications of cell cycle regulators, a developmental shift occurs in that transcription of the zygotic genome becomes active. At this time in development, S phase regulators can now be controlled at a transcriptional level, a mode of regulation characteristic of the somatic cell cycle.

A. MBT in Xenopus

The cell cycle of Xenopus is modified during the midblastula transition (MBT) which occurs after the first 6 hours of development. The early rapid cycles of S and M phase slow, giving rise to a longer cell cycle in which both gap phases are added to the cell cycle. This process then allows for the resumption of zygotic transcription (Kimelman et al., 1987). This slowing of the cell cycle is thought to occur due to a mitotic initiation factor that becomes rate-limiting at the MBT (Kirschner et al., 1985; Newport et al., 1987). Blastula cleavage becomes less synchronous, cells become motile, and zygotic transcription turns on. Recent results determined that the excess of histones present in the early embryo is responsible for the repression of transcription prior to the MBT (Prioleau et al., 1994).

As mentioned previously, the developmental regulation of S phase is altered at two different levels during the midblastula transition in Xenopus. First, similar to Drosophila, origin activation presumably becomes asynchronous giving rise to a lengthened S phase. DNA replication becomes asynchronous as evidenced by a variation of PCNA staining, with some nuclei showing a peripheral staining and others showing a homogenous staining (Leibovici et al., 1992). This is in contrast to the early cycles in which PCNA staining is homogeneous throughout S phase. Second, the entry into S phase is altered such that a G1 phase is added to the cell cycle. Zygotic transcription resumes, permitting S phase regulators to become transcriptionally controlled.

B. Addition of G1 in Drosophila

The developmental shift to asynchronous origin activation has already occurred by cycle 14 of the Drosophila embryo. The second developmental control placed on S phase occurs following cycle 16 when a G1 phase is added to the cell cycle. Following cycle 16, mitotic embryonic cells will either arrest in G1 and divide later in development (imaginal cells) or continue to divide (neural cells), whereas cells giving rise to most of the larval tissues become polytene. Recent results show that the down regulation of cyclin E is needed for the arrest of cells in G1, and that cyclin E is then necessary for the G1/S transition (Richardson et al., 1993; Knoblich et al., 1994). Both the cyclin E transcript and protein are down regulated following the last mitotic division of epidermal cells during cycle 16, and remain off in these G1 arrested cells (Knoblich et al., 1994; H. Richardson and R. Saint, personal communication). This down regulation of cyclin E is necessary not only for the arrest in G1, but also for the addition of G1 to the cell cycle.

During Drosophila development, the transcriptional control of cyclin E during the added G1 phase is a new mode of S phase regulation that occurs specifically following the embryonic cell cycles. Cyclin E kinase is proposed to act as an activator of the transcription factor, E2F, which regulates many S phase genes. Several of these cyclin E-regulated gene products in Drosophila include Polymerase α, *PCNA*, and Ribonuclease reductase 1 and 2 (*RNR1, 2*) (Duronio and O'Farrell, 1994).

V. POLYPLOIDY/ POLYTENY

A. Changes in S phase Regulation

Polyploid cells exist in a number of organisms including plants, ciliates, dipteran insects as well as some mammalian cell types such as the trophoblasts that give rise to the mammalian placenta. Polyploidy is often associated with cells or tissues in which a requirement for increased protein production is needed; multiple chromosome copies is one way in which evolution has met that demand. An area of current research concerns the identification of regulators that govern the developmental transition leading to polyploidy. Similar to the developmental alterations of DNA replication already discussed, polyploid replication is controlled at two basic levels. These include changes in regulation of the cell cycle as well as alterations at the level of replication origins and other parameters used during DNA synthesis. In Drosophila, the transition to polyteny results in an altered cell cycle, termed the endo cell cycle, which consists of an alternating S phase and gap phase (Smith and Orr-Weaver, 1991). Parameters of replication also become altered, and the block to rereplication is overcome during the endo cell cycle.

Polyploid and polytene cells are defined as those in which DNA replication has become uncoupled from mitosis giving rise to cells with greater than diploid content of DNA. The degree to which replication is uncoupled from mitotic aspects of the cell cycle can vary. Polytene cells uncouple replication from all aspects of mitosis; 1000 or more chromosome copies remain synapsed forming the large polytene chromosomes characteristic of the Drosophila salivary gland. Some polyploid cells do not uncouple replication from all aspects of mitosis, and chromosome segregation or cycles of chromosome condensation still occur. This is referred to as endopolyploidy or endomitosis. Finally, multinucleate cells have been referred to as polyploid cells. The Drosophila larval polytene cells are among the best characterized in terms of alterations in S phase that occur during this developmental transition.

1. Regulators of the Endo Cell Cycle

One of the best understood examples of polyteny is in the Drosophila larval tissues (Smith and Orr-Weaver, 1991). Following embryogenesis, Drosophila larval growth is due to an increase in cell size upon polytenization, because only cells in the nervous system and imaginal tissues undergo mitosis during larval development. In Drosophila, most tissues enter the endo cell cycle during late embryogenesis, and this transition is temporally and spatially regulated (Smith and Orr-Weaver, 1991). The first transitions to the endo cell cycle occur in tissue-specific domains that replicate at characteristic times, with the salivary gland being the first tissue to enter the endo cell cycle, followed by the midgut, hindgut, and malpighian tubules. It was determined for the hindgut, that cells enter the endo cell cycle from the G1 phase of the cell cycle, whereas salivary gland cells may enter the endo cell

cycle following G2. Gap phases of the endo cell cycle can vary widely in length, from 3 hours for the midgut to 18 hours for the salivary gland (Smith and Orr-Weaver, 1991).

The spatially and temporally regulated pattern seen in Drosophila polytene tissues argues that a novel factor controls these cycles. However, regulators of the endo cell cycle could in theory be known mitotic cell cycle regulators that also act to control the endo cell cycle. In this case, the dependency between S phase and M phase must be disrupted in this altered cell cycle. In Drosophila the mitotic cell cycle regulators, cyclin A, cdc2, and the cdc25 phosphatase homolog, *string*, are not needed for the endo cell cycle. Endoreplication proceeds in embryos lacking any of these regulators (Smith and Orr-Weaver, 1991; Smith et al., 1993; Stern et al., 1993). In contrast, the endo cell cycle does not occur in embryos lacking cyclin E, and cyclin E expression parallels S phase in endoreplicating tissues (Knoblich et al., 1994). This suggests that cyclin E perhaps regulates the entry into S phase similarly to its proposed role during the mitotic cell cycle. Novel regulators as well might be involved in the control of the endo cell cycle in order to coordinate the spatial and temporal regulation seen.

An interesting gene, *escargot*, is a transcription factor that appears to maintain the diploid state of arrested imaginal cells in the Drosophila larva (Hayashi et al., 1993; Fuse et al., 1994). In certain allelic combinations, a group of imaginal cells known as the histoblast nests overreplicate. Inappropriate expression of *escargot* in the polytene salivary gland represses endoreplication. A model has been proposed by which *escargot* maintains diploidy via transcriptional repression of regulators of the endo cell cycle. However an alternate model might be that *escargot* plays a more direct role in controlling the cell fate of imaginal cells.

In the fission yeast, *S. Pombe*, the absence of cyclin B due to a *cdc13* deletion causes cells to undergo multiple rounds of S phase resulting in high levels of polyploidy (Hayles et al., 1994). These results have led to a model whereby high levels of cyclin B-cdc2 kinase promote entry into M phase, and conversely, low levels cause the entry into S phase. Thus, by simply disrupting the cyclin B kinase activity, cells are able to reset to a G1 phase and enter S phase. In this example, *S. Pombe* has created a cell cycle leading to polyploidy solely by altering the control of mitotic cell cycle regulators. The cell cycle of *S. Pombe*, however, represents a very simplified cell cycle. In higher eukaryotes a more complex set of regulators controls the cell cycle and ensures the proper coupling of replication and mitosis. Therefore, in most organisms the transition to the endo cell cycle most likely requires more than simply inactivating G2/M regulators, although this might be a necessary step for the transition to polyteny. Other regulatory changes must also occur, such as the alteration of checkpoints that act to couple S phase and mitosis, as well as the block to rereplication which must be removed.

2. Removal of the Block to Rereplication

A major alteration in the parameters of replication that must occur during the endo cell cycle is the removal of the block to rereplication. One such proposed block to rereplication involves licensing factor which might act to limit the initiation of replication. Perhaps the action of licensing factor or other factors involved in replication initiation are altered in the endo cell cycle, but the nature of this alteration is unclear. However, in the case of the multiple rounds of replication that occur in *S. Pombe* as described above, the block to rereplication was removed solely by the disruption of cyclin B.

3. Late Replicating Heterochromatin/ Underrepresentation

Another aspect of altered replication during the endo cell cycle is the temporal control of polytene replication. Fiber autoradiography of polytene chromosomes shows a pattern of late replicating heterochromatic regions (reviewed in Spradling and Orr-Weaver, 1987). In *D. virilus* the replicon size in polytene chromosomes is similar to diploid brain cells, yet the rate of fork movement is three times slower (Steinemann, 1981a; Steinemann, 1981b). Similar studies of *D. nasuta* show that shorter and slower replicons are seen in late replicating regions (Lakhotia and Sinha, 1983). Perhaps the chromatin organization of polytene heterochromatin inhibits replication fork movement. This slower replication might account for the late replication of the heterochromatin.

A second characteristic alteration of DNA replication in the endo cell cycle is that 20-30% of the genome is underrepresented, including the centric heterochromatin, and rDNA and histone repeats. Less underrepresentation of rDNA is seen however in the polyploid nurse cells, most likely due to the function of the nurse cells in producing the rRNA for the developing oocyte. Regions of euchromatin are generally replicated to the same extent during polyploidization. In Drosophila polytene chromosomes, both bands and interbands also replicate to the same extent, as confirmed by quantitative Southern blots (Spierer and Spierer, 1984).

Heterochromatic underrepresentation might be caused by incomplete replication of specific sequences during polytenization, or alternatively by elimination of sequences from the chromosome (Karpen and Spradling, 1990; Glaser et al., 1992). One hypothesis is that elimination of specific regions is caused by the excision of transposable elements. Interestingly, many transposable elements are found solely in heterochromatic regions. A testable

prediction of the elimination model is that novel DNA junctions should be formed upon elimination. The role of underrepresentation during the endo cell cycle is unclear, perhaps these sequences are not needed for the high level of protein expression characteristic of most polytene tissues.

VI. AMPLIFICATION

Amplification of specific genomic sequences is a developmentally regulated mechanism that allows for the production of large amounts of protein products in a short developmental time frame. Amplification control occurs at the level of the block to rereplication of specific sequences, whereby reinitiation of replication leads to multiple copies of genomic sequences. Amplification provides a model system to study the developmental regulation of a eukaryotic replicon. Two well characterized examples of developmental amplification are the Drosophila chorion genes and the Sciara DNA puffs.

A. Drosophila Chorion Genes

During oogenesis, the somatic follicle cells surrounding the egg chamber are responsible for the secretion of the chorionic eggshell layers encompassing the developing oocyte. Following polyploidization of these cells, a further tissue-specific mechanism ensures an increased copy number of the chorion genes, so that proteins can be made in a rapid developmental time window. The major chorion genes are organized into two chromosomal clusters present on the X and 3rd chromosomes, which amplify to levels of 15 fold and 60 fold, respectively (reviewed in Orr-Weaver, 1991). Amplification within these clusters occurs by repeated reinitiation of an

origin as shown by multiple eye forms (bubbles within bubbles) in EM spreads (Osheim et al., 1988).

Studies of both clusters have identified cis-acting regions responsible for amplification, termed the amplification control element, (ACE). ACE3 of the 3rd chromosome cluster has been delineated to a 320 bp region that acts in a distance and orientation independent manner (Orr-Weaver et al., 1989). Replication intermediates in this region have been analyzed, and a predominant replication origin has been mapped 1.5 kb downstream of ACE3. This lies in a region important for high levels of amplification known as Amplification Enhancing Region-d, (AER-d) (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). However, initiation events also occur throughout a 12 kb region surrounding both ACE3 and AER-d.

ACE3 is able to direct the autonomous amplification of sequences when inserted throughout the genome, albeit at lower amplification levels (Carminati et al., 1992). These studies suggest a model by which ACE3 controls the reinitiation of nearby origins, perhaps by capturing limiting replication factors and overcoming a block to rereplication.

B. Sciara DNA Puffs

Amplification also occurs in the fungus fly *Sciara coprophila* within puff regions of the larval salivary gland polytene chromosomes. Similar to the Drosophila chorion genes, amplification is tissue and temporally regulated. Amplification of the Sciara DNA puffs is developmentally regulated by the steroid hormone, ecdysone (Bienz-Tadmor et al., 1991; Gerbi et al., 1993). Amplification presumably allows the rapid production of proteins needed during late larval development such as those for the

formation of the pupal case. Similarly, in Rhynchosciara, puffs encode polypeptides necessary for the production of the pupal cocoon.

Amplification occurs in two major puff regions in Sciara, and puff expansion is due to a burst of transcription following amplification of these sequences to approximately 20 fold levels (DiBartolomeis and Gerbi, 1989; Wu et al., 1993). DNA amplification within one of the major puffs, II/9A, has been well characterized and is thought to occur by an onion-skin mechanism, similar to the Drosophila chorion genes. Puff II/9A encodes two genes that share 85% sequence similarity, and a major amplification origin has been mapped to a 1 kb region lying upstream of the two genes (Liang et al., 1993; Liang and Gerbi, 1994). Replication from this origin occurs bidirectionally.

VII. SUMMARY

DNA replication is regulated by a wide variety of mechanisms that act throughout development to coordinate S phase and replication with developmental transitions. Replication can be controlled both at the level of key S phase regulators and at the level of parameters of DNA synthesis, such as origin usage. By studying the unique ways that different developmental events control and alter S phase, we will broaden our understanding of the regulators and mechanisms involved in DNA replication.

In the following chapters, two areas of research are described involving the developmental regulation of DNA replication. First, studies involving ACE3 and the control of chorion gene amplification are presented. Studies are aimed at determining the mechanism by which ACE3 controls the tissue and temporal regulation of repeated rounds of reinitiation. Second, the characterization of the maternal-effect gene, *pan gu*, is addressed, in order to determine how *pan gu* acts to couple S phase with fertilization, as well as

with M phase of the early embryonic divisions. Finally, experiments are described aimed at the molecular identification of the *pan gu* gene.

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REFERENCES

Anachkova, B. and J. L. Hamlin. 1989. Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. *9*: 532-40.

Axton, J. M., F. L. Shamanski, L. M. Young, D. S. Henderson, J. B. Boyd and T. L. Orr-Weaver. 1994. The inhibitor of DNA replication encoded by the Drosophila gene *plutonium* is a small, ankyrin repeat protein. Embo J. *13*: 462-70.

Bell, S. P. and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature *357*: 128-34.

Bell, S. P., R. Kobayashi, and B. Stillman. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. Science *262*: 1844-49.

Bienz-Tadmor, B., Smith, H. S., and Gerbi, S. A. 1991. The promoter of DNA puff gene II/9-1 of *Sciara coprophila* is inducible by ecdysone in late prepupal salivary glands of *Drosophila melanogaster*. Cell Regul. *2*, 875-8.

Blow, J. J. and R. A. Laskey. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. Nature *332*: 546-8.

Blumenthal, A. B., H. J. Kriegstein and D. S. Hogness. 1973. The units of DNA replication in *Drosophila melanogaster* chromosomes. Cold Spring Harb. Symp. Quant. Biol. *38*: 205-23.

Brewer, B. J. and W. L. Fangman. 1987. The localization of replication origins on *ARS* plasmids in *S. cerevisiae*. Cell *51*: 463-71.

Buck, V., A. White and J. Rosamond. 1991. CDC7 protein kinase activity is required for mitosis and meiosis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. *227*: 452-7.

Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz and M. L. DePamphilis. 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. Cell *62*: 955-65.

Callan, H. G. 1974. DNA replication in the chromosomes of eukaryotes. Cold Spring Harb. Symp. Quant. Biol. *38*: 195-203.

Carminati, J. L., C. G. Johnston and T. L. Orr-Weaver. 1992. The Drosophila ACE3 chorion element autonomously induces amplification. Mol. Cell. Biol. *12*: 2444-53.

Chandley, A. C. 1966. Studies on oogenesis in *Drosophila melanogaster* with 3H-thymidine label. Exp. Cell Res. *44*: 201-15.

Chiang, C., P. G. Mitsis and I. R. Lehman. 1993. DNA polymerase δ from embryos of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *90*: 9105-9.

Ciapa, B., D. Pesando, M. Wilding and M. Whitaker. 1994. Cell-cycle calcium transients driven by cyclic changes in inositol trisphosphate levels. Nature *368*: 875-8.

Collins, I. and C. S. Newlon. 1994. Chromosomal DNA replication initiates at the same origins in meiosis and mitosis. Mol. Cell. Biol. *14*: 3524-34.

Cotterill, S., I. R. Lehman and P. McLachlan. 1992. Cloning of the gene for the 73 kD subunit of the DNA polymerase alpha primase of *Drosophila melanogaster*. Nucleic Acids Res. *20*: 4325-30.

Cotterill, S. M., M. E. Reyland, L. A. Loeb and I. R. Lehman. 1987. A cryptic proofreading 3'-5' exonuclease associated with the polymerase subunit of the DNA polymerase-primase from *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *84*: 5635-9.

Crevel, G. and S. Cotterill. 1991. DNA replication in cell-free extracts from *Drosophila melanogaster*. Embo J. *10*: 4361-9.

Delidakis, C. and F. C. Kafatos. 1989. Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. Embo J. *8*: 891-901.

DiBartolomeis, S. M. and S. A. Gerbi. 1989. Molecular characterization of DNA puff II/9A genes in *Sciara coprophila*. J. Mol. Biol. *210*: 531-43.

Diffley, J. F., J. H. Cocker, S. J. Dowell and A. Rowley. 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell *78*: 303-16.

Dowell, S. J., P. Romanowski and J. F. Diffley. 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science *265*: 1243-6.

Duronio, R. J. and P. H. O'Farrell. 1994. Developmental control of a G1-S transcriptional program in Drosophila. Development *120*: 1503-15.

Dynlacht, B. D., O. Flores, J. A. Lees and E. Harlow. 1994. Differential regulation of E2F trans-activation by cyclin/cdk2 complexes. Genes Dev. *8*: 1772-86.

Edgar, B. A., C. P. Kiehle and G. Schubiger. 1986. Cell cycle control by the nucleo-cytoplasmic ratio in early Drosophila development. Cell *44*: 365-72.

Edgar, B. A. and P. H. O'Farrell. 1990. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by *string*. Cell *62*: 469-80.

Edgar, B. A. and G. Schubiger. 1986. Parameters controlling transcriptional activation during early Drosophila development. Cell *44*: 871-7.

Epstein, C. B. and F. R. Cross. 1992. *CLB5*: a novel B cyclin from budding yeast with a role in S phase. Genes Dev. *6*: 1695-706.

Ferguson, B. M., B. J. Brewer, A. E. Reynolds and W. L. Fangman. 1991. A yeast origin of replication is activated late in S phase. Cell *65*: 507-15.

Ferguson, B. M. and W. L. Fangman. 1992. A position effect on the time of replication origin activation in yeast. Cell *68*: 333-9.

Foe, V. E., G. M. Odell and B. A. Edgar. 1993. Mitosis and morphogenesis in the Drosophila embryo: point and counterpoint. In *The development of Drosophila melanogaster*, ed. M. Bate and A. M. Arias, pp. 149-300. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.).

Freeman, M. and D. M. Glover. 1987. The *gnu* mutation of Drosophila causes inappropriate DNA synthesis in unfertilized and fertilized eggs. Genes Dev. *1*: 924-30.

Freeman, M., C. Nüsslein-Volhard and D. M. Glover. 1986. The dissociation of nuclear and centrosomal division in *gnu*, a mutation causing giant nuclei in Drosophila. Cell *46*: 457-68.

Furuno, N., M. Nishizawa, K. Okazaki, H. Tanaka, J. Iwashita, N. Nakajo, Y. Ogawa and N. Sagata. 1994. Suppression of DNA replication via Mos function during meiotic divisions in Xenopus oocytes. Embo J. *13*: 2399-410.

Fuse, N., S. Hirose and S. Hayashi. 1994. Diploidy of Drosophila imaginal cells is maintained by a transcriptional repressor encoded by *escargot*. Genes Dev. *8*: 2270-81.

Gerbi, S. A., Liang, C., Wu, N., DiBartolomeis, S. M., Bienz-Tadmor, B., Smith, H. S., and Urnov, F. D. 1993. DNA amplification in DNA puff II/9A of *Sciara coprophila*. Cold Spring Harb. Symp. Quant. Biol. *58*, 487-93.

Glaser, R. L., G. H. Karpen and A. C. Spradling. 1992. Replication forks are not found in a Drosophila minichromosome demonstrating a gradient of polytenization. Chromosoma *102*: 15-9.

Grell, R. F. 1973. Recombination and DNA replication in the *Drosophila melanogaster* oocyte. Genetics *73*: 87-108.

Harland, R. M. and R. A. Laskey. 1980. Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. Cell *21*: 761-71.

Hayashi, S., S. Hirose, T. Metcalfe and A. D. Shirras. 1993. Control of imaginal cell development by the *escargot* gene of Drosophila. Development *118*: 105-15.

Hayles, J., D. Fisher, A. Woollard and P. Nurse. 1994. Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. Cell *78*: 813-22.

Heck, M. M. and A. C. Spradling. 1990. Multiple replication origins are used during Drosophila chorion gene amplification. J. Cell Biol. *110*: 903-14.

Heintz, N. H. and J. L. Hamlin. 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. *79*: 4083-7.

Henderson, D. S., S. S. Banga, T. A. Grigliatti and J. B. Boyd. 1994. Mutagen sensitivity and suppression of position-effect variegation result from

mutations in *mus209*, the Drosophila gene encoding PCNA. Embo J. *13*: 1450-9.

Hennessy, K. M., C. D. Clark and D. Botstein. 1990. Subcellular localization of yeast *CDC46* varies with the cell cycle. Genes Dev. *4*: 2252-63.

Hirose, F., M. Yamaguchi, Y. Hishida, M. Masutani, H. Miyasawa, F. Hanaoka and A. Matsukage. 1991. Structure and expression during development of *Drosophila melanogaster* gene for DNA polymerase α. Nucleic Acids Res. *19*: 4991-8.

Hollingsworth, R., Jr. and R. A. Sclafani. 1993. Yeast pre-meiotic DNA replication utilizes mitotic origin *ARS1* independently of *CDC7* function. Chromosoma *102*: 415-20.

Hsieh, T. S., S. D. Brown, P. Huang and J. Fostel. 1992. Isolation and characterization of a gene encoding DNA topoisomerase I in *Drosophila melanogaster*. Nucleic Acids Res. *20*: 6177-82.

Hsieh, T. S., M. P. Lee, J. M. Nolan and E. Wyckoff. 1987. Molecular genetic analysis of topoisomerase II gene from *Drosophila melanogaster*. Nci Monogr. *4*: 7-10.

Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. el-Assouli and L. R. Davis. 1987. The in vivo replication origin of the yeast 2 microns plasmid. Cell *51*: 473-81.

Hyrien, O. and M. Mechali. 1992. Plasmid replication in Xenopus eggs and egg extracts: a 2D gel electrophoretic analysis. Nucleic Acids Res. *20*: 1463-9.

Hyrien, O. and M. Mechali. 1993. Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of Xenopus early embryos. Embo J. *12*: 4511-20.

Jackson, A. L., P. M. Pahl, K. Harrison, J. Rosamond and R. A. Sclafani. 1993. Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol. Cell. Biol. *13*: 2899-908.

Jagiello, G., W. K. Sung and J. Van't Hof. 1983. Fiber DNA studies of premeiotic mouse spermatogenesis. Exp. Cell Res. *146*: 281-7.

Johnston, L. H., D. H. Williamson, A. L. Johnson and D. J. Fennell. 1982. On the mechanism of premeiotic DNA synthesis in the yeast *Saccharomyces cerevisiae*. Exp. Cell Res. *141*: 53-62.

Karpen, G. H. and A. C. Spradling. 1990. Reduced DNA polytenization of a minichromosome region undergoing position-effect variegation in Drosophila. Cell *63*: 97-107.

Kimelman, D., M. Kirschner and T. Scherson. 1987. The events of the midblastula transition in Xenopus are regulated by changes in the cell cycle. Cell *48*: 399-407.

Kirschner, M., J. Newport and J. Gerhart. 1985. The timing of early developmental events in Xenopus. Trends Genet. *1*: 41-7.

Kitsberg, D., S. Selig, I. Keshet and H. Cedar. 1993. Replication structure of the human β-globin gene domain. Nature *366*: 588-90.

Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint and C. F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell *77*: 107-20.

Krek, W., M. E. Ewen, S. Shirodkar, Z. Arany, W. Kaelin Jr. and D. M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell *78*: 161-72.

Lakhotia, S. C. and P. Sinha. 1983. Replication in Drosophila chromosomes. X. Two kinds of active replicons in salivary gland polytene nuclei and their relation to chromosomal replication patterns. Chromosoma *88*: 265-76.

Lee, M. P., S. D. Brown, A. Chen and T. S. Hsieh. 1993. DNA topoisomerase I is essential in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *90*: 6656-60.

Leibovici, M., G. Monod, J. Geraudie, R. Bravo and M. Mechali. 1992. Nuclear distribution of PCNA during embryonic development in *Xenopus laevis*: a reinvestigation of early cell cycles. J. Cell Sci. *102*: 63-9.

Leu, T. H. and J. L. Hamlin. 1989. High-resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Mol. Cell. Biol. *9*: 523-31.

Liang, C. and S. A. Gerbi. 1994. Analysis of an origin of DNA amplification in *Sciara coprophila* by a novel three-dimensional gel method. Mol. Cell. Biol. *14*: 1520-9.

Liang, C., J. D. Spitzer, H. S. Smith and S. A. Gerbi. 1993. Replication initiates at a confined region during DNA amplification in Sciara DNA puff II/9A. Genes Dev. *7*: 1072-84.

Linskens, M. H. and J. A. Huberman. 1990. The two faces of higher eukaryotic DNA replication origins. Cell *62*: 845-7.

Lorca, T., F. H. Cruzalegui, D. Fesquet, J. C. Cavadore, J. Mery, A. Means and M. Doree. 1993. Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs. Nature *366*: 270-3.

Mahbubani, H. M., T. Paull, J. K. Elder and J. J. Blow. 1992. DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts. Nucleic Acids Res. *20*: 1457-62.

Martinez-Salas, E., D. Y. Cupo and M. L. DePamphilis. 1988. The need for enhancers is acquired upon formation of a diploid nucleus during early mouse development. Genes Dev. *2*: 1115-26.

Martinez-Salas, E., E. Linney, J. Hassell and M. L. DePamphilis. 1989. The need for enhancers in gene expression first appears during mouse development with formation of the zygotic nucleus. Genes Dev. *3*: 1493-506.

Marton, R. F., P. Thommes and S. Cotterill. 1994. Purification and characterisation of dRP-A: a single-stranded DNA binding protein from *Drosophila melanogaster*. FEBS Lett. *342*: 139-44.

McKnight, S. L. and O. L. J. Miller. 1977. Electron microscopic analysis of chromatin replication in the cellular blastoderm *Drosophila melanogaster* embryo. Cell *12*: 795-804.

Mechali, M. and S. Kearsey. 1984. Lack of specific sequence requirement for DNA replication in Xenopus eggs compared with high sequence specificity in yeast. Cell *38*: 55-64.

Minshull, J. 1993. Cyclin synthesis: who needs it? Bioessays *15*: 149-55.

Newlon, C. S. 1988. Yeast chromosome replication and segregation. Microbiol. Rev. *52*: 568-601.

Newport, J. and M. Kirschner. 1982a. A major developmental transition in early Xenopus embryos. I. Characterization and timing of cellular changes at the midblastula stage. Cell *30*: 675-86.

Newport, J. and M. Kirschner. 1982b. A major developmental transition in early Xenopus embryos. II. Control of the onset of transcription. Cell *30*: 687-96.

Newport, J., T. Spann, J. Kanki and D. Forbes. 1987. The role of mitotic factors in regulating the timing of the midblastula transition in Xenopus. Cold Spring Harb. Symp. Quant. Biol. *50*: 651-6.

Ohsumi, K., W. Sawada and T. Kishimoto. 1994. Meiosis-specific cell cycle regulation in maturing Xenopus oocytes. J. Cell Sci. *107*: 3005-13.

Orr-Weaver, T. L. 1991. Drosophila chorion genes: cracking the eggshell's secrets. Bioessays *13*: 97-105.

Orr-Weaver, T. L., C. G. Johnston and A. C. Spradling. 1989. The role of ACE3 in Drosophila chorion gene amplification. Embo J. *8*: 4153-62.

Osheim, Y. N., O. Miller Jr. and A. L. Beyer. 1988. Visualization of *Drosophila melanogaster* chorion genes undergoing amplification. Mol. Cell. Biol. *8*: 2811-21.

Peck, V. M., E. W. Gerner and A. E. Cress. 1992. Delta-type DNA polymerase characterized from *Drosophila melanogaster* embryos. Nucleic Acids Res. *20*: 5779-84.

Peck, V. M., E. W. Gerner and A. E. Cress. 1993. A DNA polymerase alpha-associated 56 kDa protein kinase. Biochem. Biophys. Res. Commun. *190*: 325-31.

Picard, A., E. Karsenti, M. C. Dabauvalle and M. Doree. 1987. Release of mature starfish oocytes from interphase arrest by microinjection of human centrosomes. Nature *327*: 170-2.

Piggott, J. R., R. Rai and B. L. A. Carter. 1982. A bifunctional gene product involved in two phases of the yeast cell cycle. Nature *298*: 391-3.

Prioleau, M. N., J. Huet, A. Sentenac and M. Mechali. 1994. Competition between chromatin and transcription complex assembly regulates gene expression during early development. Cell *77*: 439-49.

Reed, S. I. and C. Wittenberg. 1990. Mitotic role for the *Cdc28* protein kinase of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. *87*: 5697-701.

Reynolds, A. E., R. M. McCarroll, C. S. Newlon and W. L. Fangman. 1989. Time of replication of *ARS* elements along yeast chromosome III. Mol. Cell. Biol. *9*: 4488-94.

Richardson, H. E., L. V. O'Keefe, S. I. Reed and R. Saint. 1993. A Drosophila G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. Development *119*: 673-90.

Richardson, H. E., C. Wittenberg, F. Cross and S. I. Reed. 1989. An essential G1 function for cyclin-like proteins in yeast. Cell *59*: 1127-33.

Sagata, N., M. Oskarsson, T. Copeland, J. Brumbaugh and G. F. Vande Woude. 1988. Function of *c-mos* proto-oncogene product in meiotic maturation in Xenopus oocytes. Nature *335*: 519-25.

Schild, D. and B. Byers. 1978. Meiotic effects of DNA-defective cell division cycle mutations of *Saccharomyces cerevisiae*. Chromosoma *70*: 109-30.

Schwob, E., T. Bohm, M. D. Mendenhall and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40*SIC1* controls the G1 to S transition in *S. cerevisiae*. Cell *79*: 233-44.

Schwob, E. and K. Nasmyth. 1993. *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. Genes Dev. *7*: 1160-75.

Serrano, M., G. J. Hannon and D. Beach. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*: 704-7.

Shamanski, F. L. and T. L. Orr-Weaver. 1991. The Drosophila *plutonium* and *pan gu* genes regulate entry into S phase at fertilization. Cell *66*: 1289-300.

Sherr, C. J. 1993. Mammalian G1 cyclins. Cell *73*: 1059-65.

Sherr, C. J. 1994. G1 phase progression: cycling on cue. Cell *79*: 551-5.

Shinomiya, T. and S. Ina. 1991. Analysis of chromosomal replicons in early embryos of *Drosophila melanogaster* by two-dimensional gel electrophoresis. Nucleic Acids Res. *19*: 3935-41.

Shinomiya, T. and S. Ina. 1993. DNA replication of histone gene repeats in *Drosophila melanogaster* tissue culture cells: multiple initiation sites and replication pause sites. Mol. Cell. Biol. *13*: 4098-106.

Shinomiya, T. and S. Ina. 1994. Mapping an initiation region of DNA replication at a single-copy chromosomal locus in *Drosophila melanogaster* cells by two-dimensional gel methods and PCR-mediated nascent-strand analysis: multiple replication origins in a broad zone. Mol. Cell. Biol. *14*: 7394-403.

Shuster, E. O. and B. Byers. 1989. Pachytene arrest and other meiotic effects of the start mutations in *Saccharomyces cerevisiae*. Genetics *123*: 29-43.

Simchen, G. and J. Hirschberg. 1977. Effects of the mitotic cell-cycle mutation *cdc4* on yeast meiosis. Genetics *86*: 57-72.

Smith, A. V., J. A. King and T. L. Orr-Weaver. 1993. Identification of genomic regions required for DNA replication during Drosophila embryogenesis. Genetics *135*: 817-29.

Smith, A. V. and T. L. Orr-Weaver. 1991. The regulation of the cell cycle during Drosophila embryogenesis: the transition to polyteny. Development *112*: 997-1008.

Spierer, A. and P. Spierer. 1984. Similar level of polyteny in bands and interbands of Drosophila giant chromosomes. Nature *307*: 176-78.

Spradling, A. and T. Orr-Weaver. 1987. Regulation of DNA replication during Drosophila development. Ann. Rev. Genet. *21*: 373-403.

Steinemann, M. 1981a. Chromosomal replication in *Drosophila virilis*. II. Organization of active origins in diploid brain cells. Chromosoma *82*: 267-88.

Steinemann, M. 1981b. Chromosomal replication in *Drosophila virilis*. III. Organization of active origins in the highly polytene salivary gland cells. Chromosoma *82*: 289-307.

Stern, B., G. Ried, N. J. Clegg, T. A. Grigliatti and C. F. Lehner. 1993. Genetic analysis of the Drosophila *cdc2* homolog. Development *117*: 219-32.

Taira, T., S. M. M. Iguchi-Ariga and H. Ariga. 1994. A novel DNA replication origin identified in the human heat shock protein 70 gene promoter. Mol. Cell. Biol. *14*: 6386-97.

Tasheva, E. S. and D. J. Roufa. 1994. A mammalian origin of bidirectional DNA replication within the Chinese Hamster RPS14 locus. Mol. Cell. Biol. *14*: 5628-35.

Vassilev, L. and E. M. Johnson. 1990. An initiation zone of chromosomal DNA replication located upstream of the *c-myc* gene in proliferating HeLa cells. Mol. Cell. Biol. *10*: 4899-904.

Vaughn, J. P., P. A. Dijkwel and J. L. Hamlin. 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell *61*: 1075-87.

Wernette, C. M. and L. S. Kaguni. 1986. A mitochondrial DNA polymerase from embryos of *Drosophila melanogaster*. Purification, subunit structure, and partial characterization. J. Biol. Chem. *261*: 14764-70.

Whitaker, M. and R. Patel. 1990. Calcium and cell cycle control. Development *108*: 525-42.

Whitaker, M. and K. Swann. 1993. Lighting the fuse at fertilization. Development *117*: 1-12.

Williamson, D. H., L. H. Johnston, D. J. Fennell and G. Simchen. 1983. The timing of the S phase and other nuclear events in yeast meiosis. Exp. Cell Res. *145*: 209-17.

Wirak, D. O., L. E. Chalifour, P. M. Wassarman, W. J. Muller, J. A. Hassell and M. L. DePamphilis. 1985. Sequence-dependent DNA replication in preimplantation mouse embryos. Mol. Cell. Biol. *5*: 2924-35.

Wu, N., C. Liang, S. M. DiBartolomeis, H. S. Smith and S. A. Gerbi. 1993. Developmental progression of DNA puffs in *Sciara coprophila*: amplification and transcription. Dev. Biol. *160*: 73-84.

Wyckoff, E. and T. S. Hsieh. 1988. Functional expression of a Drosophila gene in yeast: genetic complementation of DNA topoisomerase II. Proc. Natl. Acad. Sci. *85*: 6272-6.

Wyckoff, E., D. Natalie, J. M. Nolan, M. Lee and T. Hsieh. 1989. Structure of the Drosophila DNA topoisomerase II gene. Nucleotide sequence and homology among topoisomerases II. J. Mol. Biol. *205*: 1-13.

Yan, H., A. M. Merchant and B. K. Tye. 1993. Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. Genes Dev. *7*: 2149-60.

Zhang, H. and J. V. Ruderman. 1993. Differential replication capacities of G1 and S-phase extracts from sea urchin eggs. J. Cell Sci. *104*: 565-72.

CHAPTER II.

The Drosophila ACE3 Chorion Element Autonomously Induces Amplification

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ABSTRACT

The chorion genes of Drosophila amplify in the follicle cells by repeated rounds of reinitiation of DNA replication. ACE3 has been identified by a series of deletion experiments as an important control element for amplification of the third chromosome chorion cluster. Several AERs, elements that quantitatively enhance amplification, also have been defined. We show that a single 440 bp ACE3 sequence is sufficient to regulate amplification with proper developmental specificity autonomously from other chorion DNA sequences and regulatory elements. Although ACE3 is sufficient for amplification, the levels of amplification are low even when ACE3 is present in multiple copies.

When controlled solely by ACE3, amplification initiates either at ACE3 or within closely linked sequences. Amplification of an ACE3 transposon insertion produces a gradient of amplified DNA that extends into flanking sequences approximately the same distance as does the amplification gradient at the endogenous chorion locus. The profile and extent of the amplified gradient imply that the low levels of amplification observed are the result of limited rounds of initiation of DNA replication. Transposon inserts containing multiple copies of ACE3 in a tandem, head to tail array, are maintained stably in the chromosome. However, mobilization of the P element transposons containing ACE3 multimers results in deletions within the array at a high frequency.

INTRODUCTION

A crucial step in understanding the mechanism of DNA replication is the identification of the sites at which DNA replication initiates. While origins have been identified for eukaryotic DNA viruses, the isolation of chromosomal origins in higher eukaryotes has proven difficult. It has been possible to map replication origins in viruses due to the many cycles of replication that initiate at defined sites on the virus genome during infection. Viral origins have been mapped both by mutational studies and by analysis of nascent DNA fragments (Stillman, 1989; Marraccino et al., 1990). In addition, an in vitro replication system for SV40 was shown to be dependent on a functional origin, permitting the minimal origin to be defined (Li et al., 1986). These studies have demonstrated specific, sequence-dependent, viral origins and have shown that in most cases the replication origins overlap with control elements regulating transcription (DePamphilis, 1988).

Chromosomal origins of replication were isolated in yeast by their ability to maintain plasmids extrachromosomally. These ARS, Autonomously Replicating Sequences, have subsequently been confirmed as sites of initiation of DNA replication on yeast chromosomes by the analysis of replication intermediates on two-dimensional gel systems (Brewer and Fangman, 1988; Huberman et al., 1988; Linsken and Huberman, 1988). However, episomal assays for replication origins have not been successful in higher eukaryotes, impeding the identification of origins.

The mapping of replication origins has been facilitated by mammalian cell culture lines in which chromosomal intervals were amplified in response to selective conditions. In these lines the selected gene can be present in up to several thousand copies. The high copy number magnifies

the signal to background ratio in techniques directed at mapping origins. Using a CHO cell line in which the DHFR gene is present in about 1000 copies, Hamlin and coworkers were able to define a 28 kb interval in which DNA replication initiates (Heintz and Hamlin, 1982; Anachkova and Hamlin, 1989; Leu and Hamlin, 1989). However, the precise mechanism of initiation within this interval is unclear. By mapping the position of Okazaki fragments, a predominant origin of bidirectional replication was delineated to a 0.45 kb sequence (Burhans et al., 1990), yet analysis of replication intermediates on two-dimensional gel electrophoresis failed to identify a specific site of replication initiation (Vaughn et al., 1990). The two techniques utilized test different aspects of replication initiation, so there are several possible ways to reconcile the seemingly different conclusions. One explanation is that while many origins within an interval may initially fire, a preferred single one may eventually predominate (Linskens and Huberman, 1990).

Amplification has proven an informative model system for the investigation of the regulation of DNA replication in Drosophila. During Drosophila follicle cell differentiation the chorion genes, which encode the eggshell proteins, are amplified; this amplification is essential to allow sufficient expression of the genes during a short developmental period (for review see Orr-Weaver, 1991). The majority of the chorion genes are localized in two clusters in the genome, one on the X chromosome and one on the third chromosome. Both of these clusters undergo amplification in the follicle cells, and several lines of investigation have shown that chorion amplification results from multiple rounds of DNA replication initiating within each cluster (Spradling, 1981; Spradling and Mahowald, 1981; Osheim and Miller, 1983; Osheim et al., 1988). Drosophila chorion gene amplification is in several ways simpler to investigate than mammalian cell culture

amplification. Amplification is temporally regulated and tissue specific, so that the process can be analyzed from its onset during follicle cell differentiation. The multiple rounds of replication result in an "onion skin" structure and secondary rearrangements do not occur, thus permitting investigation of the primary event responsible for amplification (Spradling, 1981; Osheim and Miller, 1983). Finally, and most importantly, Drosophila permits a genetic analysis of amplification.

Thus the Drosophila chorion genes provide a defined chromosomal replicon subject to developmental regulation. The cis-acting control elements necessary for amplification of the third chromosome chorion cluster have been defined by germline transformation experiments using P element transposons (Orr-Weaver, 1991). ACE3, Amplification Control Element from the third chromosome, was identified initially because it was essential for amplification of chorion transposons (de Cicco and Spradling, 1984; Orr-Weaver and Spradling, 1986; Orr-Weaver et al., 1989). Several AERs, Amplification Enhancing Regions, were identified, both in transposon constructs and by deletions generated in situ, by their ability to quantitatively increase levels of amplification (Delidakis and Kafatos, 1987; Delidakis and Kafatos, 1989). The amplification origins, the sites at which DNA replication initiates during amplification, have been mapped by two-dimensional gel analysis. A predominant origin, estimated to be utilized 70-80% of the time, is positioned about 1.5 kb away from ACE3 in the chorion cluster, in a region that functions as an AER (Delidakis and Kafatos, 1989; Heck and Spradling, 1990).

ACE3 was delineated and characterized in the context of a large (7.7 kb *Eco*RI) chorion fragment that contained three chorion genes (Orr-Weaver and Spradling, 1986; Orr-Weaver et al., 1989). A set of small deletions was used to

map ACE3 within the 7.7 kb *Eco*RI fragment by testing the effect of each deletion on amplification following transformation of the chorion fragment in a P element transposon. Deletion of ACE3 in this transposon eliminated amplification, and the deletions localized ACE3 to a 320 bp region (Orr-Weaver and Spradling, 1986; Orr-Weaver et al., 1989). ACE3 was shown to function independently of orientation with respect to the chorion gene cluster and to be composed of multiple domains (Orr-Weaver et al., 1989). It is not clear whether ACE3 has a unique function that is mechanistically distinct from that of the AERs or the amplification origin. Recently it has been found that ACE3 is not essential in some transposon constructs containing two AERs (Swimmer et al., 1989). This raises the possibility that the AERs can fulfill the same regulatory function as ACE3, but that ACE3 is merely the strongest of these elements. Moreover, it is possible that ACE3 can serve as an amplification origin. In addition to the predominant amplification origin, several minor origins were observed. One of these lies in the vicinity of ACE3, but it has not been localized with sufficient precision to know if it overlaps ACE3 (Heck and Spradling, 1990).

Due to the complexity of control elements within the chorion cluster we sought to investigate ACE3 in isolation and to test whether it was sufficient to regulate amplification with proper developmental specificity. We also wanted to determine whether ACE3 was capable of acting as an amplification origin. We describe experiments that show that ACE3 is sufficient to regulate chorion amplification, and that demonstrate that amplification either initiates within ACE3 or in nearby sequences.

METHODS

Strains

The *E. coli* JA300 recA$^-$ strain was used for plasmid constructions and LE392 was used for electroporation (Borck et al., 1976; Murray et al., 1977; Tschumper and Carbon, 1980). *Rosy*^{506} flies were used for all transformation experiments. To mobilize transposons to new locations *CyO/Sp; ry*^{506} *Sb* P[ry$^+$ Δ2-3] (99B)/*TM6, Ubx* was crossed in as a source of transposase. This stock was constructed by Engels (Robertson et al., 1988).

Plasmid constructions

The ACE3 multimer was constructed by generating a *Sal*I site at one end of ACE3 and a *Xho*I site at the other end and multimerizing this fragment. The *Sal*I and *Xho*I sites were added by using a 3.8 kb *Sal*I chorion fragment containing ACE3 and the *s18* and *s15* genes cloned into the polylinker of a pUC derived plasmid (Levine and Spradling, 1985). The *Bal*I site at -190 relative to the *s18* gene was turned into a *Xho*I site by adding linkers. The plasmid was partially digested with *Bal*I, which preferentially cleaves at the *Bal*I site at -190, *Xho*I linkers were ligated on, and a *Xho*I digest followed by ligation resulted in a *Xho*I site at -190 as well as deletion from -190 to the *Xho*I site at the 3' end of *s15*. A *Sal*I site was generated at -630 5' to the *s18* gene by making a deletion from the *Sal*I site upstream of *s18* to a *Kpn*I site at -630. This plasmid was denoted $pA_{70}O_9$.

The -630 to -190 ACE3 fragment was cut from $pA_{70}O_9$, gel purified, and ligated to create tandem head to tail repeats in a stepwise manner. The *Sal*I-*Xho*I fragment was ligated under conditions of high DNA concentration with polyethylene glycol in the presence of *Sal*I and *Xho*I enzymes. This ensured

that only direct repeats would be generated. A trimer of ACE3 was generated and cloned back into $pA_{70}O_9$. This trimer was then re-isolated and the ligation protocol was repeated to generate a 9-mer. This ligation mix was then phenol extracted and directly cloned into the Carnegie 20 transformation vector (Rubin and Spradling, 1983) cut with *Sal*I. Two inserts were obtained; one with one copy of the 9-mer, denoted P[rosy, chorion M9], and one with two copies of the 9-mer, denoted P[rosy, chorion M18]. These will be abbreviated as M9 and M18 respectively. The ACE3 repeats are in the opposite orientation relative to *rosy* in M9 and M18 (Figures 1A and 1B).

To construct P[rosy, chorion M9pUC], pUC18 was cleaved with *Sma*I and *Sal*I and ligated into the *Hpa*I and *Sal*I sites of M9. This will be referred to as M9pUC (Figure 1C).

To construct a transposon with a single copy of ACE3 flanked by pUC DNA, a derivative of the $pA_{70}O_9$ plasmid was inserted into the P element on Carnegie 20. Since $pA_{70}O_9$ contains both ACE3 and the 3' untranslated region of *s15*, the segment of chorion DNA flanking the *s15* gene was deleted by removing a *Xho*I-*Sal*I fragment. A partial *Sal*I digest of $pA_{70}O_9$ was done, and full length linear fragments were isolated from a gel and then cleaved to completion with *Xho*I. This fragment was then self-ligated, re-digested with *Xho*I and used to transform JA300 bacteria. Plasmids containing a deletion of -190 (*Xho*I) to the *Sal*I site 3' of the *s15* gene were identified by restriction mapping. This plasmid was cleaved with *Sma*I and *Sal*I which both cut in the polylinker to one side of the chorion DNA, and the entire plasmid was cloned into the *Hpa*I and *Sal*I sites of Carnegie 20 to generate P[rosy, chorion M1pUC], which will be abbreviated as M1pUC (Figure 1D).

P-element transformation

For constructs M9, M18 and M9pUC, transposons were coinjected with a transposase gene into ry^{506} embryos and the resulting flies crossed to ry^{506} flies to identify transformants as described (Orr-Weaver and Spradling, 1986). For injection of the M1pUC construct, the transposase activity was supplied by a transposase-containing chromatographic fraction (the "TdT 0.3M" fraction) as described (Kaufman and Rio, 1991). 1 µl of DNA at 1mg/ml was added to a 3 µl aliquot of transposase at 1.5 mg/ml and an aliquot of this was injected into ry^{506} embryos. 268 embryos were injected and 1 independent line was obtained.

To generate new lines of M18 and M1pUC, $rosy^+$ transformants on the second chromosome were crossed to *CyO/Sp*; ry^{506} *Sb* P[ry^+ Δ2-3] (99B)/*TM6, Ubx* in order to mobilize the transposon (Robertson et al., 1988). Males that were ry^+, Sb, and either CyO or Sp were crossed to ry^- females. Lines in which the transposon had mobilized off the second chromosome were detected by ry^+ flies that were CyO or Sp in the next generation, and the *Sb* marker was used to select against the presence of the transposase gene in these new insertion lines. For M9pUC an X-linked transformant was mobilized by crossing ry^+ females to the same transposase stock to obtain ry^+ males bearing the Δ2-3 transposase gene. Hops off the X were identified by ry^+ males among the progeny obtained from crossing these males to ry^- females.

Independent transformant insertions were confirmed by Southern analysis of DNA from transformed flies (Orr-Weaver and Spradling, 1986). DNA from transformants was also restriction mapped to determine whether the transposon had rearranged. In all but one case single insertion lines were used for the amplification assays; one of the M1pUC lines (insertions 2 and 3)

analyzed had two separate insertions that could be differentiated in an amplification blot.

Amplification assays

Amplification of the transposons was assayed as described previously by comparing stage 1-8 egg chamber DNA with amplifying stage 13 egg chamber DNA on a quantitative Southern blot (Orr-Weaver and Spradling, 1986). Amplification initiates in stage 9 of egg chamber development and reaches maximal levels at stage 13. The ratio of intensity of a transposon fragment to a host non-amplifying fragment in stage 13 egg chambers was compared to the same ratio from stage 1-8 egg chamber DNA. Southern blots were hybridized as previously described (Orr-Weaver and Spradling, 1986), or the DNA was transferred to nylon membranes (GeneScreen, NEN), UV crosslinked using a UV Stratalinker (Stratagene), and hybridization was performed as described (Church and Gilbert, 1984). Probes were labeled either by nick-translation or by random priming of DNA fragments that had been isolated from low melt agarose gels. Quantitation of amplification levels for most of the transposon insertion lines was done using a Molecular Dynamics Densitometer with ImageQuant software and preflashed film. The amplification gradients and the M1pUC amplification levels were quantified on a Molecular Dynamics PhosphorImager with ImageQuant software. For most blots, two *rosy*^{506} host bands were used as non-amplifying standards, and two transposon bands were quantified. Amplification was determined for both bands relative to the two standards, and the average amplification value taken. Amplification values of 1.5 or higher were scored as amplification positive. Fragments quantified as amplifying 1.5 or higher by visual inspection were clearly amplified.

Gradient Analysis

Flanking DNA was cloned from both lines M9-2 and M9pUC-3 in order to analyze the amplification gradient profile of the flanking regions. A lambda genomic library was made from M9-2 using the Lambda Fix vector (Stratagene). 34 kb of DNA containing the transposon and flanking sequences was isolated, subcloned into pGEM vectors (Promega), and restriction enzyme mapped. The pUC sequence in M9pUC-3 was used to clone the region of this transposon directly into *E. coli* by plasmid rescue. To clone the transposon as well as flanking sequences to the left of the insert, Drosophila genomic DNA from M9pUC-3 was digested with *Kpn*I, which cleaves in pUC at the end of the transposon and in the flanking DNA to the left of the transposon. The DNA was ligated to recircularize the fragments, and electroporated (Gene Pulser, BioRad) into *E. coli* LE392 cells. The plasmid containing the transposon and flanking sequences was isolated using ampicillin selection. Thus the transposon as well as 5 kb of flanking DNA to the left was cloned directly into *E. coli* and subsequently plasmid purified. Similarly, *Bam*HI and *Sal*I digests allowed us to clone pUC DNA within the transposon and sequences to the right side of the insert. 18 kb of flanking DNA was cloned, purified, and mapped with restriction enzymes. For both gradients analyzed, the transposon and flanking sequences were used as probes in quantitative Southerns to determine amplification values as described above.

For both M9-2 and M9pUC-3 heterozygous flies balanced over *CyO* were used to obtain stage 1-8 and stage 13 egg chamber DNA. Both lines are homozygous lethal and thus had to be tested as heterozygotes. PhosphorImager quantitation of each Southern blot was repeated four times to determine amplification values for all fragments analyzed on the blot. The

four amplification values for each fragment were then averaged. For fragments unique to the transposon, amplification values were determined as described. However, the amplification analysis of flanking regions was complicated by the fact that fragments that were not unique to the transposon would be detected both in the amplifying chromosome and in the nonamplifying homolog. Therefore a single amplifying band would contain one copy of the fragment from the non-amplifying homolog. This fragment would also contribute to the signal in the stage 1-8 band, giving the following: observed amplification value= (stage 13 amplifying band + 1)/ (stage 1-8 band + 1). To correct for the contribution of the non-amplifying chromosome in both stage 1-8 and stage 13 DNA, the following formula was used: Amplification value= (2 X observed amplification value) - 1.

Several different digests and Southern blots were performed to analyze the region spanning the gradients. Digests were chosen to obtain overlapping fragments along the gradient. In Figure 3, each line represents the average quantitation of a band from a single blot. The same type of line denotes all fragments probed in a single blot. For line M9-2, four different blots were used in the quantitation, and for line M9pUC-3, three blots were quantified. Statistical analysis was performed using the Wilcoxon's rank sum test (Lindren et al., 1978) to determine if the differences in amplification values along the gradient were statistically significant.

Two Dimensional Gel Analysis

Stage 10 egg chamber DNA was purified as previously described (Orr-Weaver and Spradling, 1986), except that 400-1000 stage 10 egg chambers were used for each digest. To separate replicating intermediates, two dimensional gel analysis was performed as described (Brewer and Fangman, 1987). The

first dimension was run through a 0.4% agarose gel in TBE buffer at 1V/cm for 36 hours. The second dimension was then run through 1% agarose containing 0.3 μg/ml ethidium bromide in TBE buffer with 0.3 μg/ml ethidium bromide. The second dimension gel was run with circulating buffer at 5V/cm for 10-12 hours at 4°C. The gel was transferred and hybridized as described above.

RESULTS

ACE3 is Sufficient to Regulate Amplification with the Proper Developmental Specificity

To characterize further the role of ACE3 in the regulation of amplification, we wished to determine whether ACE3 as an autonomous chorion element has the ability to induce amplification. An 845 bp chorion fragment containing ACE3 had been demonstrated to undergo amplification (Delidakis and Kafatos, 1989), but we wanted to test the ACE3 element alone. Previously, it had been shown that transposon constructs containing chorion fragments are particularly sensitive to position effects in their ability to amplify (Orr-Weaver and Spradling, 1986). Constructs containing larger amounts of chorion DNA were more resistant to these position effects (Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987). Therefore, we constructed a transposon containing multiple copies of ACE3. We reasoned that this multimer would be able to act as a larger chorion fragment in buffering itself against position effects, yet it would contain ACE3 as the only chorion DNA.

Two constructs were made containing multimers of ACE3. The multimers were created by ligating ACE3 in a sequential manner into head to tail repeats (see Methods). Although ACE3 had been defined to 320 bp, for ease of construction the multimers were made with a 440 bp fragment containing ACE3. P[rosy, chorion M9], which will be abbreviated as M9, contains 9 copies of ACE3 whereas P[rosy, chorion M18], or M18, contains 18 copies (Figure 1A and B). Independent transformants were obtained for each of the constructs and their structures were subsequently verified by Southern blots of transformant fly DNA. The constructs underwent some

rearrangements, presumably due to crossover events in the ACE3 repeats, which will be discussed later. However, in most of the initial transformant lines the transposon contained the starting number of ACE3 repeats.

Both the M9 and M18 multimer constructs underwent amplification when integrated into the genome, revealing that ACE3 is sufficient to initiate amplification even when it is the only chorion DNA present (Table I). To assay amplification, a single rosy$^+$ transformed male was crossed to rosy$^-$ females and amplification was measured in the rosy$^+$ progeny. This was done because we were uncertain of the frequencies with which unequal crossing over would rearrange the transposon. Amplification was assayed by comparing band intensities of DNA from dissected stage 13 egg chambers to that from stage 1-8 egg chambers on a quantitative Southern (Figure 2A and Methods). Overall amplification levels ranged from 1.5 to 5.9 fold. Thus as previously shown for other chorion constructs, amplification was dependent on the position of integration (de Cicco and Spradling, 1984; Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987).

The amplification values observed were considerably lower than the 60 fold level of endogenous amplification that occurs at the third chromosome

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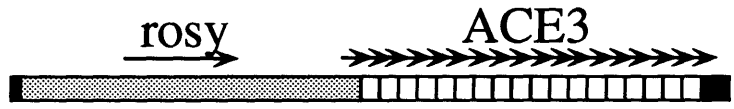
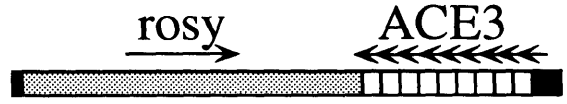
Fig. 1. ACE3 transposon constructs. The position of the ACE3 repeats within the constructs is shown relative to the rosy selectable marker. (A) M18 contains 18 copies of ACE3 in head to tail repeats (see Methods). (B) M9 contains 9 copies of ACE3 in the opposite orientation relative to rosy (see Methods). (C) M9pUC contains pUC sequences flanking the ACE3 repeats. (D) M1pUC contains a single ACE3 element flanked to either side by *rosy* and pUC sequences. Black box, P element DNA; open box, ACE3; gray box, *rosy*; stippled box, pUC. The arrow over the *rosy* gene indicates the direction of transcription; the arrows over the ACE3 repeats indicate orientation, with the arrowhead being the side of the ACE3 element closest to the *s18* gene ($^-$190).

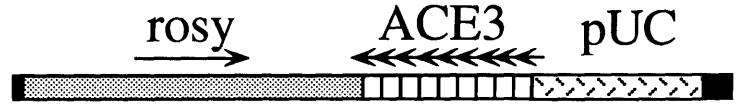
A. M18



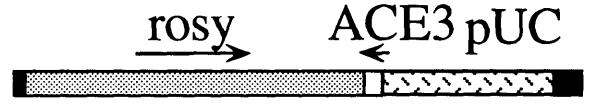
B. M9



C. M9pUC



D. M1pUC



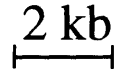


Table I. ACE3 multimer constructs

Transformant Line	Amplification Level	Amplification Frequency
M18		
1	2.5	1/5
3	1.3	
2	1.2	
4	1.0[a]	
5	1.0[a]	
M18; deleted line[b]		
1	2.9	1/1
M9		
2	5.9	4/11
10	3.2	
8	2.5	
3	1.5	
1	1.4	
4	1.4	
11	1.4	
5	1.2	
7	1.1	
9	1.1	
6	0.9	

[a] No amplification was seen by visual inspection of Southern blots, however amplification values were not determined.

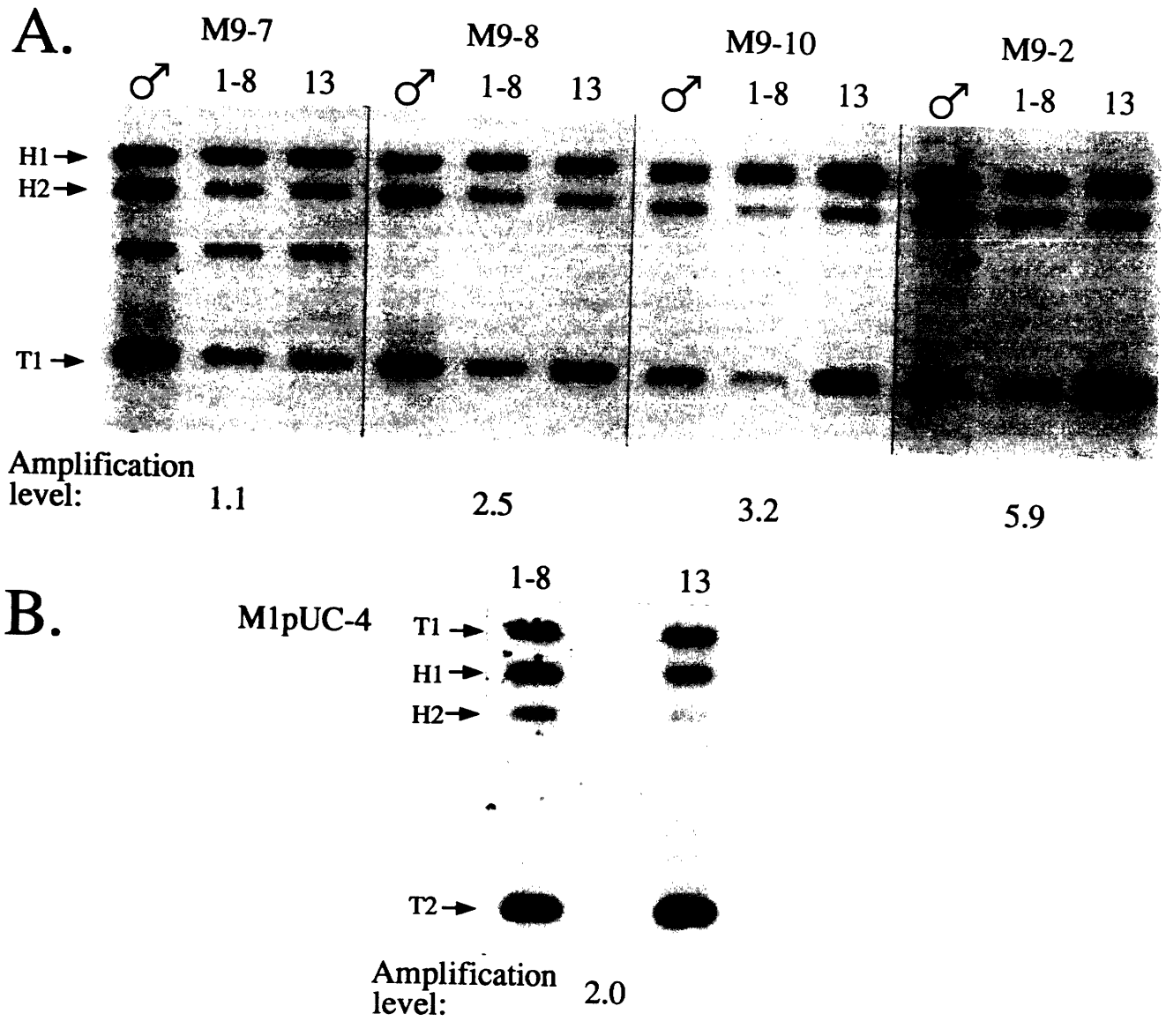
[b] Sequences within the ACE3 repeats were deleted leaving 1-6 copies of ACE3 present in this transformant.

chorion cluster. They were also lower than the average levels of amplification observed in transposons with larger chorion fragments that include both ACE3 and several AERs (Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987; Delidakis and Kafatos, 1989; Orr-Weaver et al., 1989). However, as can be seen in Figure 2, amplification values of 2.0 fold were clearly distinguishable from no amplification (1 fold), as well as from higher levels of 3.2 and 5.9 fold. This demonstrates that while amplification values were low, the differences between these low levels could be measured accurately. Moreover, we previously analyzed 32 transformant lines bearing a chorion transposon which was deleted for ACE3 and never observed amplification levels higher than 1.4 (Orr-Weaver and Spradling, 1986; Orr-Weaver et al., 1989), thus the quantitation is accurate enough to detect 2.0 fold levels of amplification.

It might have been expected that the additional copies of ACE3 in M18 would increase the levels of amplification as compared to M9; however we found that the M18 transformant lines did not amplify to higher levels than the M9 lines. Furthermore, an M18 line in which the transposon was deleted, leaving between 1 and 6 copies of ACE3, showed a similar level of amplification as the M9 and M18 lines (Table I). The orientation of the ACE3 repeats within the construct did not appear to be critical for amplification; the M9 and M18 constructs have opposite orientations of the ACE3 multimer in relation to the *rosy* marker.

The developmental specificity of amplification was examined by comparing male and stage 1-8 egg chamber DNA to stage 13 egg chamber DNA. Amplification occurred only in female egg chambers and was not detectable in male tissue, although amplification in a rare tissue could have been missed. Amplification also occurred with correct temporal control in

Figure 2. Quantitation of amplification levels. Stage 1-8 and stage 13 egg chamber DNA was isolated from females heterozygous for the transposon insertions. DNA was also prepared from whole male flies. DNA was digested with *Bam*HI and Southerns were probed with *rosy*$^{+}$ sequences. Two *rosy*^{506} host bands, H1 and H2 (4.7 and 4.5 kb, respectively), were used as non-amplifying controls. An internal 2.8 kb transposon fragment, T1, and in some experiments a second transposon fragment containing flanking sequences, T2, were used to determine amplification levels. The position of T2 is unique in each transposon insertion. The ratio of T1 to H1 in stage 13 DNA is compared to the same ratio in stage 1-8 DNA. The same ratio was determined using the H2 standard and the average amplification value taken. When possible, amplification of T2 was also determined using H1 and H2 and these values averaged as well. (A) Amplification values of four M9 transposon lines were determined. In two lines the transposon fragment containing flanking DNA, T2, can be seen. In M9-7 T2 runs above T1, and in M9-10 T2 comigrates with H1. For M9-10, T2 and H1 were not used in quantitation, rather the ratio of T1 was compared to H2 as the non-amplifying standard. Amplification levels are shown. M9-7 did not undergo amplification (1.1 fold), whereas M9-8, M9-10, and M9-2 amplified to increasing levels. (B) Amplification of M1pUC-4 is shown. T2 is the internal 2.8 kb transposon band. Amplification values were determined from the ratio of T1 to H1 in stage 13 as compared to stage 1-8 DNA.


A.
M9-7
M9-8
M9-10
M9-2
♂
1-8
13
H1
H2
T1
Amplification level:
1.1
2.5
3.2
5.9
B.
M1pUC-4
1-8
13
T1
H1
H2
T2
Amplification level:
2.0

that stage 1-8 egg chambers did not amplify whereas stage 13 egg chambers underwent amplification. Therefore, the ACE3 control element regulated amplification with the correct tissue and temporal specificity independent of other chorion sequences.

A third transposon was constructed containing pUC sequences adjacent to the ACE3 multimer and designated P[rosy, chorion M9pUC], or M9pUC (Figure 1C). pUC sequences were added to facilitate cloning of the DNA adjacent to the insertion site. The presence of pUC DNA in the transposon did not appear to inhibit or stimulate amplification. Amplification occurred in one out of seven initial transformants, with an amplification level of 3.0 fold. Four out of nine insertions produced by mobilizing an X-linked insert to new insertion sites also underwent amplification (data not shown).

A Single Copy of ACE3 is Sufficient for Amplification

The ACE3 multimer constructs were able to undergo amplification, and from the M9 and M18 data, it appeared that the ACE3 elements did not act in an additive fashion to regulate amplification. Yet, it was possible that a minimum number of ACE3 elements were needed for amplification. To test the ability of a single ACE3 element to regulate amplification, we made a construct in which non-chorion DNA was used as a buffer against position effects. Because pUC appeared to neither stimulate nor inhibit amplification in the M9pUC construct, pUC DNA was used to flank one side of a single copy of ACE3. The construct P[rosy, chorion M1pUC], which will be abbreviated M1pUC (Figure 1D), also contains the *rosy* selectable marker on the other side of ACE3.

One copy of the ACE3 element was able to direct amplification in stage 13 egg chambers (Figure 2B and Table II). Again, amplification appeared to be

specific to egg chamber DNA; amplification did not occur in male tissue in five lines that were tested. The overall amplification levels of the single copy construct were slightly lower than those obtained from the multimer lines tested; two fold amplification was the highest attained. This level of amplification is clearly detectable as can be seen in Figure 2B, and low levels of amplification were reproducibly quantified. Therefore, a single ACE3 element contains all of the information necessary to regulate amplification with the correct tissue and temporal specificity.

Mapping the Origin of Amplification Used in ACE3 Transformants

Studies of the endogenous third chromosome chorion cluster have mapped the predominant origin used during amplification (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). This origin lies 1.5 kb downstream

Table II. ACE3 single copy construct

Transformant Line: M1pUC	Amplification Level	Amplification Frequency
4	2.0	3/10
9	1.8	
6	1.5	
2	1.3	
8	1.3	
5	1.2	
7	1.2	
1	1.1	
10	1.1	
3	0.9	

of ACE3 and is located between the *s18* and *s15* chorion genes. ACE3 is therefore distinct from the major amplification origin at the endogenous locus, although it is possible that ACE3 could still be acting as an origin at a lower frequency. Using the multimer transformants that amplify under the sole control of ACE3, we wanted to determine whether ACE3 served both as the amplification origin and the regulatory element of amplification. Alternatively, ACE3 might have functioned as a regulatory element that activated fortuitous origins near the transposon insertion site in the genome.

The initiation site for amplification could be localized by examining the profile of the amplification gradient. At both endogenous chorion loci, amplification results in a gradient of amplified DNA that is centered over the chorion cluster and extends 50 kb in either direction (Spradling, 1981). If ACE3 acted as an origin in the multimer transposon inserts, the gradient peak would be centered over ACE3. Conversely, if ACE3 activated an origin outside of the transposon, the gradient peak would be offset from the transposon and centered over the activated origin. The exact position of the origin within the peak would depend on whether replication fork movement was bidirectional.

The gradient of amplification was analyzed in two ACE3 multimer transformants, M9-2 and M9pUC-3. The M9-2 line amplified six fold, and the transposon localized by in situ hybridization to salivary gland chromosome bands 56F14 to 57A on the right arm of the second chromosome. The second insert analyzed, M9pUC-3, amplified three fold, and localized to salivary gland chromosome band 42B. In order to quantify the amplification levels of DNA flanking the transposon inserts, it was first necessary to clone the flanking DNA for use as probes. A lambda genomic library was generated from the M9-2 insertion line, and a 34 kb interval of DNA including the

transposon and flanking sequences was cloned from this library and restriction mapped. The pUC sequences present in the transposon of line M9pUC-3 allowed us to clone directly a 38 kb interval of DNA by plasmid rescue into *E. coli* (see Methods) which was subsequently mapped.

The level of amplification for DNA fragments spanning the transposon and flanking regions were quantified on Southern blots, and a gradient of amplification was mapped for each line. Since the gradient results from gradual differences in the extent of amplification of the fragments tested, several measures were taken to insure the results were quantitatively significant: 1) several different digests were performed to obtain overlapping fragments that spanned the transposon as well as the flanking DNA; 2) amplification blots were repeated several times; and 3) amplification values were rigorously quantified by PhosphorImager analysis of the Southern blots (see Methods).

In line M9-2, the peak of the amplification gradient was centered over the transposon (Figure 3). Quantitative Southerns using four different sets of digests were performed for this analysis. Amplification values along the gradient ranged from two to five fold; these differences can be distinguished by visual inspection of the blots as well as reproducibly quantified (Figures 2, 3). In each of the four Southern blots, the fragment with the highest amplification level was the one containing the transposon. Of the transposon fragments, those from the 3' end of the *rosy* gene were the highest amplified. The Wilcoxon's rank-sum test was used to determine if amplification values from different fragments within a single Southern blot, quantified repeatedly, were statistically different from one another. The quantitation and statistical analysis show that for M9-2 the peak of the gradient is over the *rosy* sequences

within the transposon. Thus the origin appears to be in the transposon itself or within nearby flanking sequences.

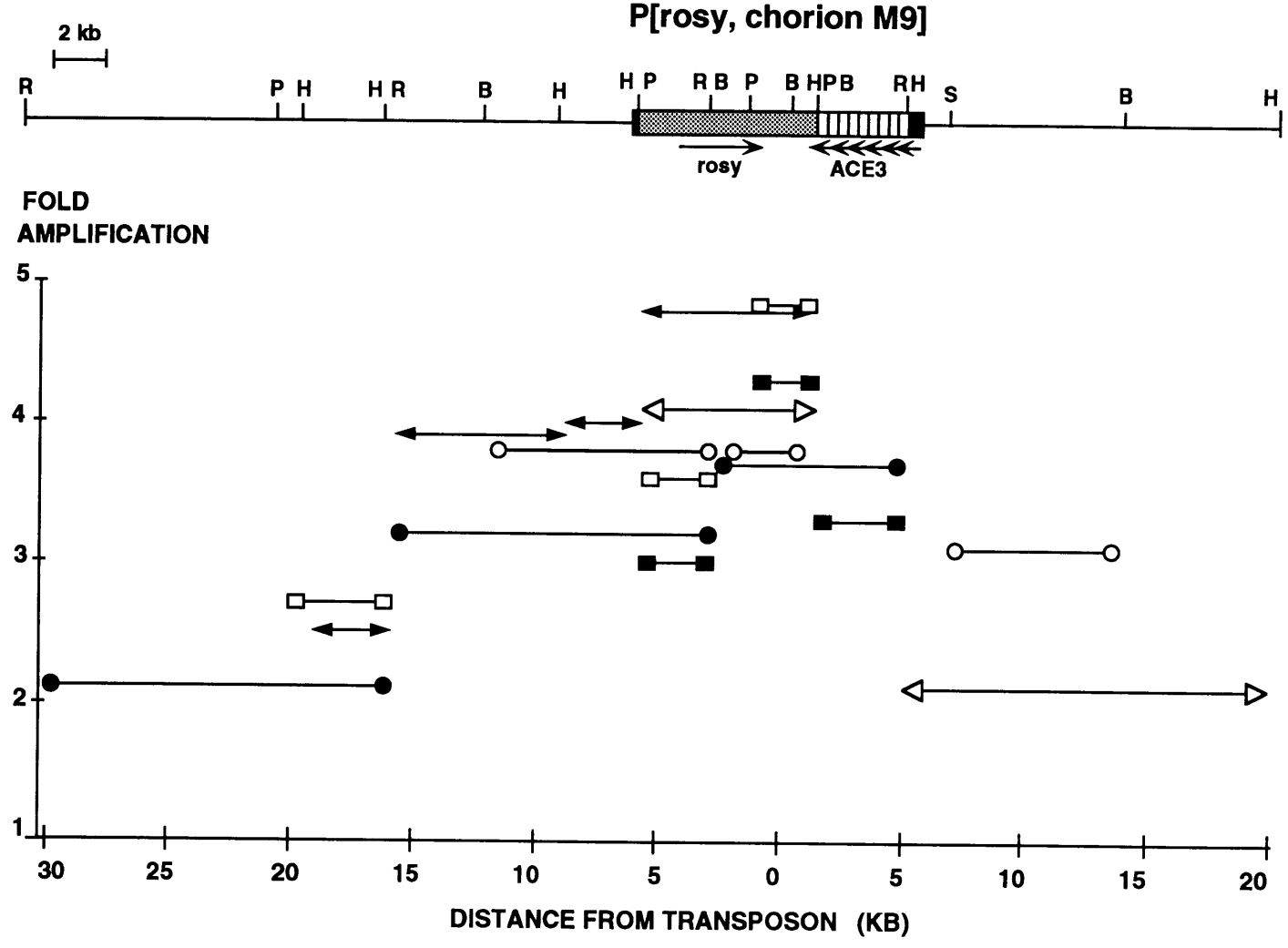
The peak of the gradient of amplification surrounding the M9pUC-3 insert was also centered over the transposon but extended 8 kb to the right and 5 kb to the left (data not shown). The peak of this gradient could not be resolved with greater precision than this 28 kb interval due to the shallow nature of this gradient. The position of this peak over the transposon suggests that the amplification origin is located in the transposon or in nearby flanking sequences.

Although amplification gradients have been characterized for the two endogenous chorion clusters, such an analysis has not been performed for any chorion transposon insertions. Our analysis of the gradient surrounding two multimer transposon inserts has revealed that in both lines, the gradient extends over the transposon and into flanking sequences. The gradient profile of M9-2 extends at least 50 kb and that of M9pUC-3 extends at least 28 kb. Thus although these two lines amplify considerably less than the endogenous loci, the gradients of amplification extend nearly as far.

Analysis of Replication Intermediates

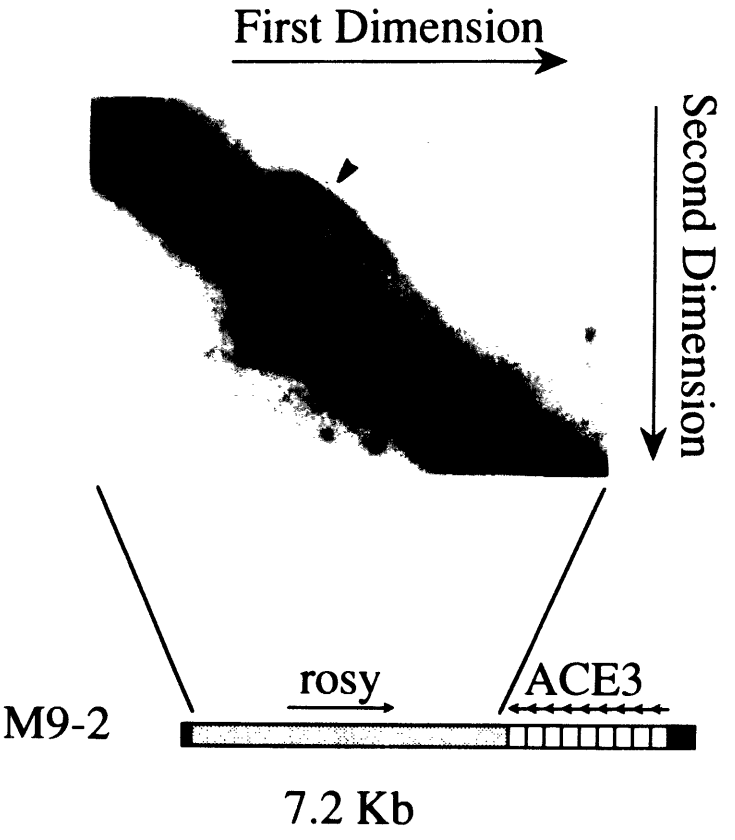
The gradient analysis of the M9-2 and M9pUC-3 lines permitted the localization of the amplification origin to the transposon or closely linked sequences. To confirm these results by an independent method, we used two-dimensional gel analysis to identify replication intermediates in this region. The two dimensional gel technique allows the separation of replication intermediates from linear non-replicating molecules (Brewer and Fangman, 1987). In the second dimension, replication intermediates run above a diagonal of linear molecules and form characteristic arcs.

Figure 3. Gradient analysis of multimer transformant M9-2. Amplification levels of transposon and flanking fragments are shown. The top line shows a map of the transposon and flanking DNA drawn to the same scale as the graph below. On the graph the level of amplification is shown for restriction fragments, designated by each line, spanning the transposon and flanking DNA. The X axis of the graph denotes the position relative to the midpoint of the transposon which was set as zero. Amplification values were determined as described, and amplification of flanking fragments was corrected using the formula described in Materials and Methods. Fragments from the same blot are shown using lines with the same shape at either end. Lines with black arrowheads denote fragments from a *Hin*dIII digest, whereas fragments with white arrowheads are from the same *Hin*dIII blot reprobed with a different set of probes. Black squares are fragments from a *Eco*R1 plus *Pst*I digest, and white squares show fragments from the same blot reprobed with different probes. White circles denote a *Sal*I and *Bam*HI digest. Black circles denote an *Eco*RI digest. On the top line black boxes, P element DNA; gray box, *rosy*; open boxes, ACE3 repeats; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I. The arrows for *rosy* and ACE3 are as in Fig. 1.


P[rosy, chorion M9]
2 kb
R
P H
H R
B
H
H P
R B P
B HPB
RH
S
B
H
rosy
ACE3
FOLD
AMPLIFICATION
5
4
3
2
1
30
25
20
15
10
5
0
5
10
15
20
DISTANCE FROM TRANSPOSON (KB)

DNA was isolated from stage 10 egg chambers in which amplification is in its early stages, and it was electrophoresed on two-dimensional gels, blotted, and hybridized to probes of various transposon and flanking sequences. Y-arcs, indicative of fragments containing replication forks, were seen on a Southern blot of transposon M9-2 probed with *rosy* (Figure 4). Y arcs were seen in several fragments tested for both lines M9-2 and M9pUC-3 (data not shown). Replication intermediates were never seen in the non-amplifying $rosy^{506}$ locus. Thus even though the amplification levels were low, amplifying DNA could be distinguished from non-amplifying by the presence of replication intermediates. These results confirmed that amplification is occurring throughout the gradient in both lines tested. Bubble arcs, characteristic of fragments containing an origin of replication, were not observed. The absence of replication bubbles, together with the shallow shape of the gradient, suggests that amplification initiates only a few times in these lines. Infrequent initiation events would not be detected on a two dimensional gel. Alternatively, it is possible that bubble arcs were not detected because they were unstable and lost during DNA purification. However, we tried several techniques such as psoralen crosslinking and BND cellulose to increase the recovery of replication bubbles and still did not see bubble arcs (data not shown).

Figure 4. Two-dimensional gel analysis of transformant M9-2. Stage 10 egg chamber DNA from transformant females was digested with *Hin*dIII and run in two dimensions. The Southern was probed with $rosy^{+}$ sequences. A Y-arc of replicating intermediates (arrow) containing replication forks can be seen starting from a position containing full length linear 7.2 kb molecules. A second spot of linear molecules can also be seen centered under the Y-arc; this is from the $rosy^{506}$ endogenous sequence which yields an 8 kb fragment. No Y-arc can be seen from this single copy locus. The map of the transposon is as in Fig. 1.


First Dimension
Second Dimension
rosy
ACE3
M9-2
7.2 Kb

Instability of Tandem Repeats During P-element Transposition in Drosophila

In generating the initial transformant lines for each ACE3 multimer transposon, we noticed that deletions occurred within the constructs. Moreover, rearrangements occurred with a greater frequency when an initial transposon transformant was mobilized to new positions in the genome. For example, 1 out of 4 of the M18 initial transformants obtained by injection contained a deletion of sequences within the ACE3 repeats. Upon mobilization, 9 out of 24 single inserts examined carried a deletion (Fig. 5B). For M9pUC, 1 out of 7 initial transformants had a rearrangement, whereas 4 out of 5 single insert lines obtained by transposition contained deletions. This instability was most likely due to crossing over within tandem repeats of ACE3 upon transposition since the deletions were within the ACE3 repeats (data not shown).

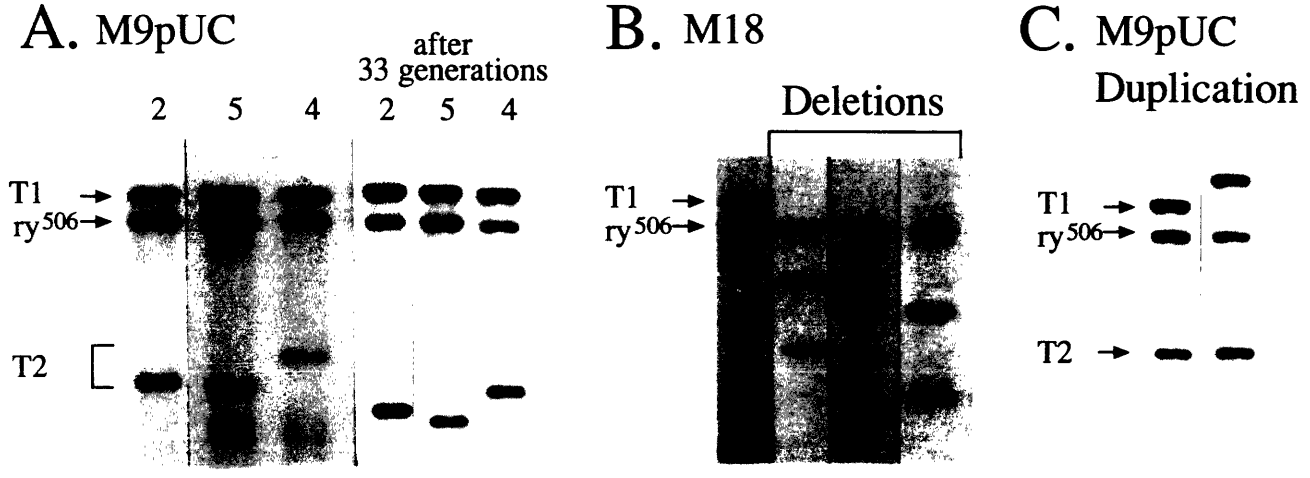
In contrast to the instability of the ACE3 multimers during transposition, the transposon containing a single copy of ACE3 and an inverted duplication of the polylinker DNA underwent deletion at a low frequency when transposed to new insertion sites. 0 out of 13 single insert lines were deleted, but one line with multiple inserts did contain a deletion.

To determine if multiple, direct repeats were stable once integrated into the chromosome, we examined transformant lines containing multimer constructs that had been maintained in the lab for three years. 24 lines were examined after being passaged from 33 to 42 generations without balancer chromosomes. All of these transposons, although containing tandem repeats, were very stable and did not undergo deletions (Figure 5A and data not shown). Since we could not select for flies in which the transposon rearranged, it is possible that rearrangements occurred at a low frequency or in a minor proportion of the flies. Nevertheless, the multimers were

considerably more stable once integrated than during transposition. This may be due to the greater susceptibility of the DNA to recombination and crossing over during the transposition event. Alternatively, these rearrangements may have been triggered directly by transposase or the transposition mechanism. If this were the case, then derivatives of P elements with internal deletions might arise via transposition, rather than as a result of incomplete gap repair at the donor site (Engels et al., 1990).

Despite the stability observed in the transformant lines maintained for many generations, we did detect a rearrangement that unambiguously arose by unequal crossing over between sister chromatids. A duplication within the ACE3 repeats occurred in an autosomal copy of one of the initial M9pUC transformants (Figure 5C). From an initial G_0 rosy$^+$ male, 10 G_1 rosy$^+$ progeny were obtained with the same insertion site; two of these contained a duplication of the ACE3 repeats. Since the duplication occurred within the same insertion site as the unrearranged transposon, an unequal crossover must have occurred after integration of the transposon. Therefore, two premeiotic events occurred; first, the transposon inserted, and second, an unequal exchange between sister chromatids generated a duplication of the ACE3 repeats.

Figure 5. Analysis of the ACE3 multimer transposon structure in transformant lines. Male DNA was purified and digested with *Eco*RI, and Southern blots were prepared and hybridized to a probe homologous to the *rosy* gene. *Eco*RI cleaves both at a site in the *rosy* gene on the transposon and at the far end of each transposon, producing a fragment internal to the transposon, denoted T1. The fragment containing the 5' end of *rosy* will also contain flanking sequences to the left of the transposon, yielding a unique fragment for each independent insert, T2. (A) The stability of M9pUC transformants after 33 generations. T1 (11 kb), the endogenous $rosy^{506}$ fragment (8 kb), and the flanking fragment (T2) for each of the transposons are shown. The first three lanes are a Southern of DNA from M9pUC lines 2, 5, and 4 shortly after their initial isolation; the last three lanes show the same lines after 33 generations. (B) Deletions occurred after mobilization of M18. An *Eco*RI digest yields the endogenous 8 kb $rosy^{506}$ band and a 12 kb transposon fragment, T1. A second unique transposon fragment can be seen as well. The first lane is an unrearranged transformant, and the last 3 lanes show deletions that occurred within the ACE3 repeats in three independent transformants. In these deleted transposon lines the T1 fragment runs at lower molecular weights. It was not possible to distinguish the deleted T1 band from the flanking T2 band. (C) A duplication within the ACE3 repeats occurred premeiotically in a G_0 male. The first lane is the full length transformant. T1 (11 kb), $rosy^{506}$ (8 kb), and T2, a smaller flanking fragment are indicated. The second lane is a different G_1 isolate in which the transposon contains a duplication of the ACE3 repeats that results in a larger T1 fragment.


A. M9pUC
after
33 generations
2 5 4 2 5 4
T1
ry506
T2
B. M18
Deletions
T1
ry506
C. M9pUC
Duplication
T1
ry506
T2

DISCUSSION

Our studies have focused on the role of ACE3 during DNA amplification in Drosophila egg chambers. Despite the complexity of the control elements present in the chorion cluster, ACE3 is sufficient to induce amplification with the proper developmental regulation. The presence of other control elements such as the major origin and the AERs are not required, although these elements probably aid in achieving the high levels of amplification which occur at the endogenous locus. ACE3 must therefore function in three ways. First, ACE3 responds to temporal signals such that amplification does not occur until after stages 1-8 of egg chamber development. Second, ACE3 confers tissue-specific regulation since amplification occurs only in female egg chambers. Third, ACE3 contains an element causing an amplification origin to undergo repeated rounds of reinitiation. All three of the above functions must be contained within the 440 bp sequence we have shown to be sufficient.

Our demonstration that ACE3 is sufficient to regulate amplification at low levels but that increasing the number of copies of ACE3 does not significantly improve amplification levels suggests that the function of ACE3 is distinct from other chorion regulatory elements. The AERs are required for high levels of amplification; the presence of more AERs correlates with higher levels of amplification (Delidakis and Kafatos, 1987; Delidakis and Kafatos, 1989). Since ACE3 did not act in an additive manner to produce high levels of amplification, the functions of ACE3 and the AERs may differ. However, it is also possible that the spacing between these regulatory elements, which are normally each separated by at least 1 kb in the third chromosome cluster, may be critical in the regulation of amplification. In

sorting out potential differences and similarities between ACE3 and the AERs it will be useful to determine if the AERs are sufficient for amplification as well. In addition, testing the activity of the major origin independent of ACE3 and the chorion cluster would be informative.

In the two lines analyzed the site of maximal amplification mapped to the transposon, and it is most probable that the origin of amplification is within the peak of the gradient of amplified DNA. Assuming bidirectional fork movement and similar fork rate movement in each direction, the origin would be centered within the peak in the *rosy* gene on the transposon. However, with unequal fork rate movement, or with unidirectional fork movement, the origin would still be within the peak of maximal amplification, but it could lie anywhere along the area of the peak or at either end of the peak. ACE3 may be acting as the origin if one assumes unequal fork rate movement, as could sequences to the left of the transposon, since they lie at either end of the peak of the gradient. Therefore, ACE3 may be acting either as the origin of amplification, or alternatively ACE3 may be activating an origin within the transposon or nearby flanking region. At the endogenous locus, the predominant origin used is 1.5 kb away from ACE3. It is a likely hypothesis that ACE3 may act to control the initiation of DNA replication at this origin as well as initiations at other minor origins.

Chorion gene amplification is very sensitive to position effects, such that transformant lines are observed both in which the transposon fails to amplify and in which amplification levels are low. Presumably one type of position effect is the inhibition of initiation at the amplification origin. An additional potential source of inhibition would be the presence of replication fork terminators adjacent to the transposon insertions. Such replication fork barriers have been documented in the yeast *rDNA* gene cluster (Brewer and

Fangman, 1988). If terminators or barriers blocked fork movement, the stalled replication forks could impede further reinitiations at the origin. Replication terminators would be detected as sites at which the gradient of amplification would drop off sharply. At the two transposon insertion sites we analyzed, the gradients extended to at least 28 and 50 kb. Both of these gradients were shallow and dropped off gradually rather than sharply. The extent and profile of these gradients suggest that terminator sequences are not responsible for the lowered amount of amplification observed at these two insertion sites.

Despite the fact that the two transformant lines amplify 10 to 20 fold less than the third chromosome chorion cluster, the gradients extend to similar distances. This indicates that the low levels of amplification result from initiation occurring only two to three times, and that the gradient is a consequence of the rate of replication fork movement and the length of time during follicle cell differentiation when amplification occurs. At the endogenous loci, chorion gene amplification results in a gradient of amplified DNA extending approximately 100 kb. In analyzing the rate of replication fork movement at the third chromosome chorion gene cluster, Spradling and Leys found that the profile of the gradient could be explained by the slow rate of fork movement, without invoking the presence of specific sites of replication termination (Spradling and Leys, 1988). Thus the shallow gradients we observed surrounding the two transposon insertions can be explained by the replication forks from the first initiations of amplification moving at approximately the same rate and for the same period of follicle cell development as at the endogenous locus. However, only a few rounds of reinitiation take place on the transposon, and then initiation must cease. Both the absence of AERs and inhibitory position effects could reduce the

number of rounds of initiation. The postulated limited number of activations of the amplification origin is consistent with our failure to detect replication bubbles in the amplification gradients. Although the absence of bubbles on the two dimensional gel could have been a consequence of their instability during purification, we also failed to detect them after stabilization by psoralen crosslinking.

Knowing that the 440 bp element ACE3 has all the controls necessary to regulate amplification, it is now possible to dissect this control element and to define the cis-acting elements responsible for the tissue and temporal specificity as well as for origin activation. Our demonstration that ACE3 contains all of the necessary regulatory elements sets the foundation for identifying the trans-acting factors that interact with ACE3 in order to delineate further the mechanism of amplification of the chorion genes. Elucidation of the mechanism by which DNA replication is regulated by ACE3 in response to developmental signals will provide a framework for understanding the control of chromosomal replicons in higher eukaryotes.

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REFERENCES

Anachkova, B. and J. L. Hamlin. 1989. Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. *9*: 532-540.

Borck, K., J. D. Beggs, W. J. Brammar, A. S. Hopkins and N. E. Murray. 1976. The construction in vitro of transducing derivatives of phage lambda. Mol. Gen. Genet. *146*: 199-207.

Brewer, B. J. and W. L. Fangman. 1987. The localization of replication origins on ARS plasmids in *S. cerevisiae*. Cell *51*: 463-471.

Brewer, B. J. and W. L. Fangman. 1988. A replication fork barrier at the 3' end of yeast ribosomal RNA genes. Cell *55*: 637-643.

Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz and M. L. DePamphillis. 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. Cell *62*: 955-965.

Church, G. and W. Gilbert. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. *81*: 1991-1995.

de Cicco, D. and A. Spradling. 1984. Localization of a cis-acting element responsible for the developmentally regulated amplification of Drosophila chorion genes. Cell *38*: 45-54.

Delidakis, C. and F. C. Kafatos. 1987. Amplification of a chorion gene cluster in Drosophila is subject to multiple cis-regulatory elements and to long range position effects. J. Mol. Biol. *197*: 11-26.

Delidakis, C. and F. C. Kafatos. 1989. Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. EMBO J. *8*: 891-901.

DePamphilis, M. L. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell *52*: 635-638.

Engels, W. R., D. M. Johnson-Schlitz, W. B. Eggleston and J. Sved. 1990. High-frequency P element loss in Drosophila is homolog dependent. Cell *62*: 515-525.

Heck, M. and A. Spradling. 1990. Multiple replication origins are used during Drosophila chorion gene amplification. J. Cell. Biol. *110*: 903-914.

Heintz, N. H. and J. L. Hamlin. 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. *79*: 4083-4087.

Huberman, J., J. Zhu, L. R. Davis and C. S. Newlon. 1988. Close association of a DNA replication origin and an *ARS* element on chromosome III of the yeast, *Saccharomyces cerevisiae*. Nucl. Acids Res. *16*: 6373-6383.

Kaufman, P. D. and D. C. Rio. 1991. Germline transformation of *Drosophila melanogaster* by purified P element transposase. Nucl. Acids Res. *19*: 6336.

Leu, T. and J. L. Hamlin. 1989. High-resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Mol. Cell. Biol. *9*: 523-531.

Levine, J. and A. Spradling. 1985. DNA sequence of 3.8 kilobase pair region controlling Drosophila chorion gene amplification. Chromosoma *92*: 136-142.

Li, J. J., K. W. Peden, R. A. Dixon and T. Kelly. 1986. Functional organization of the simian virus 40 origin of DNA replication. Mol. Cell. Biol. *6*: 1117-1128.

Lindren, B.W., G.W. McElrath, and D.A. Berry. 1978. Probability and Statistics. (MacMillan, New York, N. Y.).

Linsken, M. and J. Huberman. 1988. Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *8*: 4927-4935.

Linskens, M. and J. A. Huberman. 1990. The two faces of higher eukaryotic DNA replication origins. Cell *62*: 845-847.

Marraccino, R. L., R. Fotedar, G. D'Urso and J. M. Roberts. 1990. Control of DNA replication. Curr. Opinion Cell Biol. *2*: 262-268.

Murray, N. E., N. J. Brammar and K. Murray. 1977. Lambdoid phages that simplify the recovery of in vitro recombinants. Mol. Gen. Genet. *150*: 53-61.

Orr-Weaver, T. and A. Spradling. 1986. Drosophila chorion gene amplification requires an upstream region regulating *s18* transcription. Mol. Cell. Biol. *6*: 4624-4633.

Orr-Weaver, T. L. 1991. Drosophila chorion genes: cracking the eggshell's secrets. BioEssays *13*: 97-105.

Orr-Weaver, T. L., C. G. Johnston and A. C. Spradling. 1989. The role of ACE3 in *Drosophila* chorion gene amplification. EMBO J. *8*: 4153-4162.

Osheim, Y. N. and O. L. Miller. 1983. Novel amplification and transcriptional activity of chorion genes in *Drosophila melanogaster* follicle cells. Cell *33*: 543-553.

Osheim, Y. N., O. L. Miller and A. L. Beyer. 1988. Visualization of *Drosophila melanogaster* chorion genes undergoing amplification. Mol. Cell. Biol. *8*: 2811-2821.

Robertson, H., C. Preston, R. Phillis, D. Johnson-Schlitz, W. Benz and W. Engels. 1988. A stable genomic source of P element transposase in *Drosophila melanogaster*. Genetics *118*: 461-470.

Rubin, G. M. and A. C. Spradling. 1983. Vectors of P element-mediated gene transfer in Drosophila. Nucl. Acids Res. *11*: 6341-6351.

Spradling, A. 1981. The organization and amplification of two clusters of Drosophila chorion genes. Cell *27*: 193-202.

Spradling, A. C. and E. Leys. 1988. Slow replication fork movement during *Drosophila* chorion gene amplification. In *Cancer Cells*, ed. T. Kelly and B. Stillman, pp. 305-309. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.).

Spradling, A. C. and A. P. Mahowald. 1981. A chromosome inversion alters the pattern of specific DNA replication in *Drosophila* follicle cells. Cell *27*: 203-209.

Stillman, B. 1989. Initiation of eukaryotic DNA replication in vitro. Ann. Rev. Cell Biol. *5*: 197-245.

Swimmer, C., C. Delidakis and F. C. Kafatos. 1989. Amplification-control element ACE-3 is important but not essential for autosomal chorion gene amplification. Proc. Natl. Acad. Sci. *86*: 8823-8827.

Tschumper, G. and J. Carbon. 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and the *TRP1* gene. Gene *10*: 157-166.

Vaughn, J. P., P. A. Dijkwel and J. L. Hamlin. 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell *61*: 1075-1087.

CHAPTER III.

Replication of Chorion DNA; In Vitro Assays

INTRODUCTION

Replication studies have been approached using numerous assays in many different organisms. In the yeast *S. cerevisiae*, autonomously replicating sequences (ARS elements) have been identified by the ability of certain sequences to allow the autonomous replication of plasmids (Brewer and Fangman, 1987; Huberman et al., 1987). Recently, an origin recognition complex, (ORC), has been identified that binds to the essential region of ARS elements (Bell and Stillman, 1992). This complex consists of six proteins that remain bound throughout the cell cycle, and interactions with other factors may be necessary for replication initiation (Bell et al., 1993; Diffley et al., 1994).

In Xenopus, replication has been studied using both eggs and extracts that are able to replicate added template DNA. Plasmid replication in Xenopus eggs and extracts is under cell cycle control, and initiation does not occur at specific sequences but instead occurs at random sites throughout the plasmid (Harland and Laskey, 1980; Mechali and Kearsey, 1984; Hyrien and Mechali, 1992; Mahbubani et al., 1992). Using Xenopus extracts, the control that limits replication to once per cell cycle has also been examined. A postulated licensing factor is involved in this regulation, and variations of this replication assay are currently being used to purify licensing factor (Blow and Laskey, 1986; Blow, 1993; Coverley et al., 1993).

Similar to studies in Xenopus, replication has also been examined using both Drosophila embryos and extracts. When injected into Drosophila embryos, plasmids have been shown to become enclosed in a nucleus-like structure, and undergo one round of replication or repair synthesis (Steller and Pirrotta, 1985; Roth, 1991). Drosophila extracts from 0-2 hour embryos have also been used to assay replication of added Xenopus sperm nuclei

(Crevel and Cotterill, 1991). Recently Drosophila tissue culture cells have been used to study origin usage during DNA replication. Using two-dimensional gel analysis, a 10 kb initiation region was identified downstream of the DNA polymerase α gene, and multiple initiation sites were found within the histone repeats (Shinomiya and Ina, 1993; Shinomiya and Ina, 1994).

Finally, mammalian cell-free extracts have been used extensively in studying the replication of SV40 DNA. This in vitro model system has allowed the biochemical fractionation and identification of many essential replication polymerases and accessory factors (reviewed in Stillman, 1994). The recent reconstitution of SV40 replication with purified proteins and T antigen has allowed the biochemical analysis of events occurring during DNA synthesis at the replication fork (Waga and Stillman, 1994).

In Drosophila, amplification of the chorion genes has provided a model system in which to study a developmentally regulated replicon. Germ-line mediated transformation of chorion constructs has identified several cis-acting elements important for tissue-specific and temporally regulated amplification. The amplification control element for the third chromosome, ACE3, is sufficient to induce amplification and is composed of functionally redundant subdomains (Orr-Weaver et al., 1989). Several cis-acting elements important for high levels of amplification are the amplification enhancing regions (AERs) (Fig.1) (reviewed in Orr-Weaver, 1991). Finally, a major initiation site for amplification within the chorion cluster is located within AER-d, however minor initiations are also seen throughout a larger 12 kb region (Delidakis and Kafatos, 1989; Heck and Spradling, 1990).

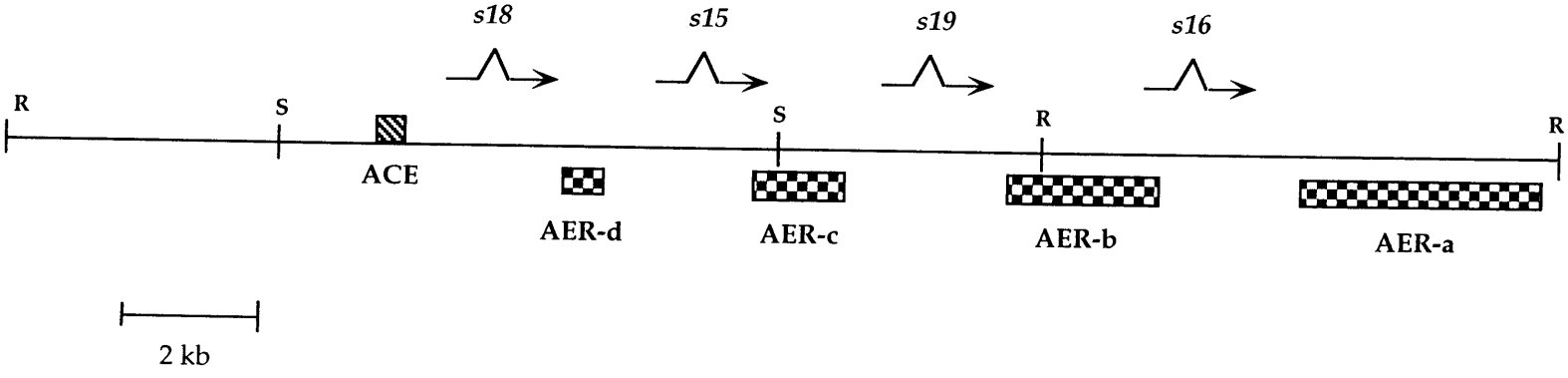
How ACE3 and the AER elements act together to regulate amplification is not known. Within the third chromosome chorion cluster, ACE3 is functionally redundant in that larger constructs deleted for ACE3 are able to amplify. Perhaps AER elements might also be sufficient to induce amplification similar to ACE3. Alternatively ACE3 might be unique in its interaction with the amplification origin, and the AER elements might be necessary to stimulate this interaction. Finally, whether ACE3 itself acts as an amplification origin remains uncertain.

The germ-line transformation approach previously used to define cis-acting elements is limited in several respects. Transformants of chorion constructs are subject to position effects in their ability to amplify. Many independent transformant lines must be analyzed to determine the effect of a single construct on amplification. Furthermore, already low levels of amplification in combination with position effects make it difficult to compare the effects of different constructs. Chorion constructs containing a 7.7 kb *Eco*R1 fragment amplify from 2 to 12 fold, with an average level of 5.1 fold (Orr-Weaver et al., 1989). ACE3 multimer constructs amplify to slightly lower levels, showing between 2 and 6 fold amplification. Further studies to delineate the AERs more precisely and compare their role to ACE3 would be difficult given the limitations of this in vivo assay.

In order to facilitate further studies of ACE3 and AER function, attempts were made to develop an in vitro assay for replication of chorion DNA. One approach assayed replication in Drosophila Schneider L-2 cells following transient transfection of chorion plasmids. A second approach assayed replication following injection of chorion plasmids into embryos. Several key questions of chorion amplification could be addressed using an in vitro replication system. First, whether replication uses the same

predominant origin used during amplification could be determined. Second, constructs containing only ACE3 or AERs would determine whether these sequences are able to function as replication origins, and constructs could be made to study the effect of having more than one origin present per plasmid. Combinations of ACE3 and AERs might illuminate interactions between these different control elements. Finally the development of an in vitro system might facilitate the biochemical identification of trans-acting factors involved in amplification.

Figure 1. Structure the third chromosomal chorion cluster. The 12 kb region contains the four major chorion genes as depicted by lines with arrows. The stripped box represents the ACE3 region, defined from -630-310 5' to the *s18* gene. The checkered boxes represent the AERs (amplification enhancing regions). A predominant amplification origin is located within AER-d.


s18
s15
s19
s16
R
S
S
R
R
ACE
AER-d
AER-c
AER-b
AER-a
2 kb

METHODS

Strains

The *Escherichia coli* JA300 RecA$^-$ strain was used for plasmid constructions, and plasmids were purified from *E. coli* strain JA300 and a *dam*$^-$ *dcm*$^-$ strain RK1007.

Plasmid Constructions

Plasmid construction of $A_{54}O_{18}$ and $A_{48}O_{28}$ are described in (Orr-Weaver et al., 1989), and plasmids $A_{10}O_{31}$ and $A_{22}O_{31}$ are described in (Orr-Weaver and Spradling, 1986). The Carnegie 3 plasmid is described in (Rubin and Spradling, 1983). Plasmids $A_{31}H_{18}$, $A_{31}H_{18}\Delta_5$, $A_{75}O_8$, $A_{77}O_6$, $A_{78}O_8$, and $A_{68}O_9$ were constructed by T. Orr-Weaver (personal communication). The remaining AER-c region was deleted in plasmids $A_{77}O_6$ and $A_{78}O_8$ to generate $A_{80}O_9$ and $A_{81}O_{17}$ respectively. A *Sal*I partial digest, followed by a *Xho*I digest and religation created a 270 bp *Sal*I-*Xho*I deletion of AER-c.

Schneider L-2 Cell Culture Transfections

Schneider L-2 cells were grown at 25°C in M3 medium supplemented with 10% heat inactivated fetal calf serum, 100 u/ml penicillin and 100 μg/ml streptomycin. Cells were passaged every 3 days. 30 ml cultures were cotransfected with plasmids, after which 5 ml aliquots were taken every 24 hours up to 120 hours following transfection. 25 μg of each plasmid DNA was used to cotransfect cells by $CaCl_2$ transfection as described (Ashburner, 1989). Cells were washed 24 hours after transfection and the first aliquot taken. For heat shock experiments cells were heat shocked for one hour at 37°C every 12 hours following the initial 24 hour period. Plasmid DNA was

isolated from aliquots by the Hirt supernatant protocol (Ausubel et al., 1987), EtOH precipitated, and samples were frozen until all aliquots were taken.

Plasmids were digested with various enzymes either resistant or sensitive to *dam* or *dcm* methylation. *Dpn*I digested only fully methylated DNA, whereas *Cla*I digested only fully unmethylated DNA. *Dpn*I is a 4 bp-sequence recognition enzyme and cleaved DNA into many small fragments. Either of these enzymes were used in combination with enzymes that did not have methylated sites so that both unreplicated and replicated plasmid fragments would run at distinct positions on Southern blots. pUC, *lac Z*, *rosy*, or chorion sequences were used as probes to detect fragments from plasmids on Southern blots. Southern blots were performed as described in Ch. II.

Replication Assay in Embryos

A mixture of two plasmids, each at 250 µg/ml in injection buffer was injected into embryos. Plasmid injection was performed as described for P-element transformation in Ch. II, except that Canton S flies were used. 50-100 embryos were injected for each experiment, and a 5-10 fold greater volume than normally used for P-element transformation was injected. Embryos aged 0-30 minutes were injected and allowed to develop for 3 hours under oil at 18°C before replication was assayed. Embryos were rinsed in heptane to remove the oil, and collected in an eppendorf tube. 50 µl lysis buffer was added and nuclear fractionation was performed as described (Steller and Pirrotta, 1985), except that a vertical rotor eppendorf was used to spin down nuclei.

Cytoplasmic and nuclear DNA were digested with *Dpn*I and *Bgl*I, and Southern blots were done as described in Ch. II. *Bgl*I was insensitive to methylation and digested either methylated or unmethylated DNA, whereas

*Dpn*I digested only methylated DNA. A *Bgl*I digest resulted in an internal 3.2 kb fragment for $A_{31}H_{18}$ and a linearized 3.6 kb Carnegie 3 plasmid, when probed with pUC sequences. *Dpn*I digestion resulted in many small fragments.

RESULTS

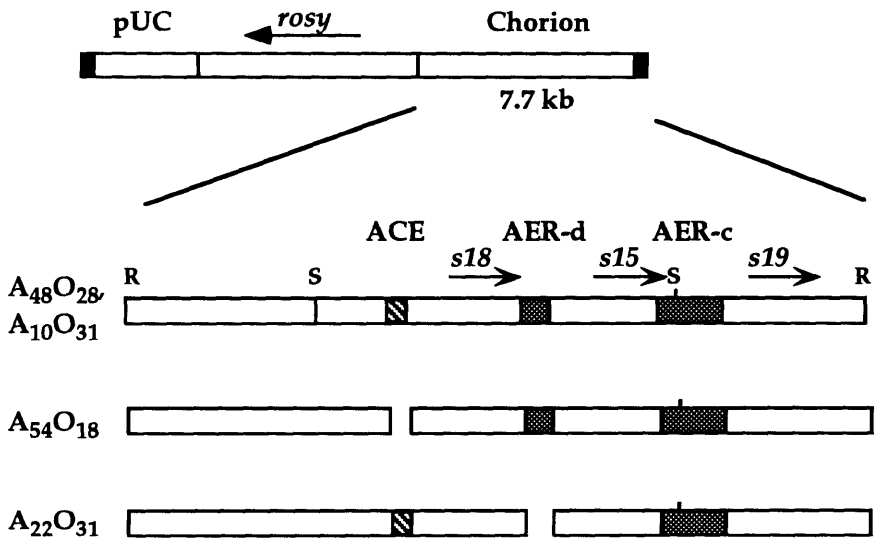
Replication of Chorion DNA is Dependent on the hsp70 Enhancer in Tissue Culture Cells

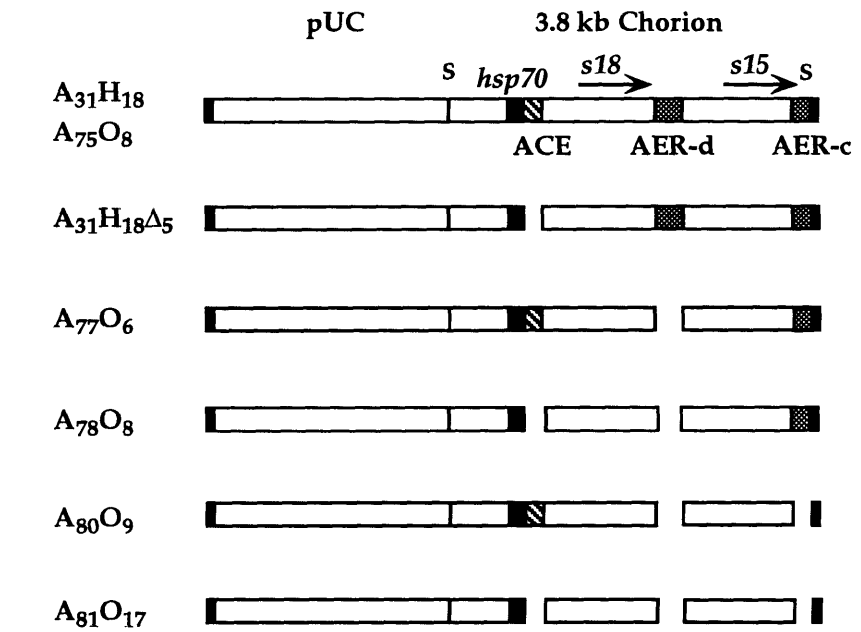
In order to test whether chorion constructs replicated following transient transfection into Drosophila Schneider L-2 cells, plasmids were cotransfected, and aliquots were assayed for replication following transfection. Equal amounts of two chorion plasmids were transfected into culture cells to provide an internal control for the efficiency of transfection, and replication of the two constructs was compared directly. Following transfection, plasmid DNA was isolated at 24 hour intervals for five days, digested and Southern blotted. Digests and probes were chosen so that both unreplicated and replicated fragments from each plasmid ran as distinct bands on Southern blots.

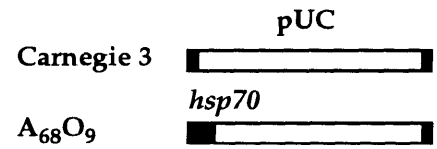
Replication was assayed by digesting DNA with restriction enzymes sensitive to methylation. *Dpn*I cleaved fully methylated DNA whereas *Cla*I digested fully unmethylated DNA. Plasmid DNA when isolated from bacteria was initially methylated by bacterial methylases. Following transfection into tissue culture cells, DNA became hemimethylated after one round of replication and was completely unmethylated following two complete rounds of replication. *Dpn*I resistant plasmid DNA was therefore diagnostic for one round of replication or repair synthesis. Following two rounds of replication or repair synthesis, plasmid DNA became sensitive to *Cla*I digestion.

Several different chorion constructs were tested for replication in pairwise combinations (Fig. 2 and Table I). The first pair, $A_{54}O_{18}$ and $A_{48}O_{28}$, compared two constructs containing a 7.7 kb *Eco*RI chorion fragment, as well

Figure 2. Chorion constructs used in replication studies. All constructs shown contain pUC sequences as well as P-element sequences depicted at the ends of the constructs by black boxes. Plasmids are deleted for either ACE3 (stripped box), AER-d or AER-c (gray boxes) as indicated. The top four constructs contain a 7.2 kb fragment containing the *rosy* gene, and a 7.7 kb *Eco*RI fragment containing the first three chorion genes of the third chromosome cluster. Plasmids $A_{48}O_{28}$ and $A_{54}O_{18}$ contain a 500 bp *rosy* sequence tag in *s18*. Plasmid $A_{10}O_{31}$ contains a 3.1 kb *lacZ* tag in *s18* and $A_{22}O_{31}$ contains *lacZ* in *s15*. The next seven constructs do not have the *rosy* gene and contain a 3.8 kb *Sal*I fragment containing the first two chorion genes. These constructs contain a 250 bp dimer of the *hsp70* enhancer (⁻107-⁻37) (black dotted box), inserted at the border of ACE3 (⁻630 5′ of *s18*). Plasmids $A_{31}H_{18}$, and $A_{31}H_{18}\Delta_5$, also contain a 500 bp *rosy* sequence tag in *s18*. No sequence tags are present in $A_{75}O_8$, $A_{77}O_6$, $A_{78}O_8$, $A_{80}O_9$, and $A_{81}O_{17}$. Carnegie 3 contains pUC and P-element sequences, and a derivative, $A_{68}O_9$, has the *hsp70* enhancer inserted into the polylinker of Carnegie 3. Deletions of ACE3 extend from ⁻630-⁻310 5′ of *s18*, and deletions of AER-d are at position ⁻820-⁻390 5' of *s15*. The rest of the AER-c region (270 bp) was deleted up to a *Xho*I site in *s15*.


pUC
rosy
Chorion
7.7 kb
ACE
AER-d
AER-c
s18
s15
s19
R
S
S
R
A48O28
A10O31
A54O18
A22O31


pUC
3.8 kb Chorion
S
hsp70
s18
s15
S
A31H18
A75O8
ACE
AER-d
AER-c
A31H18Δ5
A77O6
A78O8
A80O9
A81O17


pUC
Carnegie 3
hsp70
A68O9

as *rosy* and pUC sequences. $A_{54}O_{18}$ differed from $A_{48}O_{28}$ in that ACE3 sequences (⁻310-⁻630 5' of *s18*) were deleted. The second set compared $A_{22}O_{31}$ with $A_{10}O_{31}$. $A_{10}O_{31}$ was similar to $A_{48}O_{28}$ with the exception of sequence tags present in the chorion genes (Fig. 2). $A_{22}O_{31}$ contained a deletion of AER-d (⁻390-⁻820 5' of *s15*), the region containing the major amplification origin. Replication did not occur for any of these four constructs following transfection. It was possible that the 7.2 kb *rosy* fragment inhibited replication of these plasmids. However preliminary experiments suggested that similar constructs lacking this fragment did not replicate or replicated to very low levels.

Replication is often influenced by transcription, and enhancers are sometimes required in cis for replication, as is the case with polyoma viral replication (DePamphilis, 1988). Because chorion constructs did not replicate in tissue culture cells, we wished to determine whether an enhancer could stimulate replication. The *hsp70* enhancer was chosen so that heat shock of tissue culture cells might activate this enhancer. In all constructs containing *hsp70*, the enhancer was inserted at the border of ACE3 (⁻630 5' to *s18*). Pairwise combinations of constructs were tested (Fig. 2, Table I), and cell cultures were heat shocked every 12 hours following an initial 24 hour period after transfection. Transfections were also performed in which cells were not heat shocked.

In contrast to the first group of constructs, chorion constructs containing *hsp70* replicated in SL-2 cells. The first pair, $A_{31}H_{18}$ and $A_{31}H_{18}\Delta_5$, differed only in a deletion of ACE3, and both plasmids replicated under heat shock and non-heat shock conditions. The second pair, $A_{75}O_8$ and $A_{77}O_6$, compared a complete chorion fragment with a deletion of AER-d. $A_{75}O_8$ was similar to $A_{31}H_{18}$ and differed only in sequence tags present in chorion genes

(Fig. 2). Again, both plasmids replicated with or without heat shocking. Therefore, the presence of *hsp70* enhancer in cis was necessary for chorion DNA replication in vitro.

In order to test whether known cis-acting amplification regions were required for replication, several deletion constructs were tested. Plasmids were deleted for either ACE3, AER-d, or AER-c (Fig. 2). $A_{78}O_8$ contained a deletion of both ACE3 and AER-d, whereas $A_{80}O_9$ was deleted for AER-d and AER-c, and $A_{81}O_{17}$ was deleted for all three regulatory regions. All pairwise combinations replicated with or without heat shock (Fig. 3, Table I). These results showed that replication was not dependent on the known cis-acting amplification control elements.

All replicating plasmids showed similar levels of replication in relation to the amount of input plasmid, and therefore the ratio of replication for pairwise combinations was not determined. Furthermore, at least two rounds of replication or repair synthesis occurred as shown by the digestion of fully unmethylated DNA with *Cla*I. The replication of plasmids containing

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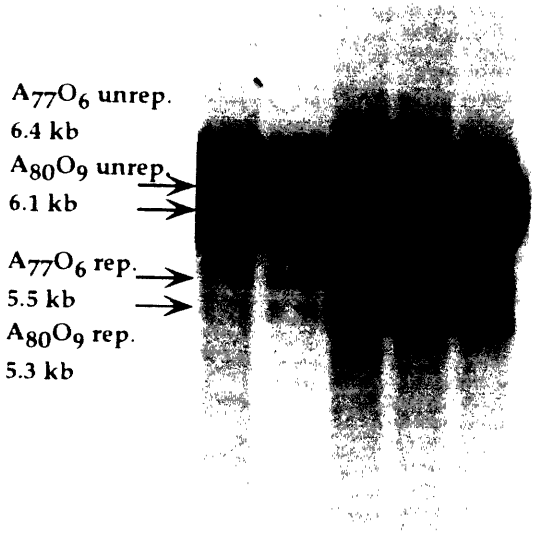
Fig. 3 Replication of chorion plasmids in SL-2 tissue culture cells. Three pairwise combinations of chorion plasmids are shown. Replicated plasmids that had undergone two rounds of replication or repair synthesis were cleaved at an internal *Cla*I site resulting in smaller fragments than the corresponding fragments from unreplicated plasmids. Plasmids were isolated every 24 hours following transfection for five time points. Examples are shown of plasmids isolated from cells that were either heat shocked or not heat shocked. Both experiments were done for all sets of plasmids and similar results were obtained in either case. **A.** $A_{77}O_6$ and $A_{80}O_9$ were deleted for AER-d and both AER-d and AER-c, respectively. **B.** $A_{81}O_{17}$ was deleted for ACE3, AER-d and AER-c. **C.** $A_{78}O_8$ was deleted for ACE3 and AER-d.

A. $A_{77}O_6 + A_{80}O_9$

No Heat Shock

Hours: 24 48 72 96 120

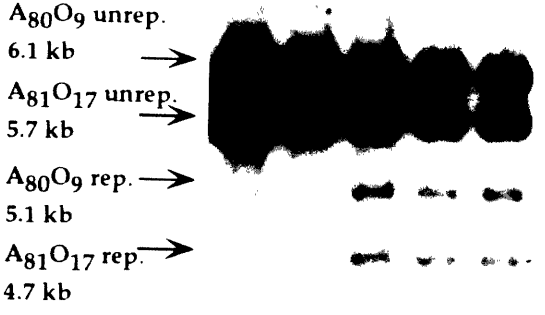


EcoRI + ClaI
probe: pUC

B. $A_{80}O_9 + A_{81}O_{17}$

No Heat Shock

24 48 72 96 120

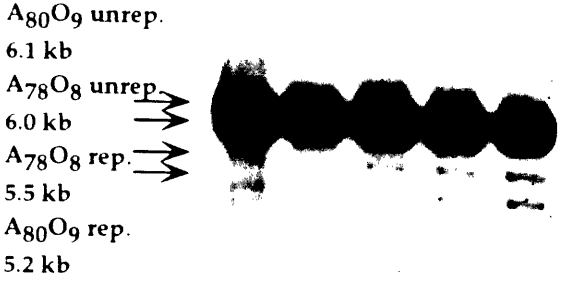


MluI + ClaI
probe: pUC

C. $A_{78}O_8 + A_{80}O_9$

Heat Shock

24 48 72 96 120



EcoRI + ClaI
probe: pUC

hsp70 enhancer occurred in transfections that were either heat shocked or not heat shocked, suggesting that the transfection alone activated *hsp70*. *hsp70* was inserted into a 3.8 kb *Sal*I chorion fragment, in contrast to the 7.7 kb fragment tested in the first group of plasmids. It was possible that the larger chorion fragment contained sequences inhibitory to replication. However, preliminary results suggested that larger constructs also replicated when *hsp70* was inserted next to the ACE3 element.

Efficient Replication is Dependent on Chorion DNA

In the above experiments, constructs contained both pUC and P-element derived sequences as well as chorion DNA. In order to determine whether replication was dependent on chorion DNA, constructs containing pUC and P-element sequences were assayed for replication. The Carnegie 3 and $A_{68}O_9$ plasmids consisted of P-element ends and pUC DNA, and $A_{68}O_9$ also contained the *hsp70* enhancer inserted in the polylinker (Fig. 2). These two plasmids were compared in their ability to replicate following heat shock. A small amount of replication occurred for both plasmids as indicated by weak *Dpn*I resistant bands. Whether more than one round of replication occurred was not tested. These results indicated that efficient replication was dependent on chorion DNA. An alternative possibility was that replication was dependent on plasmid size, which was not investigated further.

Although replication of chorion DNA containing *hsp70* enhancer occurred in tissue culture cells, the replication was not dependent on ACE3 or AER elements. Therefore, these amplification regulatory regions could not be studied further in this in vitro system.

Table I. Pairwise Cotransfections in Schneider L-2 Tissue Culture Cells

Cotransfected Plasmids		Replication[a]
$A_{54}O_{18}$	$A_{48}O_{28}$	-/-
$A_{10}O_{31}$	$A_{22}O_{31}$	-/-
$A_{31}H_{18}$	$A_{31}H_{18}\Delta_5$	+/+
$A_{75}O_8$	$A_{77}O_6$	+/+
$A_{77}O_6$	$A_{78}O_8$	+/+
$A_{77}O_6$	$A_{80}O_9$	+/+
$A_{78}O_8$	$A_{81}O_{17}$	+/+
$A_{78}O_8$	$A_{80}O_9$	+/+
$A_{80}O_9$	$A_{81}O_{17}$	+/+
Carnegie 3	$A_{68}O_9$	+/+[b]

[a] Replication of plasmids was roughly equivalent for all plasmids tested.

[b] Both plasmids replicated only to very low levels.

Replication of Plasmids Injected into Embryos

A second in vitro assay system was developed to study chorion DNA replication. In this system plasmids were coinjected into embryos and replication was assayed following 3 hours of development. Plasmids were recovered from a nuclear DNA preparation and digested with *Dpn*I and *Bgl*I. Unreplicated methylated DNA was digested with *Dpn*I and *Bgl*I, whereas replicated hemimethylated or unmethylated DNA was only sensitive to *Bgl*I digestion. DNA resistant to *Dpn*I and sensitive to *Bgl*I digestion underwent at least one round of replication or repair synthesis. Whether further rounds of replication occurred in this assay system was not determined.

The first combination of plasmids analyzed was the $A_{31}H_{18}$ chorion construct and Carnegie 3 which did not contain chorion DNA. In three independent experiments embryos were injected, and both supernatant and nuclear fractions were analyzed for plasmid replication. The majority of plasmid DNA was recovered in nuclear fractions, consistent with previous reports of injected plasmids becoming enclosed in nuclear structures (Steller and Pirrotta, 1985). For both plasmids, some plasmid became resistant to *Dpn*I and sensitive to *Bgl*I digestion, indicating that at least one round of replication or repair synthesis had occurred (Fig. 4). Quantitation of fragments from replicated plasmids indicated that $A_{31}H_{18}$ replicated from 2.2 to 4 times as much as Carnegie 3. The greater replication of $A_{31}H_{18}$ was either due to specific chorion sequences or to a larger plasmid size.

To determine whether replication of chorion DNA was dependent on amplification control elements, Carnegie 3 was next compared to $A_{31}H_{18}\Delta_5$ which contains a deletion of ACE3. One experiment was performed, and both plasmids replicated to a very slight extent. Quantitation indicated that replication was equivalent for the two plasmids. Therefore, in contrast to the results obtained in tissue culture, replication of chorion DNA in embryos might be dependent on ACE3.

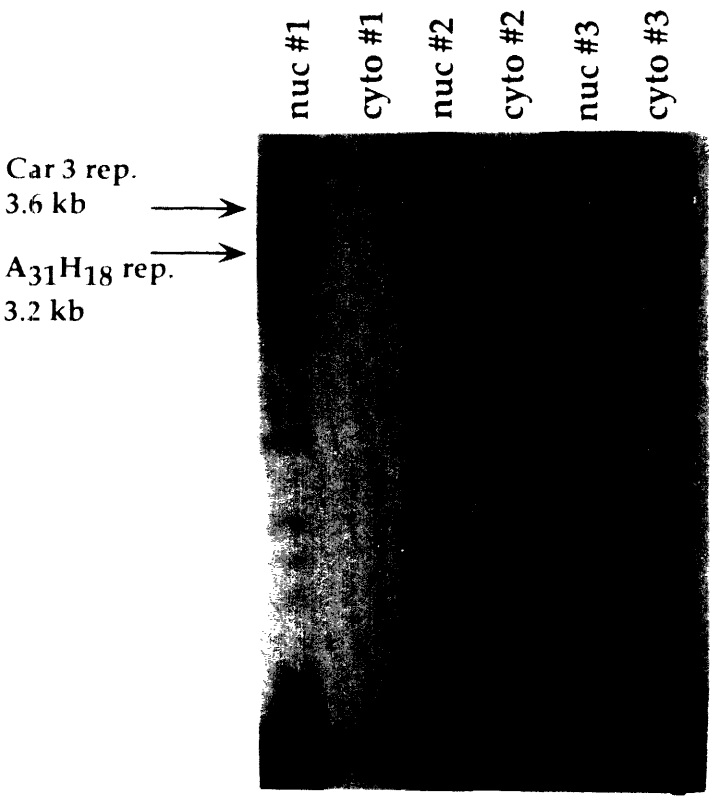
Two other pairwise injections were performed to determine whether hsp70 enhancer was necessary for chorion replication, and also to compare a deletion of ACE3 with a plasmid deleted for both ACE3 and AER-d. However, plasmids were not recovered in these experiments. Lowered plasmid recovery might be due to variable nuclear formation surrounding plasmids, plasmid degradation within embryos, or problems inherent in the nuclear DNA preparation. These variations might have also been responsible for the differences in replication efficiencies between the chorion construct, the ACE3

deletion, and the Carnegie 3 plasmid. Because of these variations in plasmid recovery, further analysis of replication in embryos was not performed.

Fig. 4 Replication of plasmids injected into embryos. Carnegie 3 and $A_{31}H_{18}$ were coinjected into embryos, and nuclear and cytoplasmic fractions were prepared from three independent experiments (nuc, nuclear fraction; cyto, cytoplasmic fraction). Plasmids were digested with *Bgl*I and *Dpn*I, and plasmids that underwent one round of replication or repair synthesis were resistant to *Dpn*I cleavage. The 3.6 kb and 3.2 kb fragments for Carnegie 3 and $A_{31}H_{18}$, respectively, are from replicated plasmids that were not cleaved by *Dpn*I.

Replication in Embryos:

Carnegie 3 + $A_{31}H_{18}$



*Bgl*I + *Dpn*I
probe: pUC

DISCUSSION

An in vitro assay for replication in Drosophila SL-2 cells, was found to be dependent on chorion DNA and the presence of the *hsp70* enhancer inserted at ACE3. The effect of *hsp70* inserted at ACE3 might be due to altered chromatin structure, perhaps due to trans-acting factors bound at *hsp70* following transfection. This alteration might enable replication factors to bind origins present in chorion DNA and promote replication. Alternatively, *hsp70* could activate transcription of chorion genes present on the plasmid, and transcription might then enhance replication in vitro. In vivo, the transcriptional controls for the chorion genes are separable from the cis-acting amplification control region ACE3 (Orr-Weaver et al., 1989), yet it remains possible that replication in vitro is different in this regard.

The stimulatory effect of the *hsp70* enhancer on replication is only seen with chorion DNA. Carnegie 3 replicates to a small extent, yet *hsp70* does not increase the efficiency of replication. One possibility is that Drosophila DNA or specific chorion sequences are necessary for efficient replication in tissue culture cells in combination with *hsp70*. However, another possibility is that replication of plasmids is size dependent as well as dependent on *hsp70*. This is true for plasmid replication in Xenopus eggs where replication efficiency correlates with plasmid size (Mechali and Kearsey, 1984). All chorion constructs that replicated were 6 to 7 kb, whereas less efficient replication occurred with the 3 to 4 kb Carnegie 3 and $A_{68}O_9$ plasmids. Perhaps a minimum size greater than 4 kb is necessary for efficient replication. Preliminary results of Delidakos and Kafatos (personal communication) also indicate that replication in embryos is size dependent. They compared chorion plasmids to pUC and found that replication of chorion plasmids

increased as the plasmids increased in size from 4 kb to 16 kb. However, the replication efficiency of larger non-chorion plasmids was not determined. It might be that the increased amount of Drosophila DNA or specific chorion DNA was responsible for more efficient replication.

Replication of chorion DNA however is not dependent on the three regions known to be important for the control of amplification. ACE3 is essential for amplification of the 7.7 kb *Eco*RI chorion fragment, and AER-d and -c are necessary for high levels of amplification. AER-d also contains the predominant origin used during amplification. These three cis-acting elements do not appear to have any effect on replication in vitro. One explanation for this is that these elements are only necessary for amplification, whereas replication proceeds via the control of distinct cis-acting regions. Alternatively, these elements might control DNA replication, yet multiple redundant elements might be present in chorion DNA, similar to the redundancy seen for amplification cis-acting elements. Deletions of some of these elements may not have an effect on replication, whereas an effect on amplification is seen. Perhaps in vitro replication is not an appropriately sensitive assay to see quantitative effects of cis-acting controls.

Whether any of the deleted amplification elements contain a replication origin was not determined. Deletions had no effect on replication, yet replication in vitro might occur at multiple origins present in the plasmid. An alternative explanation is that replication in vitro is not dependent on sequence-specific replication origins. This is true for the replication of plasmids in Xenopus embryos or extracts, where replication initiates at random sequences throughout the plasmid (Hyrien and Mechali, 1992; Mahbubani et al., 1992). This is also true of initiations within the chromosomal histone repeats in Drosophila embryos and tissue culture cells

as shown by two-dimensional gel analysis (Shinomiya and Ina, 1991; Shinomiya and Ina, 1993). If replication of chorion DNA in tissue culture cells also occurs at multiple initiation sites, then deletions of any particular region would not have an effect on replication.

Replication of plasmids when injected into Drosophila embryos occurs at low levels. At least one round of replication or repair synthesis occurs, yet plasmid recovery is variable. Replication of chorion plasmids is more efficient than control plasmids, and increased efficiency might also be due to either chorion sequences or a larger plasmid size. It was not determined if replication was dependent on known amplification regulatory sites due to the variabilities inherent in this assay system.

REFERENCES

Ashburner, M. 1989. Drosophila. A Laboratory Manual. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.).

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. 1987. Current Protocols In Molecular Biology. K. Janssen, (John Wiley & Sons, New York, N. Y.).

Bell, S. P. and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature *357*: 128-34.

Bell, S. P., R. Kobayashi, and B. Stillman. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. Science *262*: 1844-49.

Blow, J. J. 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. J. Cell Biol. *122*: 993-1002.

Blow, J. J. and R. A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. Cell *47*: 577-87.

Brewer, B. J. and W. L. Fangman. 1987. The localization of replication origins on ARS plasmids in S. cerevisiae. Cell *51*: 463-71.

Coverley, D., C. S. Downes, P. Romanowski and R. A. Laskey. 1993. Reversible effects of nuclear membrane permeabilization on DNA replication: evidence for a positive licensing factor. J. Cell Biol. *122*: 985-92.

Crevel, G. and S. Cotterill. 1991. DNA replication in cell-free extracts from *Drosophila melanogaster*. Embo J. *10*: 4361-9.

DePamphilis, M. L. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell *52*: 635-8.

Delidakis, C. and F. C. Kafatos. 1989. Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. Embo J. *8*: 891-901.

Diffley, J. F., J. H. Cocker, S. J. Dowell and A. Rowley. 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell *78*: 303-16.

Harland, R. M. and R. A. Laskey. 1980. Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. Cell *21*: 761-71.

Heck, M. M. and A. C. Spradling. 1990. Multiple replication origins are used during Drosophila chorion gene amplification. J. Cell Biol. *110*: 903-14.

Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. el-Assouli and L. R. Davis. 1987. The in vivo replication origin of the yeast 2 microns plasmid. Cell *51*: 473-81.

Hyrien, O. and M. Mechali. 1992. Plasmid replication in Xenopus eggs and egg extracts: a 2D gel electrophoretic analysis. Nucleic Acids Res. *20*: 1463-9.

Mahbubani, H. M., T. Paull, J. K. Elder and J. J. Blow. 1992. DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts. Nucleic Acids Res. *20*: 1457-62.

Mechali, M. and S. Kearsey. 1984. Lack of specific sequence requirement for DNA replication in Xenopus eggs compared with high sequence specificity in yeast. Cell *38*: 55-64.

Orr-Weaver, T. L. 1991. Drosophila chorion genes: cracking the eggshell's secrets. Bioessays *13*: 97-105.

Orr-Weaver, T. L., C. G. Johnston and A. C. Spradling. 1989. The role of ACE3 in Drosophila chorion gene amplification. Embo J. *8*: 4153-62.

Orr-Weaver, T. L. and A. C. Spradling. 1986. Drosophila chorion gene amplification requires an upstream region regulating s18 transcription. Mol. Cell. Biol. *6*: 4624-33.

Roth, G. E. 1991. Replication analysis of plasmid DNAs injected into Drosophila embryos. Chromosoma *100*: 267-77.

Rubin, G. M. and A. C. Spradling. 1983. Vectors for P element-mediated gene transfer in Drosophila. Nucleic Acids Res. *11*: 6341-51.

Shinomiya, T. and S. Ina. 1991. Analysis of chromosomal replicons in early embryos of *Drosophila melanogaster* by two-dimensional gel electrophoresis. Nucleic Acids Res. *19*: 3935-41.

Shinomiya, T. and S. Ina. 1993. DNA replication of histone gene repeats in *Drosophila melanogaster* tissue culture cells: multiple initiation sites and replication pause sites. Mol. Cell. Biol. *13*: 4098-106.

Shinomiya, T. and S. Ina. 1994. Mapping an initiation region of DNA replication at a single-copy chromosomal locus in *Drosophila melanogaster* cells by two-dimensional gel methods and PCR-mediated nascent-strand analysis: multiple replication origins in a broad zone. Mol. Cell. Biol. *14*: 7394-403.

Steller, H. and V. Pirrotta. 1985. Fate of DNA injected into early Drosophila embryos. Dev. Biol. *109*: 54-62.

Stillman, B. 1994. Smart machines at the DNA replication fork. Cell *78*: 725-8.

Waga, S. and B. Stillman. 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. Nature *369*: 207-12.

CHAPTER IV.

Characterization of Early Embryonic Defects in *pan gu;* a Germ-line Dependent Gene Required to Inhibit DNA Replication

INTRODUCTION

DNA replication is controlled at several levels to ensure a single round of replication during each cell cycle. First, S phase onset is coupled to mitosis, and second, origins of replication are activated only once during a single S phase. Rereplication of origins does not normally occur during S phase, although some exceptions include the amplification of the chorion genes, as discussed previously. Rereplication is prevented by allowing only one initiation event to occur per replication origin. How this mechanism works at a molecular level is not yet known, although one hypothetical model involves a licensing factor that directly modifies the DNA allowing only one initiation event (Blow and Laskey, 1988; Blow, 1993; Coverley et al., 1993). This putative licensing factor is inactivated following one round of replication, and only reenters the nucleus upon nuclear envelope breakdown during mitosis.

Other mechanisms exist to couple replication and mitosis. Checkpoints and feedback controls ensure the proper coupling of S phase and M phase. They act to prevent DNA replication until the completion of mitosis, and analogous controls prevent mitosis during DNA replication. Examples of checkpoints that block mitosis during S phase include *CDC6* in *S. cerevisiae*, and *cdc18*$^+$, *cdt1*$^+$, and *cut5*$^+$ in *S. pombe* (Bueno and Russell, 1992; Kelly et al., 1993; Saka and Yanagida, 1993; Hofmann and Beach, 1994). These genes are also essential for S phase, and mutants are blocked at the entry into S phase before attempting subsequent mitotic divisions. Similarly, *rum1*$^+$ in *S. pombe* acts earlier, as a checkpoint during G1, that blocks mitosis (Moreno and Nurse, 1994). The *rum1*$^+$ checkpoint may prevent mitosis by blocking cdc2 kinase activation (reviewed in Nurse, 1994). Consistent with this, cells

overexpressing *rum1*$^+$ undergo multiple rounds of replication, as if the cell is continually reset into G1 phase. In *S. pombe*, simply disrupting cyclin B kinase activity by a cyclin B (*cdc13*) deletion also results in multiple rounds of S phase (Hayles et al., 1994). These results indicate that high levels of cyclin B kinase are necessary for entry into M phase, and low levels result in entry into S phase.

Similar controls exist that repress S phase during mitosis, one example being the *ts41* gene product in hamster cells (Handeli and Weintraub, 1992). The *ts41* gene product may also be required for mitosis, and cells mutant for *ts41* undergo multiple rounds of S phase. *ts41* may be necessary for cyclin B kinase activation in hamster cells, analogous to the above regulation in *S. pombe*. Thus, checkpoint controls ensure the proper sequential ordering of events during the cell cycle.

Checkpoints and feedback mechanisms may control regulators of the cell cycle, such as cyclin-cdk complexes. Throughout the cell cycle, cyclin kinases control the entry into S phase and M phase. Cyclins A and B in complex with cdc2 are the predominant M phase kinases, whereas cyclins E, D, and A in complex with cdk2, 4, and 6 are the predominant S phase kinases (reviewed in Sherr, 1993; King et al., 1994; Sherr, 1994). Regulators of cyclin-cdk complexes have been identified as cdk inhibitors, (cdis), and include the proteins p21^{Cip1}, p27^{Kip1}, and p16^{Ink4} from higher eukaryotes and p40^{Sic1} from *S. cerevisiae* (Schwob et al., 1994; reviewed in Sherr, 1994). Checkpoints may regulate these inhibitors to repress cell cycle events until their proper time of action. For example, the cdk inhibitor p21 is induced by a mammalian DNA damage checkpoint, p53, and inhibits G1/S cyclin kinases (El-Deiry et al., 1993). Alternatively checkpoint controls may regulate proteins needed for replication or mitosis, such as initiation factors required for DNA

synthesis. The cdk inhibitor p21 also binds directly to PCNA and inhibits DNA replication, but not repair synthesis, in response to the p53 checkpoint (Li et al., 1994).

In order to examine the regulation of DNA replication during Drosophila development, I chose to study the gene, *pan gu* (*png*), which is a maternal-effect gene involved in this regulation. The cell cycle during the first 13 divisions of the Drosophila embryo consists of a rapid cycle alternating between S phase and M phase. Zygotic transcription does not occur, and S phase regulators are post-transcriptionally regulated (Edgar and Schubiger, 1986; Richardson et al., 1993; Knoblich et al., 1994). In addition, unique regulators may be present during these early cell cycles. This is in contrast to the later canonical cell cycle, where cell cycle regulators are transcriptionally controlled. Drosophila presents a useful genetic system in which to study cell cycle regulation. The simplified cell cycle of the early embryo in which nuclei remain in a common syncytium makes the identification of regulators of DNA replication accessible to genetic study.

In Drosophila, the stage 14 oocyte is arrested at metaphase of meiosis I, and following passage through the uterus, meiosis is completed. The egg becomes rehydrated upon passage through the uterus, and this rehydration is thought to release the oocyte from a metaphase I arrest (reviewed in Foe et al., 1993). How this arrest is maintained and overcome at a molecular level is not known. In Drosophila, the completion of meiosis is not dependent on fertilization, and unfertilized eggs complete meiosis and arrest with four meiotic products, the three polar bodies and a female pronucleus. When fertilized, the three polar bodies form a characteristic bouquet arrangement at the anterior dorsal surface. Two bouquet arrangements are often formed, one consisting of two polar bodies and the other containing the third polar body.

When fertilized, the female pronucleus migrates to the interior of the anterior portion of the egg where the male pronucleus is located. The three polar bodies persist until cycle 10, and are subsequently degraded (Rabinowitz, 1941).

The genes *pan gu* (*png*), *plutonium* (*plu*), and *giant nuclei* (*gnu*) are good candidates for unique regulators of the early embryo. All are maternal-effect genes that undergo inappropriate DNA replication in mutant unfertilized eggs, leading to giant, polyploid nuclei (Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). Mutant stage 14 oocytes do not contain overreplicated DNA, whereas in early mutant unfertilized eggs, four overreplicated nuclei are present often in the location of polar bodies (Shamanski and Orr-Weaver, 1991). Therefore, meiosis is completed properly, and the four meiotic products undergo inappropriate DNA replication. As these nuclei continue to overreplicate, they often fuse to form one giant polyploid nucleus (Fig. 1A). The mutant phenotype in unfertilized eggs suggests that these genes normally act as negative regulators of DNA replication in the unfertilized egg, and make the restart of S phase dependent on fertilization. Fertilization must overcome the action of these genes in order to resume the cell cycle.

These genes may also regulate S phase in the early cycles following fertilization. In mutant fertilized embryos, giant polyploid nuclei also form suggesting that DNA replication is not properly coupled to mitosis following fertilization (Fig. 1A). Both the polar bodies and female and male pronuclei overreplicate and again fuse to form one to five giant nuclei (Freeman et al., 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). Although fertilized, these embryos fail to properly couple S phase and M phase, and some aspects of mitosis such as centrosome duplication continue

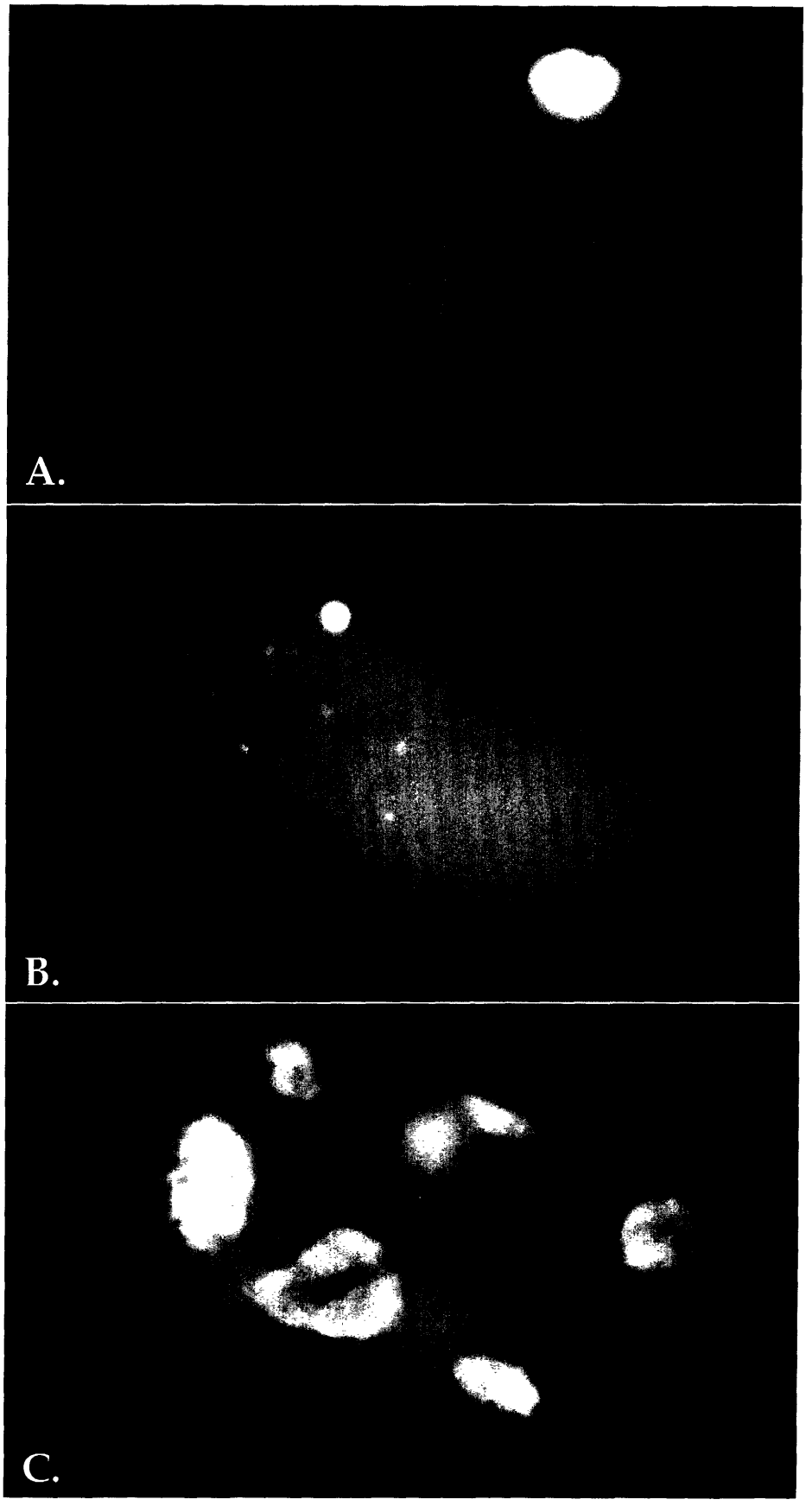
to cycle independently from nuclear division (Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). Genetic interactions have been detected among these three genes, suggesting that they regulate a common pathway or regulatory function (Shamanski and Orr-Weaver, 1991).

Among these genes, *png* is unique in that two phenotypes are seen in fertilized mutant embryos (Shamanski and Orr-Weaver, 1991). Three *png* alleles, $png^{13\text{-}1058}$, $png^{13\text{-}1920}$, and $png^{12\text{-}158}$, result in embryos containing up to five giant nuclei. The four meiotic products and the male pronucleus overreplicate and often fuse to form a single giant nucleus (Fig. 1A). This class of alleles will be referred to as the single nucleus class. Two alleles, $png^{12\text{-}2786}$ and $png^{12\text{-}3318}$, result in 30-50% of embryos containing multiple giant nuclei (6 -20 nuclei) (Fig. 1C). Younger embryos sometimes contain small diploid-appearing nuclei, suggesting that some mitotic divisions occur resulting in multiple giant nuclei (Fig. 1B). This class of alleles will be referred to as the multiple nuclei class and represents presumably leaky *png* alleles. Therefore, *png* likely acts during the early cycles to ensure that replication and mitosis occur in the correct temporal order.

The unfertilized and fertilized phenotypes of *png* can be explained by the proposal that *png* acts to negatively regulate DNA replication. In unfertilized eggs *png* functions to make S phase dependent on fertilization, whereas in fertilized embryos *png* ensures that S phase is dependent on the proper completion of mitosis. Both *plu* and *gnu* act similarly to negatively regulate DNA replication, yet at what level these genes act is unclear. They might control the entry into S phase and couple this to both fertilization as well as the completion of mitosis during the early cell cycles. Alternatively, they might control either the block to rereplication, or other aspects of the cell cycle such as chromosome condensation that link mitosis to replication.

Experiments are described that characterize the phenotype of *png* embryos and show that *png* functions in the germ line to regulate replication prior to and following fertilization. We show that defects in fertilized *png* embryos of the multiple nuclei class of alleles, occur as early as the first mitotic division. Replication and mitosis are transiently coupled, suggesting that *png* is required following fertilization to make S phase dependent on the proper completion of mitosis. The interaction between *png* and several maternal-effect mutations, including *plu* and *gnu*, is examined. Interactions with known cell cycle regulators might elucidate how *png* regulates replication and mitosis in the Drosophila embryo.

Fig. 1. Single and multiple nuclei *png* phenotype as shown by DAPI staining. A. A single giant nucleus in unfertilized eggs from $png^{13\text{-}1058}$. This phenotype is seen in unfertilized eggs of all alleles, and for fertilized embryos from $png^{12\text{-}158}$, $png^{13\text{-}1058}$, and $png^{13\text{-}1920}$. B. Small diploid-appearing nuclei are seen in a fertilized embryo from the multiple nuclei allele, $png^{12\text{-}3318}$. C. Multiple giant nuclei form in fertilized embryos of $png^{12\text{-}3318}$ and $png^{12\text{-}2786}$. Shown here is $png^{12\text{-}3318}$. Photos were taken by F. Shamanski and T. Orr-Weaver.


A.
B.
C.

METHODS

Mutations and Strains

pan gu EMS induced mutant strains (isolation number M2) came from a maternal-effect mutant collection of J. Dawson Mohler (Mohler, 1977; Mohler and Carroll, 1984). Balancers and mutant strains are described in (Lindsley and Zimm, 1992).

Embryo Fixation and Staining

Embryos 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. Alternatively, embryos were fixed using formaldehyde; embryos were shaken for 15 minutes in a 1:1 mixture of heptane and 3.7% formaldehyde (in 0.1 M KPO_4). Embryos were then devitellinized using methanol. Following either fixation protocol, embryos to be stained with antibody or DAPI were rehydrated through a PBS/MeOH series to a final 1X PBS (136 mM NaCl, 2.7 mM KCl, 6.5 mM $NaHPO_4$, 1.5 mM KH_2PO_4).

To visualize DNA, embryos were stained using DAPI (4', 6-diamidino-2-phenylindole) at 1 μg/ml in 1X PBS for 10 minutes. Embryos were then washed several times in 1X PBS before mounting in 70% glycerol in 1X PBS.

DNA and spindles were visualized by double labeling with antibodies to both histones and tubulin. Embryos were rinsed in 1X PBST (1X PBS + 0.3% Triton X-100) and blocked in 1X PBST + 1% BSA for 1 hour. Antibodies were diluted in 1X PBST + 0.1% BSA. A 1:500 dilution of a mouse anti-histones monoclonal antibody (Chemicon International, Inc.) was incubated at 4°C overnight followed by several washes in 1X PBST. A secondary

polyclonal antibody, fluorescein-conjugated goat anti-mouse (Jackson Immunoresearch Laboratories, Inc.), was then incubated at a 1:500 dilution for 3 hours at room temperature, washed, and blocked for 1 hour in 1:100 normal mouse serum (Jackson Immunoresearch Laboratories, Inc.). Finally, a rhodamine-conjugated mouse anti α-tubulin monoclonal antibody (gift from Bill Theurkauf) was incubated at a 1:250 dilution overnight at 4°C, followed by several washes. Embryos were then dehydrated by rinsing several times in 100% methanol, and were cleared and mounted in 50 µl of a 2:1 solution of benzyl benzoate: benzyl alcohol, containing 50 mg/ml propyl gallate as an anti-bleaching agent.

Microscopy

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

Epistasis Analysis

Epistasis analysis was performed previously using $png^{12\text{-}3318}$, *plu* and *gnu*, and both *plu* and *gnu* as either heterozygotes or homozygotes affected the multiple nuclei phenotype of *png*. *plu* and *gnu* are epistatic to the $png^{12\text{-}3318}$, and result in the single giant nucleus phenotype (Shamanski and Orr-Weaver, 1991). Experiments were performed in order to test the interaction of *plu* and *gnu* with the second multiple nuclei *png* allele, $png^{12\text{-}2786}$.

To test interactions between *png*, *plu*, and *gnu*, epistasis analysis was performed as described in (Shamanski and Orr-Weaver, 1991), using the allele, $png^{12\text{-}2786}$. $png^{12\text{-}2786}$*/FM0;* plu^3*/CyO* and $png^{12\text{-}2786}$*/FM0; gnu/TM3* stocks were made by T. Orr-Weaver (personal communication). 0-6 hour embryo collections were done from double homozygous females, double heterozygous females, or females homozygous for one mutation and heterozygous for the other. Embryos were fixed and stained with DAPI as described above. To confirm that the interaction was specific to plu^3 and *gnu*, $png^{12\text{-}2786}$ was reisolated from *gnu* or plu^3 by crossing double heterozygous females with FM0 balancer males. $png^{12\text{-}2786}$*/FM0; +/CyO* or $png^{12\text{-}2786}$*/FM0; +/TM3* progeny were crossed *inter se* to produce homozygous *png* females in the presence of either balancer. 0-8 hour embryos were collected from these females, fixed and stained with DAPI. The multiple nuclei phenotype of reisolated $png^{12\text{-}2786}$ confirmed a specific interaction with both plu^3 and *gnu*.

Epistasis analysis was done with both classes of *png* alleles and the maternal-effect mutant, $fs(1)Ya^2$. Both mutations are on the *X* chromosome, and recombinants were obtained by crossing *y* $png^{13\text{-}1920}$ *cv v f/FM0* virgins to y^2 $fs(1)Ya^2$ *w spl* sn^3*/Y* males and subsequently crossing the non-balancer virgins to balancer males. The progeny were scored for *y w* recombinants, and independent recombinant stocks were made and tested for non-complementation for both *png* and $fs(1)Ya^2$ in order to obtain *y png* $fs(1)Ya^2$ *w* recombinant chromosomes. Similar crosses were done using $png^{12\text{-}2786}$ to obtain a double mutant chromosome with $fs(1)Ya^2$. One recombinant stock of each *png* allele in combination with $fs(1)Ya^2$ was used to examine the phenotype of the homozygous double mutant. Homozygous females and heterozygous controls were mated to sibling males, and resulting embryos

were fixed and stained with DAPI as described above. Embryos were aged for 30 min., 3 hours, or 7.5 hours (see Table II).

Females homozygous for either *png* allele and heterozygous for *fs(1)Ya* were obtained by crossing homozygous *png* virgins to double mutant *png fs(1)Ya* males. The resulting females were mated and used for embryo collections. Similarly, females heterozygous for either *png* allele and homozygous for *fs(1)Ya* were obtained by similar crosses using *fs(1)Ya* virgins and double mutant males. 0-3.5 hour embryos were collected from the resulting females and DAPI stained. All combinations showed the expected phenotype for the homozygous mutation. Double heterozygous mutants were also examined by DAPI, and showed wild-type staining.

Effect of Increased Dosage of *plu* on *png* Mutants

A *y png* w^{1118} recombinant strain was constructed for $png^{12\text{-}3318}$ and $png^{13\text{-}1058}$. The crosses were done as described above for *fs(1)Ya* recombinants; *y w* recombinant lines were obtained and tested for non-complementation over *png* in order to identify *y png w* recombinants. A transformant insertion on the third chromosome containing a 6.5 kb genomic fragment carrying both *plu* and *PCNA*, B130-1, was crossed into a *y png w* mutant strain. A stock was made using both alleles, $png^{13\text{-}1058}$ and $png^{12\text{-}3318}$, in combination with the *plu* rescue fragment, by scoring for w^+ progeny carrying the insertion. 0-5 hour embryos from homozygous *png* females containing one or two copies of the *plu* rescue fragment were analyzed by DAPI staining as described.

Analysis of Germ-line Clones

y png$^{13\text{-}1058}$ *cv v f/FM0* or *y png*$^{12\text{-}3318}$ *cv v f/FM0* virgins were crossed to *ovo*^{D1} males and collection bottles were set up to obtain embryos. 12 hour collections at 25°C were allowed to age for another 30 hours, so that the larvae would be 36 $\pm$ 6 hours old. These larvae were then given 1000 rad X-rays from a Torrex 150 X-ray machine; 167 sec. @ 100 kV and 5 mA on shelf 7. Control experiments were also performed with un-irradiated larvae. A mitotic recombination event proximal to *ovo*^{D1} would result in a germ-line clone of *png* (*ovo*^{D1} map position is 10.2 cm, between *png* at 1 cm and *cv* at 13.7 cm). Larvae were allowed to develop and resulting non-balancer females were collected and mated 5 per vial to *png* males. Females carrying germ-line clones were scored by the ability to lay eggs. Females from egg-laying vials were then separated to one per vial to determine the number of germ-line clones obtained. 9 clones from 265 *png*$^{12\text{-}3318}$ females were obtained (3.4%), whereas one clone from 31 *png*$^{13\text{-}1058}$ females was obtained (3.2%). One fertile *ovo*^{D1} revertant was also obtained from *png*$^{13\text{-}1058}$ females; revertants were determined by the progeny being heterozygous for the markers *yellow* (*y*), crossveinless (*cv*) and *forked* (*f*) along the X chromosome. However, progeny were also homozygous for *vermilion* (*v*) indicating that a double recombination event occurred on either side of *vermilion*, and an independent event gave rise to an *ovo*^{D1} reversion.

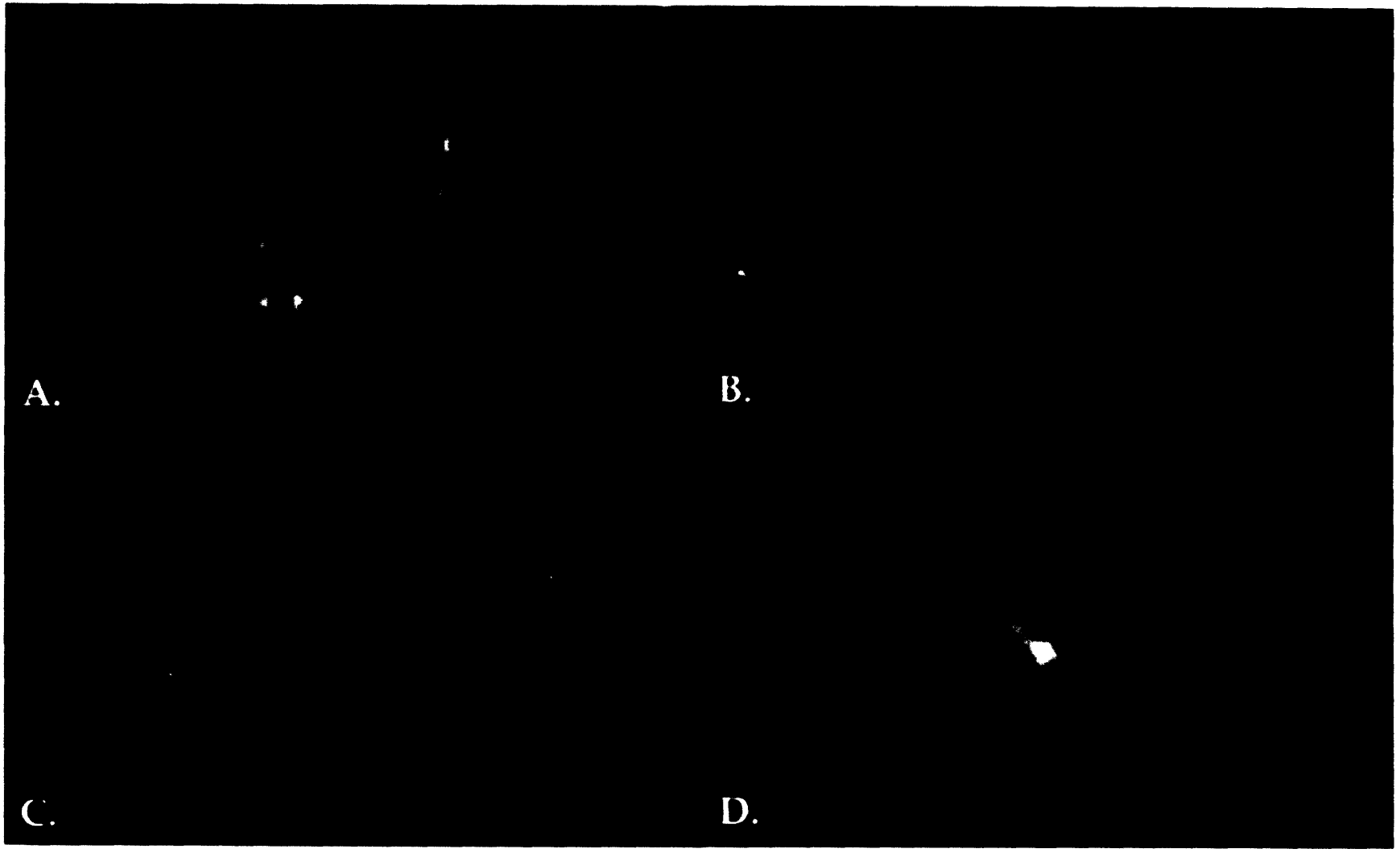
RESULTS

Characterization of Early Defects in *png* Embryos

Fertilized *png* embryos contained predominantly giant polyploid nuclei, yet smaller diploid-appearing nuclei formed in a small number of embryos (Fig. 1B) (Shamanski and Orr-Weaver, 1991). This suggested that some mitotic divisions occurred in these embryos before giving rise to giant nuclei. In order to characterize the early defects in *png* embryos and determine whether mitotic divisions did occur, we wished to examine the first divisions following fertilization. 10-30 minute collections of fertilized wild-type, $png^{12\text{-}3318}$, and $png^{13\text{-}1058}$ embryos were fluorescently labeled using histone and tubulin antibodies as markers for DNA and spindles, respectively (see Methods).

In wild-type embryos, the progression through meiosis and the first mitotic divisions have been extensively characterized (reviewed in Foe et al., 1993). In 10-30 minute collections of wild-type embryos, the three polar bodies and the male and female pronuclei could be seen at various stages following the completion of meiosis. Following meiosis, the three polar bodies decondense and then recondense forming a characteristic bouquet structure (Fig. 2A). These polar bodies are presumably in interphase when decondensed, and replicate prior to arresting in this condensed state. The three polar bodies in Fig. 2C were just starting to recondense and stained with histone antibodies. Two or three polar bodies formed a characteristic bouquet arrangement that persisted until cycle 10 (Fig. 2A). In later embryos (1-2 hours), polar bodies degrade following the cleavage divisions, and replicated chromosome arms appeared to unwind during degradation (Fig. 2A) (Rabinowitz, 1941).

Fig. 2. The fate of the four meiotic products in wild-type embryos. A. Three polar bodies are shown condensed into two bouquet arrangements on the dorsal anterior side of the embryo. Two polar bodies form the larger bouquet. Distinct chromosomes can be seen and the fourth chromosomes appear as small dots in the middle of the lower bouquet structure. Chromosome arms can be seen unraveling as polar bodies start to degrade following cycle 13. B. Following meiosis and fertilization, the two pronuclei meet in the interior of the embryo. Histone labeling reveals the two interphase pronuclei. C. Three polar bodies and the two pronuclei are shown following the first interphase. Chromosomes are beginning to condense as shown by histone staining. The two pronuclei condense as separate units during the first cell cycle. Polar bodies condense into the bouquet structure shown in A. D. The first (gonomeric) division spindle showing the two separate pronuclear units. All panels are double labeled with histone and tubulin antibodies. Only the histone staining is shown in A, and in the other panels, histone labeling is green and tubulin labeling is red. All images were obtained by confocal microscopy.


A.
B.
C.
D.

In the interior of the embryo, the first mitotic division of the male and female pronuclei (gonomeric division) occur before fusing to form two zygotic nuclei (reviewed in Foe et al., 1993). Prior to this first division, the juxtaposed, interphase pronuclei also stained with histone antibodies (Fig. 2B). Before the first mitosis, the male and female pronuclei condensed as separate units (Fig. 2C), and chromosomes from the two distinct pronuclei were segregated on a single mitotic spindle (Fig. 2D) (Foe et al., 1993). Following the formation of two zygotic nuclei, interphase and mitosis of later divisions were also observed in early embryo collections.

In contrast to wild type, replication and mitosis were not coupled in $png^{13\text{-}1058}$ embryos (Table I). In all embryos examined, nuclei stained with histone antibodies. Most nuclei were in the correct location for polar bodies or internal pronuclei, and most embryos contained one to five small polyploid nuclei. However, two embryos contained 10-15 small polyploid nuclei, suggesting that the polar bodies or pronuclei had divided. Some nuclei were paired as if divisions had occurred, yet no tubulin staining was detected. Whether these divisions occurred with a normal mitotic spindle could not be determined. 10-30% of older $png^{13\text{-}1058}$ embryos showed tubulin aster staining, and mitotic asters and centrosomes cycled independently of nuclear divisions (Shamanski and Orr-Weaver, 1991). However, no mitotic spindles or asters were observed in the early embryos (Table I).

In contrast to the single nucleus class of *png* alleles, replication and mitosis were transiently coupled in the multiple nuclei allele, $png^{12\text{-}3318}$. Several phenotypes were seen as shown in Table I and Fig. 3. 56% of the embryos only stained with histone antibody, similar to $png^{13\text{-}1058}$. In contrast to $png^{13\text{-}1058}$, 44% of the embryos also stained with tubulin antibody. 9.8% showed tubulin staining around the nuclei, yet had no recognizable spindles

or mitotic asters. 29.3% of the embryos contained mitotic figures or spindles, usually in the interior of the embryo. Overreplicated polar bodies were also present (Fig. 3B).

Aberrant mitotic figures formed during the first or second division. In one embryo, a normal first mitotic division occurred, in which the male and female chromosomes remained separate on the spindle (Fig. 3A). All other mitotic figures were aberrant, ranging from slight to severe phenotypes. Spindle abnormalities included unipolar, tripolar, and tetrapolar spindles. Mitotic figures also contained improperly aligned or condensed DNA, and lagging or lost chromosomes (Fig. 3C). As embryos developed, aberrant mitotic figures formed throughout the embryo and abnormalities became more severe. In two older embryos, nuclei were quite polyploid and independent tubulin asters were present throughout the embryo (Fig. 3D). However, some asters remained clustered around smaller polyploid nuclei. Aspects of replication and mitosis therefore became progressively uncoupled.

Fig. 3. Early defects in fertilized *png*$^{12\text{-}3318}$ embryos. A. A normal first (gonomeric) division spindle and pronuclear chromosome units are seen in a mutant embryo. B. The three polar bodies (left) are already overreplicated in an embryo containing abnormal zygotic nuclei. C. Abnormal mitotic figures are seen early on. One chromosome is not aligned with the metaphase plate in this second division spindle. Microtubules can be seen extending to this chromosome forming a tripolar spindle. D. An older *png12-3318* embryo is shown containing giant nuclei and independent tubulin asters. Asters can be seen associated with smaller DNA fragments. Histone labeling for all panels is shown in green, and tubulin labeling is red.

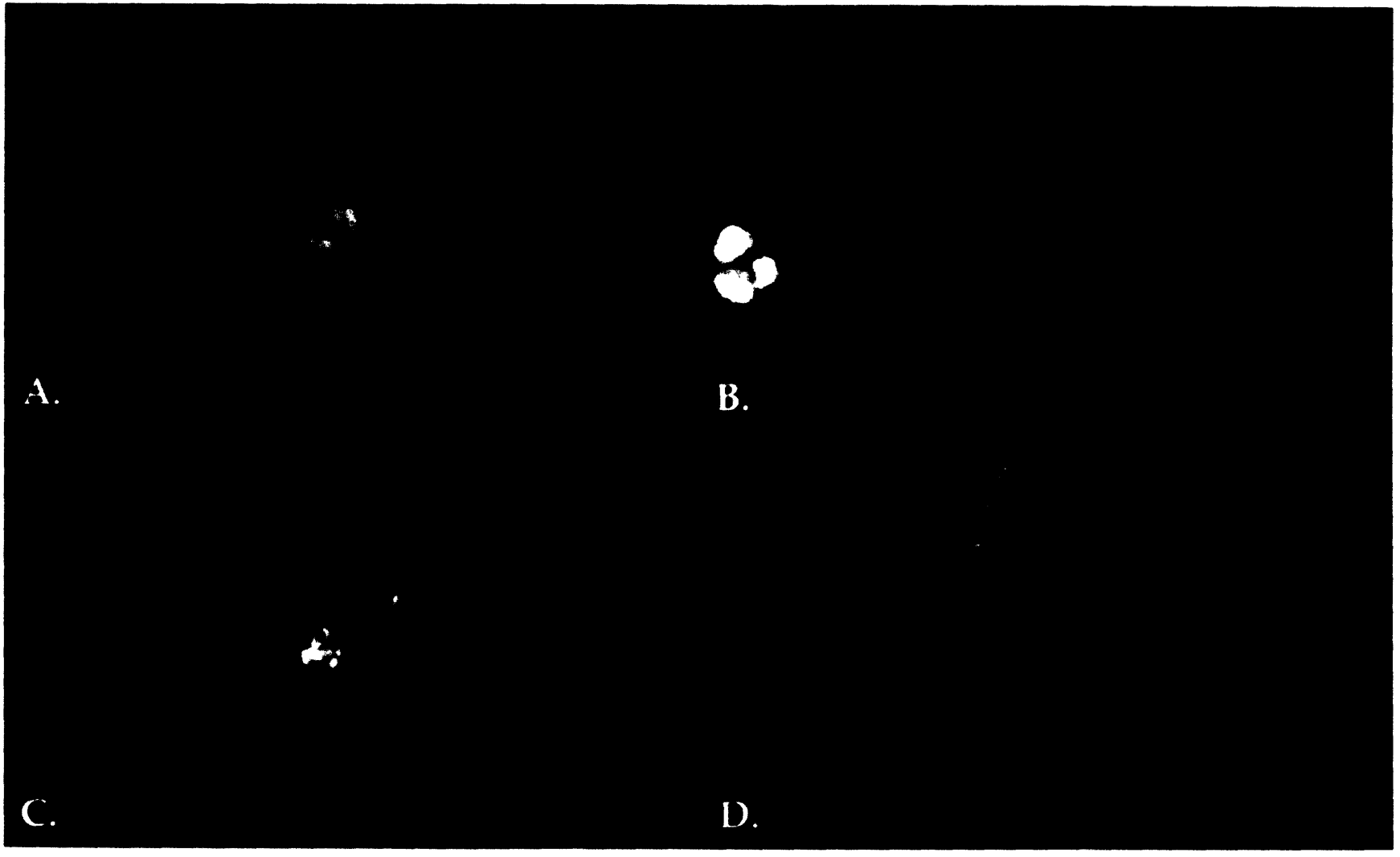

A.
B.
C.
D.

Table I. Cytological Observations in *png* Mutant Embryos

Genotype	No. of Embryos With the Observed Staining[a] (Percentages Shown in Parenthesis)			
	DNA alone	DNA + Tubulin[b]	DNA + spindles (mitotic figures)[c]	DNA + asters[d]
$png^{12\text{-}3318}$	23 (56.0)	4 (9.8)	12 (29.3)	2 (4.9)
$png^{13\text{-}1058}$	11 (100)	-	-	-

[a] 41 embryos from $png^{12\text{-}3318}$ and 11 from $png^{13\text{-}1058}$ were examined.

[b] Tubulin staining surrounded nuclei. No recognizable mitotic spindles or asters were formed.

[c] In most cases, mitotic figures showed aberrant DNA or tubulin morphology.

[d] Mitotic asters were observed in older embryos and cycled independently of nuclear divisions. Some asters did remain clustered around smaller nuclei.

$png^{12\text{-}2786}$ Interacts With *plu* and *gnu* in Regulating Replication

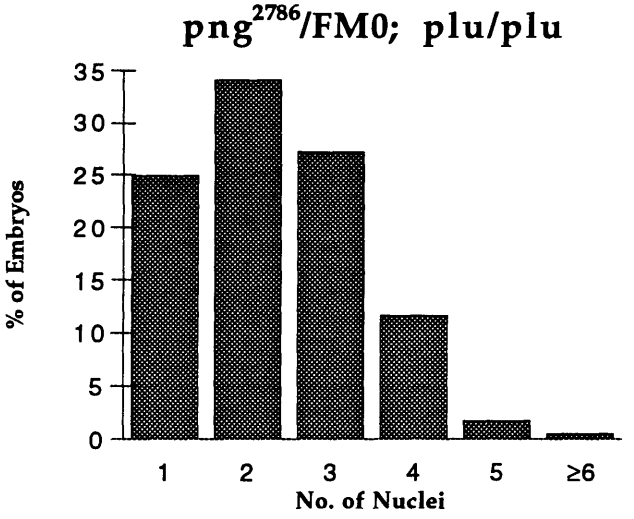
The three genes *png*, *plu*, and *gnu* have similar phenotypes in the unfertilized egg and fertilized embryo, and therefore might interact in regulating DNA replication. Genetic interactions were detected among the three genes, suggesting that these genes act in a common pathway or control a common regulatory function (Shamanski and Orr-Weaver, 1991). The distinct multiple nuclei phenotype of *png* allowed epistasis analysis with both *plu* and *gnu*, which show a single giant nucleus phenotype. Double homozygotes of $png^{12\text{-}3318}$ and plu^{3} or *gnu* resulted in embryos containing a single giant nucleus, instead of the multiple giant nuclei normally present in

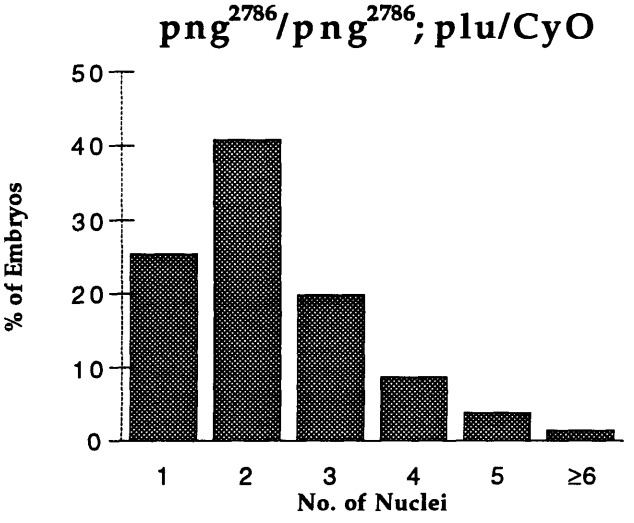
png$^{12\text{-}3318}$ homozygotes (Shamanski and Orr-Weaver, 1991). Similarly when heterozygous, *plu*^{3} or *gnu*, affected the phenotype of homozygous *png*$^{12\text{-}3318}$, embryos. Lowering the dosage of *plu* or *gnu* therefore abolished the transient coupling of replication and mitosis in *png*$^{12\text{-}3318}$ embryos.

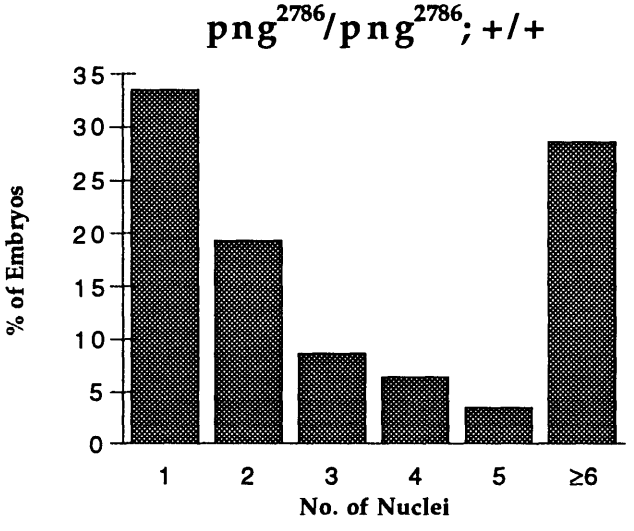
In order to characterize the second multiple nuclei allele, *png*$^{12\text{-}2786}$, and determine whether this allele similarly interacts with *plu* and *gnu*, epistasis analysis was performed with double mutants (see Methods). Both heterozygous or homozygous *plu*^{3} or *gnu* were epistatic to *png*$^{12\text{-}2786}$, causing a shift from the multiple nuclei to the single giant nucleus phenotype (Figs. 4, 5). In order to determine whether this interaction was specific to the *plu*^{3} and *gnu* mutations, *png*$^{12\text{-}2786}$ was reisolated from *gnu* and *plu*. The reisolated *png* allele regained the multiple nuclei phenotype, confirming a specific interaction with *plu*^{3} and *gnu* (Figs. 4, 5, and Methods). *plu* and *gnu* therefore interacted similarly with both multiple nuclei *png* alleles.

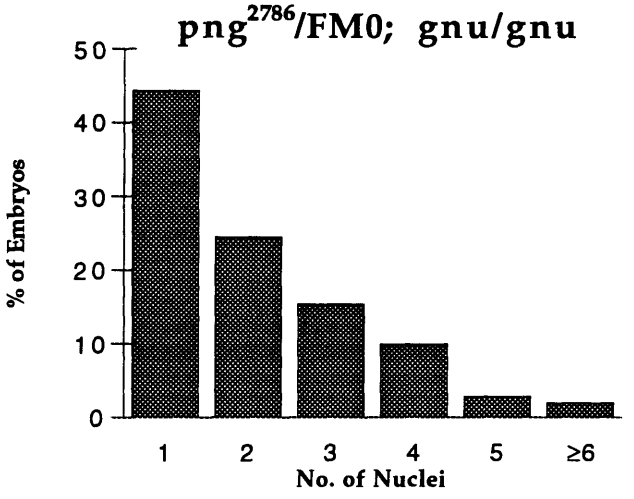
Fig. 4. Epistasis Analysis of *png*$^{12\text{-}2786}$ and *plu*^{3}. The percentage of embryos is graphed against the number of giant nuclei formed. Homozygous *plu* (top graph), or heterozygous *plu* in combination with homozygous *png*$^{12\text{-}2786}$ (middle graph), lack the class of embryos showing multiple nuclei (six or more). When reisolated from *plu*^{3}, *png*$^{12\text{-}2786}$ regains the multiple nuclei phenotype, where approximately 30% have greater than five giant nuclei.

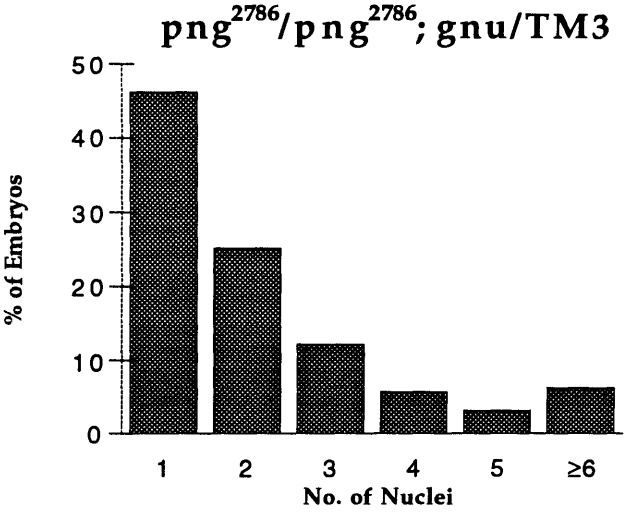
Fig. 5. Epistasis Analysis of *png*$^{12\text{-}2786}$ and *gnu*. The percentage of embryos containing one to six or more giant nuclei is shown. Homozygous *gnu* (top graph), or heterozygous *gnu* in combination with homozygous *png*$^{12\text{-}2786}$ (middle graph), lack the class of embryos showing multiple nuclei (six or more). *png*$^{12\text{-}2786}$ when reisolated from *gnu* regains the multiple nuclei phenotype.

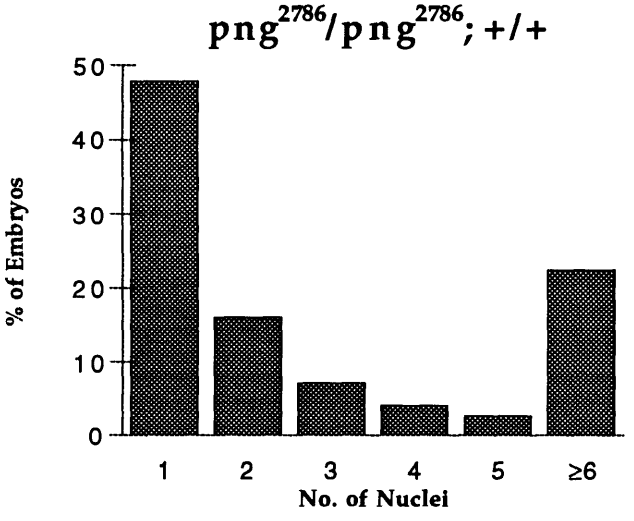

png2786/FM0; plu/plu
% of Embryos
35
30
25
20
15
10
5
0
1
2
3
4
5
≥6
No. of Nuclei


png2786/png2786; plu/CyO
% of Embryos
50
40
30
20
10
0
1
2
3
4
5
≥6
No. of Nuclei


png2786/png2786; +/+
% of Embryos
35
30
25
20
15
10
5
0
1
2
3
4
5
≥6
No. of Nuclei


png2786/FM0; gnu/gnu
% of Embryos
50
40
30
20
10
0
1
2
3
4
5
≥6
No. of Nuclei


png2786/png2786; gnu/TM3
% of Embryos
50
40
30
20
10
0
1
2
3
4
5
≥6
No. of Nuclei


png2786/png2786; +/+
% of Embryos
50
40
30
20
10
0
1
2
3
4
5
≥6
No. of Nuclei

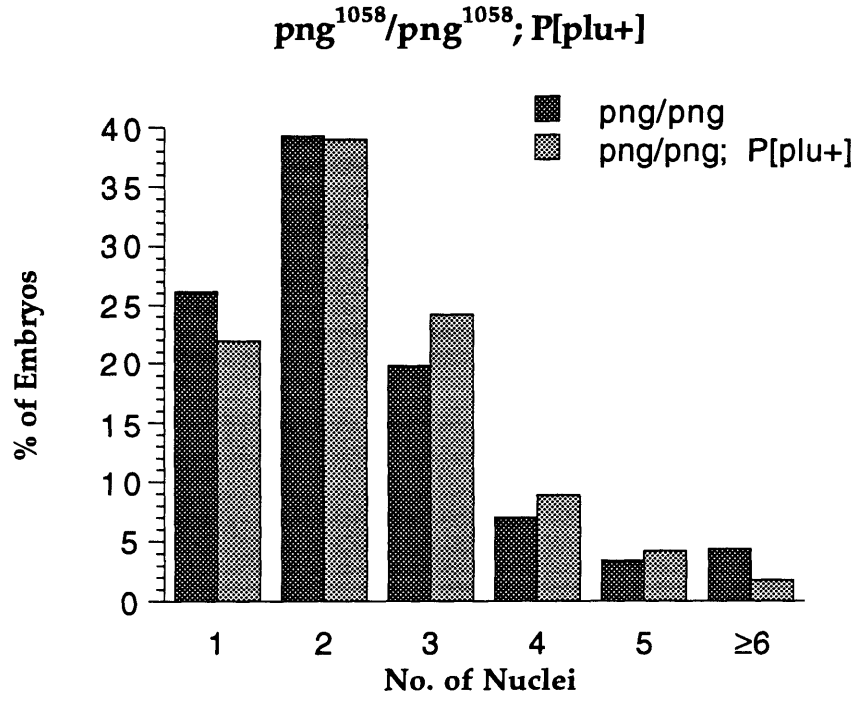
Effect of Increased Dosage of Wild-type *plu* on *png* Mutants

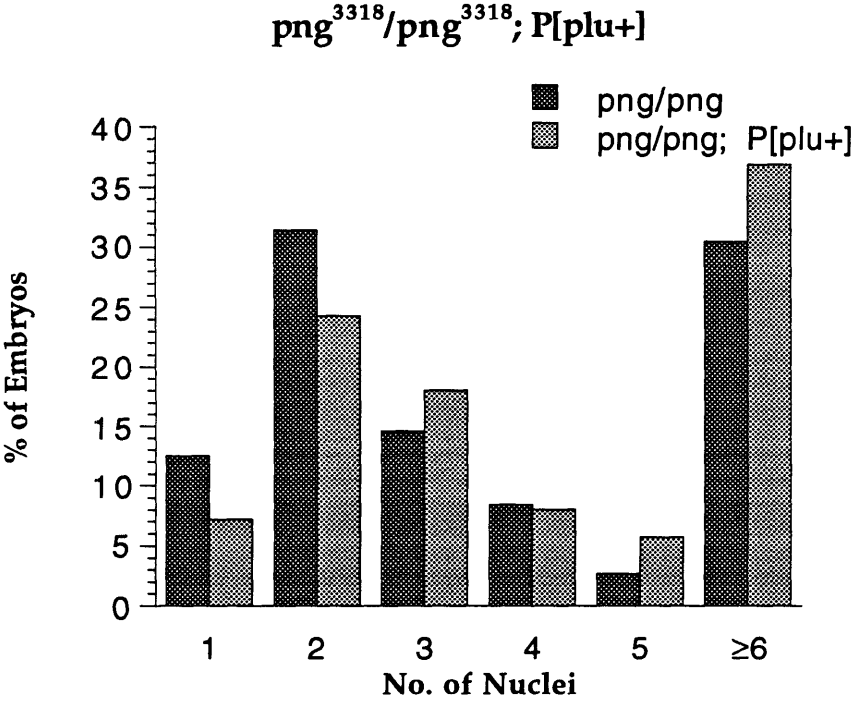
A single copy of mutant plu^3 worsened the phenotype of the multiple nuclei *png* alleles. It was therefore tested whether one or two additional copies of wild-type *plu* could rescue the *png* phenotype. Perhaps added *plu* function would allow more mitotic divisions in either class of alleles. A transformant line containing a 6.5 kb *plu* rescue fragment was tested, either heterozygous or homozygous, in combination with $png^{12\text{-}3318}$ and $png^{13\text{-}1058}$ homozygotes, and embryos were analyzed by DAPI staining (see Methods). The *plu* transformant construct did not rescue the *png* phenotype or increase the number of giant nuclei formed (Fig. 6). There may have been a slight increase in the percentage of $png^{12\text{-}3318}$ embryos containing multiple giant nuclei, however, this varied in control collections as well (data not shown). Therefore, one or two copies of *plu* was not sufficient to rescue or lessen the *png* phenotype.

fs(1)Ya*, a Gene Required for the First Embryonic Division, is Epistatic to *png

The *fs(1)Ya* gene product is needed for the first embryonic division, and the majority of mutant embryos are arrested at the pronuclear stage following the completion of meiosis (Lin and Wolfner, 1991). 25% escapers perform aberrant mitotic divisions in the null allele, $fs(1)Ya^2$. Preliminary results suggested that the null allele of *fs(1)Ya* was epistatic to both *plu* and *gnu*, resulting in the absence of giant nuclei (F. Shamanski, unpublished results;

Fig. 6. Dosage effect of added *plu* function. Homozygous *png* embryos are compared to homozygotes containing a P-element construct containing *plu*. Added *plu* function shows no significant effect in either a $png^{13\text{-}1058}$ background (top graph), or a $png^{12\text{-}3318}$ background (bottom graph).


png^1058/png^1058; P[plu+]
png/png
png/png; P[plu+]
% of Embryos
40
35
30
25
20
15
10
5
0
1
2
3
4
5
≥6
No. of Nuclei


png^3318/png^3318; P[plu+]
png/png
png/png; P[plu+]
% of Embryos
40
35
30
25
20
15
10
5
0
1
2
3
4
5
≥6
No. of Nuclei

M. Wolfner, personal communication). In order to determine the interaction between *fs(1)Ya* and *png* in the early embryo, epistasis analysis was performed. Both genes were on the *X* chromosome, and a *png fs(1)Ya* recombinant chromosome was constructed (see Methods). Epistasis analysis was performed using the null allele, $fs(1)Ya^{2}$, and both classes of *png* alleles, $png^{13\text{-}1920}$ and $png^{12\text{-}3318}$.

Embryos from mothers, homozygous mutant for both genes were DAPI stained, and showed a range of phenotypes as embryos developed over longer periods (Table II). Consistent with the *plu* and *gnu* results, short collections of double mutant embryos (30 minutes or 3 hours) showed predominantly the *fs(1)Ya* phenotype, being arrested at the pronuclear stage. However, nuclei appeared slightly less condensed than in the *fs(1)Ya* single mutant, and occasionally embryos contained giant nuclei (Table II). This observation prompted the analysis of longer collections of embryos (0-7.5 hours) in which giant nuclei formed in a greater proportion of embryos.

In longer embryo collections of the double homozygote, $png^{13\text{-}1920}$ $fs(1)Ya^{2}$, approximately 50% of the embryos contained predominantly two giant nuclei (Table II). Similarly, approximately 50-75% of the $png^{12\text{-}3318}$ $fs(1)Ya^{2}$ embryos developed either single or multiple giant nuclei. However, these nuclei were smaller than normally formed in a *png* single mutant. Embryos homozygous for either *png* or *fs(1)Ya* and heterozygous for the other showed the phenotype expected for the homozygous mutation (see Methods). Thus, *fs(1)Ya* was epistatic to *png*, yet given enough time, giant nuclei were formed in double mutant embryos. This is perhaps due to controls necessary for the *fs(1)Ya* arrest breaking down, and allowing overreplication to occur due to the *png* mutation.

Table II. Epistasis Analysis of *png* and *fs(1)Ya*

Genotype	Phenotype observed		
	30 min. collection	**3 hour collection**	**7.5 hour collection**
$\frac{fs(1)Ya^2}{fs(1)Ya^2}$	fs(1)Ya	fs(1)Ya	fs(1)Ya
$\frac{png^{13\text{-}1920}\ fs(1)Ya^2}{png^{13\text{-}1920}\ fs(1)Ya^2}$	fs(1)Ya	fs(1)Ya[b]	50 % png[d]
$\frac{png^{12\text{-}3318}\ fs(1)Ya^2}{png^{12\text{-}3318}\ fs(1)Ya^2}$	fs(1)Ya[a]	fs(1)Ya[c]	50-75 % png[e]

[a] 1/50 embryos contains one giant nucleus.

[b] Nuclei appear more spherical and less condensed than in $fs(1)Ya^2$ single mutants. 2 embryos out of several hundred contain giant nuclei.

[c] Similar to [b]. 12 embryos contain 1-4 giant nuclei and 1 embryo contains multiple giant nuclei, out of several hundred embryos.

[d] Embryos contain predominantly 2 giant nuclei, yet are not as large as typical *png* nuclei.

[e] Embryos contain single and multiple giant nuclei, yet are not as large as typical *png* nuclei.

png is Required in the Germ Line to Regulate DNA Replication

png is a maternal-effect gene that affects both unfertilized eggs and fertilized embryos. During oogenesis somatic follicle cells surround the oocyte, and it was possible that *png* is needed in these somatic cells in order to regulate replication, perhaps via a signal from the somatic cells to the oocyte. Alternatively, *png* might be required in the germ line to regulate replication

in the egg and embryo. Interestingly, a *C. elegans* mutation, *ceh-18*, results in oocytes containing giant nuclei, yet the *ceh-18* gene product is thought to be required in the somatic sheath cells surrounding the oocyte (Greenstein et al., 1994).

In order to determine whether *png* was required in the germ line, mosaic analysis was performed to examine the effect of a *png* mutant germ line in a wild-type somatic background. Germ-line clones were obtained following X-ray irradiation of *png/ovo*^{D1} larvae. Females carrying the germ-line dependent dominant mutation *ovo*^{D1} were sterile and mature eggs were not formed. However, X-ray induced mitotic recombination events resulted in *png* homozygous germ cells, which matured to unfertilized eggs or fertilized embryos. Nine germ-line clones of *png*$^{12\text{-}3318}$ and one of *png*$^{13\text{-}1058}$ were isolated (see Table III). One *ovo*^{D1} revertant was diagnosed by progeny that were heterozygous for several markers examined (see Methods and Table III). All clones were sterile, as expected for a requirement of *png* in the germ line. Embryo collections from germ-line clones of *png*$^{12\text{-}3318}$ were examined by DAPI staining and contained both single and multiple giant nuclei. These results showed that *png* is required in the germ line to regulate replication in the unfertilized egg and early embryo.

Table III. Analysis of X-ray Induced Germ-line Clones of *png*

Genotype of irradiated females[a]	X-ray dose (rads)	No. of females tested	No. of germ-line clones	Progeny from germ-line clones
$png^{12\text{-}3318}/ovo^{D1}$	1000	265	9	Sterile[d]
$png^{12\text{-}3318}/ovo^{D1}$	0	326	1[b]	
$png^{13\text{-}1058}/ovo^{D1}$	1000	31	2[c]	Sterile
$png^{13\text{-}1058}/ovo^{D1}$	0	38	0	

[a] The *png* chromosome contained the following markers; *y png cv v f.*

[b] One female laid one egg that did not hatch.

[c] One fertile revertant of ovo^{D1} was isolated (see Methods).

[d] Embryos contained single and multiple giant nuclei.

DISCUSSION

The *png* Gene Product Couples S phase and M phase in the Early Embryo

The phenotype of *png* unfertilized eggs suggests that *png* is needed to inhibit DNA replication until fertilization occurs. Mutant *png* eggs undergo inappropriate DNA replication prior to fertilization, giving rise to a giant polyploid nucleus. In three *png* alleles, a giant polyploid nucleus also forms in fertilized *png* embryos. One interpretation of the fertilized phenotype is that overreplication is already underway by the time the male pronucleus enters the embryo, and therefore mitosis and replication cannot be properly coupled. In the two multiple nuclei *png* alleles, some mitotic divisions occur before replication and mitosis become uncoupled, resulting in multiple giant polyploid nuclei. These alleles might represent leaky alleles, and residual *png* function might slow the onset of overreplication, thus allowing some coupling of mitotic and S phase events. However, this interpretation is unlikely, as overreplication occurs with the same timing and to the same extent in unfertilized eggs from both classes of *png* alleles.

An alternative and more likely explanation for the phenotype in fertilized embryos is that *png* is needed following fertilization during the early cell cycles, as well as being required prior to fertilization. *png* might be needed to both couple DNA replication to fertilization, and to mitosis following fertilization. The multiple class of *png* alleles might therefore contain weak or residual function that couples S phase and M phase for a limited time. This transient coupling of replication and mitosis does not occur with the single nucleus class of alleles, presumably representing strong or possibly null alleles.

The phenotype in *png* embryos was characterized by staining early embryos with tubulin and histone antibodies. The single nucleus class of *png* alleles did not contain mitotic figures, and showed no staining with tubulin antibodies. This is consistent with these alleles lacking any coupling of replication and mitosis. Later embryos however were reported to contain independent tubulin asters in 10-30% of embryos (Shamanski and Orr-Weaver, 1991). Perhaps the small numbers of $png^{13\text{-}1058}$ obtained from 10-30 minute collections prevented the observation of this class of embryos.

The transient coupling of replication and mitosis was similarly characterized in early embryos from the multiple nuclei class of *png* alleles. The phenotypic characterization of $png^{12\text{-}3318}$ embryos shows an interesting correlation in that 44% of embryos stain with both tubulin and histone antibodies. In older embryos, 30-50% form multiple giant nuclei, whereas the remaining embryos contain a single giant nucleus. Thus, the early embryos containing detectable tubulin staining presumably result in the formation of multiple giant nuclei. This conclusion is strengthened by mitotic figures present in these early embryos. Aberrant mitotic figures were formed as early as the first mitotic division in these embryos, and the severity of the uncoupling increased with subsequent divisions.

Condensed individual chromosomes are not seen in giant nuclei of older *png* embryos. In contrast, condensed chromosomes are associated with mitotic figures in early $png^{12\text{-}3318}$ embryos. Therefore, the transient coupling of replication and mitosis in weak *png* alleles results in mitotic figures in which some or all aspects of mitosis occur properly. In aberrant mitotic figures, defects in both spindle and DNA morphology occur during early divisions. Whether one or the other is a primary defect, causing subsequent aberrations is not known. The phenotypic characterization of early *png*

embryos suggests that *png* is required to couple S phase and M phase during the early cell cycle.

Interactions With Mutations Affecting Early Embryonic Divisions

Genetic interactions between *png*, *plu*, and *gnu* show that both *plu* and *gnu* interact with the multiple nuclei *png* alleles and result in the formation of only single giant nuclei. This suggests that *plu* and *gnu* also act in coupling S phase and M phase following fertilization. These results therefore support the conclusion that all three genes interact in their role to regulate DNA replication in both the unfertilized egg and fertilized embryo. However, increasing the copy number of *plu* is not sufficient to rescue the *png* phenotype.

The *fs(1)Ya* gene product is also required during the early divisions in Drosophila, and 75% of mutant embryos arrest at the pronuclear stage of the first division. The *fs(1)Ya* gene encodes a nuclear envelope protein required for mitotic divisions. *fs(1)Ya* is epistatic to the three genes, *png*, *plu*, and *gnu*. However, with *png*, *fs(1)Ya* double mutant embryos, polyploid nuclei are formed if given enough time. Both *fs(1)Ya* embryos blocked at the pronuclear stage and escaper embryos result in giant nuclei in the presence of *png* mutations. Greater than 25% of the double mutant embryos form giant nuclei, suggesting that embryos blocked at the pronuclear stage by *fs(1)Ya* also overreplicate. In contrast to the interaction between the three giant nuclei mutants, *png* and *fs(1)Ya* do not show a dominant interaction. Heterozygous *png* or *fs(1)Ya* does not affect the phenotype of the other homozygous mutation.

These results show that *fs(1)Ya* is epistatic to *png*, although giant nuclei form in embryos allowed to develop for longer periods. This suggests that the

initial arrest of *fs(1)Ya* is not permissive for overreplication. Perhaps *fs(1)Ya* embryos are arrested in mitosis and a checkpoint ensures that replication does not occur during mitosis. However, this checkpoint may eventually break down, allowing the overreplication of nuclei due to the *png* mutation.

Germ-line Requirement of *png*

Mutations in other organisms exist that also result in giant nuclei, such as the *ts41* mutation of hamster cells (Handeli and Weintraub, 1992). The *ceh-18* mutation, (a POU homeo box gene), in *C. elegans* results in oocytes that fail to arrest at meiosis I and instead undergo inappropriate DNA replication resulting in a polyploid nucleus (Greenstein et al., 1994). Interestingly, the *ceh-18* gene product is not expressed in the oocyte, and instead is expressed in the sheath cells surrounding the developing oocyte. The authors propose that *ceh-18* plays a role in sending a signal from the sheath cells to the oocyte, thus controlling the meiotic arrest or a meiotic checkpoint. Consistent with this model, laser ablation of the sheath cells also results in oocytes containing giant nuclei (Greenstein et al., 1994).

In contrast to the role of *ceh-18*, *png* is required in the germ line in order to regulate DNA replication in the unfertilized egg and fertilized embryo. Germ-line clones of *png* alleles resulted in embryos containing giant polyploid nuclei. Whether *png* also has a role in the somatic cells surrounding the oocyte was not determined. Although unlikely, *png* might be required in both the soma and germ line, and somatic clones might also result in the *png* phenotype.

The *png* gene product regulates DNA replication

The unfertilized and fertilized phenotypes of *png*, *plu*, and *gnu*, suggest that these genes inhibit DNA replication. In unfertilized eggs *png* functions to make S phase dependent on fertilization, whereas in fertilized embryos the *png* gene product makes S phase dependent on the proper completion of mitosis. These genes might inhibit replication by controlling either the entry into S phase or the block to rereplication. Preliminary results suggest that replication in *png* embryos is cyclic, consistent with a role in the entry into S phase (Shamanski and Orr-Weaver, 1991). These genes might control the entry into S phase either by inhibiting licensing factor or other factors required for DNA initiation. Alternatively, they might regulate and inhibit S phase cyclin kinases, either directly or through the action of cdk inhibitors. If these genes do control the entry into S phase, the completion of fertilization or mitosis would then inactivate these gene products allowing subsequent replication.

Alternatively these genes might control other aspects of the cell cycle that link mitosis to replication. Perhaps cyclic replication is dependent on chromosome condensation such that replication does not occur until chromosomes are decondensed. These genes might be involved in chromosome condensation or the maintenance of condensation, as well as the transmission of a signal that represses replication while chromosomes are condensed. Inappropriate replication might occur due to the inability of DNA to remain condensed. In unfertilized eggs, DNA condensation might play a role in the arrest of the four meiotic products, and improper condensation would again result in inappropriate replication.

REFERENCES

Blow, J. J. 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. J. Cell Biol. *122*: 993-1002.

Blow, J. J. and R. A. Laskey. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. Nature *332*: 546-8.

Bueno, A. and P. Russell. 1992. Dual functions of *CDC6*: a yeast protein required for DNA replication also inhibits nuclear division. EMBO J. *11*: 2167-76.

Coverley, D., C. S. Downes, P. Romanowski and R. A. Laskey. 1993. Reversible effects of nuclear membrane permeabilization on DNA replication: evidence for a positive licensing factor. J. Cell Biol. *122*: 985-92.

Edgar, B. A. and G. Schubiger. 1986. Parameters controlling transcriptional activation during early Drosophila development. Cell *44*: 871-7.

El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. Cell *75*: 817-25.

Foe, V. E., G. M. Odell and B. A. Edgar. 1993. Mitosis and morphogenesis in the Drosophila embryo: point and counterpoint. In *The development of Drosophila melanogaster*, ed. M. Bate and A. M. Arias, pp. 149-300. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.).

Freeman, M. and D. M. Glover. 1987. The *gnu* mutation of Drosophila causes inappropriate DNA synthesis in unfertilized and fertilized eggs. Genes Dev. *1*: 924-30.

Freeman, M., C. Nüsslein-Volhard and D. M. Glover. 1986. The dissociation of nuclear and centrosomal division in *gnu*, a mutation causing giant nuclei in Drosophila. Cell *46*: 457-68.

Greenstein, D., S. Hird, R. H. A. Plasterk, Y. Andachi, Y. Kohara, B. Wang, M. Finney and G. Ruvkun. 1994. Targeted mutations in the *Caenorhabditis elegans* POU homeo box gene *ceh-18* cause defects in oocyte cell cycle arrest, gonad migration, and epidermal differentiation. Genes Dev. *8*: 1935-48.

Handeli, S. and H. Weintraub. 1992. The *ts41* mutation in Chinese Hamster cells leads to successive S phases in the absence of intervening G2, M, and G1. Cell *71*: 599-611.

Hayles, J., D. Fisher, A. Woollard and P. Nurse. 1994. Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. Cell *78*: 813-22.

Hofmann, J. F. X. and D. Beach. 1994. *cdt1* is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. EMBO J. *13*: 425-34.

Kelly, T. J., G. S. Martin, S. L. Forsburg, R. J. Stephen, A. Russo and P. Nurse. 1993. The fission yeast *cdc18+* gene product couples S phase to START and mitosis. Cell *74*: 371-82.

King, R. W., P. K. Jackson and M. W. Kirschner. 1994. Mitosis in transition. Cell *79*: 563-71.

Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint and C. F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell *77*: 107-20.

Li, R., S. Waga, G. J. Hannon, D. Beach and B. Stillman. 1994. Differential effects by the p21 CDK inhibitor on PCNA dependent DNA replication and repair. Nature *371*: 534-7.

Lin, H. and M. F. Wolfner. 1991. The Drosophila maternal-effect gene *fs(1)Ya* encodes a cell cycle-dependent nuclear envelope component required for embryonic mitosis. Cell *64*: 49-62.

Lindsley, D. L. and G. G. Zimm. 1992. The genome of *Drosophila melanogaster*. (Academic Press, Inc., New York, N. Y.).

Mohler, J. D. 1977. Developmental genetics of the Drosophila egg. I. Identification of 59 sex-linked cistrons with maternal effects on embryonic development. Genetics *85*: 259-72.

Mohler, J. D. and A. Carroll. 1984. Sex-linked female-sterile mutations in the Iowa collection. Dros. Inf. Serv. *60*: 236-41.

Moreno, S. and P. Nurse. 1994. Regulation of progression through the G1 phase of the cell cycle by the *rum1+* gene. Nature *367*: 236-42.

Nurse, P. 1994. Ordering S phase and M phase in the cell cycle. Cell *79*: 547-50.

Rabinowitz, M. 1941. Studies on the cytology and early embryology of the egg in *Drosophila melanogaster*. J. Morph. *69*: 1-49.

Richardson, H. E., L. V. O'Keefe, S. I. Reed and R. Saint. 1993. A Drosophila G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. Development *119*: 673-90.

Saka, Y. and M. Yanagida. 1993. Fission yeast *cut5+*, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene *rad4+*. Cell *74*: 383-93.

Schwob, E., T. Bohm, M. D. Mendenhall and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40*SIC1* controls the G1 to S transition in *S. cerevisiae*. Cell *79*: 233-44.

Shamanski, F. L. and T. L. Orr-Weaver. 1991. The Drosophila *plutonium* and *pan gu* genes regulate entry into S phase at fertilization. Cell *66*: 1289-300.

Sherr, C. J. 1993. Mammalian G1 cyclins. Cell *73*: 1059-65.

Sherr, C. J. 1994. G1 phase progression: cycling on cue. Cell *79*: 551-5.

CHAPTER V.

The Molecular Characterization of the *pan gu* locus

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INTRODUCTION

The molecular characterization of G1/S regulators of the cell cycle has made great progress in recent years. The identification of both cyclins and cyclin-dependent kinases (cdks) from different organisms has illuminated key regulatory events during this transition. In mammalian systems, cyclins D and E have been shown to be rate-limiting regulators of the G1/S transition (Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994). In Drosophila, cyclin E is a key regulator of this transition, and the down regulation of cyclin E is necessary for the addition of G1 following the first 16 cell cycles in the early embryo (Richardson et al., 1993; Knoblich et al., 1994). This down regulation is also necessary for the arrest of mitotic embryonic cells in G1. Biochemical experiments have identified Dmcdc2c as the cdk2 homolog complexed with cyclin E (Knoblich et al., 1994). Downstream targets of cyclin E-cdk2 as well as regulators of the kinase will lead to a more complete picture of cell cycle regulation at the G1/S transition.

One downstream target of cyclin D and E kinases includes the retinoblastoma protein, pRb (reviewed in Sherr, 1994). Unphosphorylated pRb is complexed with the inactive form of the transcription factor, E2F. The cyclin D kinase directly phosphorylates pRb, causing its inactivation and releasing E2F, which then regulates many S phase genes. In Drosophila, cyclin E-regulated S phase genes include Polymerase α, PCNA, and Ribonuclease reductase 1 and 2 (*RNR1, 2*) (Duronio and O'Farrell, 1994). Whether cyclin E acts through E2F in Drosophila is not known. Cyclin A kinase may then inactivate E2F once its transcriptional program is complete (Dynlacht et al., 1994; Krek et al., 1994). Other downstream targets of S phase cyclins may include origin binding factors themselves. Cyclin kinases are also

found in association with replication protein-A, (RP-A), at replication foci and perhaps promote unwinding of origin DNA (Cardoso et al., 1993; reviewed in Heichman and Roberts, 1994).

Cyclin-cdk complexes are regulated by several different mechanisms. Phosphorylation or dephosphorylation either activates or inhibits kinase activity. Upstream phosphatases (*cdc25*) and kinases, (*wee1, mik1*) act at different times throughout the cycle to modulate cyclin B kinase activity in *S. pombe*, (reviewed in Minshull, 1993). Mammalian cyclin D-cdk4 and cyclin E-cdk2, are activated by cdk-activating kinase, (CAK), which itself is composed of MO15 (cdk7), and cyclin H (Fisher and Morgan, 1994). Inhibitors of cdks have also been identified that regulate cyclin-cdk complexes (reviewed in Sherr, 1994). Both inhibitors, $p21^{CIP1}$ and $p27^{KIP1}$, act during the G1/S transition and form complexes with cyclin kinases (Sherr, 1994). Stoichiometric levels of these inhibitors may regulate their inhibitory effect on cdks.

Negative regulators of replication have been identified genetically in Drosophila by mutants that undergo inappropriate DNA replication, and include the genes *pan gu* (*png*), *plutonium* (*plu*), and *giant nuclei* (*gnu*) (Freeman et al., 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). The *png* gene product is required to couple DNA replication to fertilization as well as to mitosis during the early cell cycles. The *plu* and *gnu* gene products act in a similar fashion and all may control a common regulatory pathway (Shamanski and Orr-Weaver, 1991). These genes may inhibit replication directly or may affect other aspects of the cell cycle that couple S phase and M phase, such as chromosome condensation.

The molecular characterization of *png* will enable further experimentation in order to determine at what level *png* regulates

replication. The *plu* gene product encodes a 19 kD protein containing 3 ankyrin repeats (Axton et al., 1994). Molecular data confirms that *plu* is required solely early in development and not during later canonical cell cycles. RNA null alleles are maternal-effect alleles and the *plu* transcript is not expressed later in development (Axton et al., 1994). Interestingly, another small ankyrin-repeat protein is the cdk4 inhibitor, $p16^{INK4}$, that acts to inhibit cdk4, thus disrupting its association with the cyclin D protein during the G1/S transition (Serrano et al., 1993). *plutonium* might similarly repress DNA replication by possibly inhibiting S phase cyclin kinases.

The molecular characterization of the *png* gene is described. Transformation rescue experiments identify a 39 kb cosmid containing the *png* locus. Denaturing gradient gel electrophoresis, (DGGE), is used to identify DNA mutations in *png* alleles, and Northern blot analysis identifies putative *png* transcripts that are altered in *png* alleles.

METHODS

Mutations and Strains

Deficiencies, duplications, lethals, and balancer stocks used are described in (Lindsley and Zimm, 1992). P-element stocks were obtained from the Bloomington, Indiana stock center (Flybase). All *prickly pear* (*prp*) alleles and excision stocks were from S. Misra and K. Anderson (personal communication). *In(1)GA120* was from the laboratory of N. Perrimon.

Genetic Localization of *png*

png was mapped using deficiencies between *y* and *w* on the *X* chromosome (T. Orr-Weaver, personal communication). Complementation tests of duplications with both the single and multiple nuclei classes of *png* alleles were done as follows; *Dp(1;f)101* y^+; *In(1)sc*^{8}, *Df(1)sc*^{8}, w^a/*Y* males were crossed to *png/FM0* virgins, and resulting males of the genotype *Dp(1;f)101* y^+; *png/Y* were again crossed to *png/FM0* virgins. Homozygous *png* females carrying the duplication were then tested for rescue of maternal-effect lethality. The same crosses were performed using *Dp(1;f)RA* y^+; *In(1)sc*^{l1}/*Y* males. Similar crosses were performed to test *Dp(1;3)E1* and *Dp(1;3)sta*, starting with males of the following genotypes; *Df(1)su(s)*^{83} *y cho ras*^{2} *v/Y*; *Dp(1;3)E1* y^+/+ and *l(1)3.12/Y*; *Dp(1;3)sta/CxD* (stock generated by M. Winberg, personal communication).

Complementation tests were done using both classes of *png* alleles and 11 recessive lethal complementation groups contained within *Dp(1;3)sta* (listed as the first 11 entries in Table I). Most lethals were tested against all *png* alleles. Lethal balanced females were crossed to *png/Y* males, and *l(1)/png* females were tested for maternal-effect lethality. Similarly,

complementation tests were done with $png^{13\text{-}1058}$ and three recessive lethal alleles of *prickly pear* (*prp*). The allele, prp^{75P}, failed to complement $png^{13\text{-}1058}$, and was found to be a large deletion in the region (S. Misra, K. Anderson, personal communication).

Complementation tests were also performed with *png* and 8 P-element insertions in the region 1E-2B, and a P-element allele of $prp^{15.236}$ (dominant temperature-sensitive). Complementation tests of $png^{13\text{-}1058}$ and $prp^{15.236}$ was done at both 25°C and 29°C, and assayed by DAPI staining (see Ch. IV, Methods). Complementation tests with 24 revertants of $prp^{15.236}$ as well as an inversion, *In(1)GA120/FM7*, were done with both $png^{13\text{-}1058}$ and $png^{13\text{-}1920}$.

Cytology

Salivary gland chromosome squashes of heterozygous $png^{12\text{-}2786}$, $png^{12\text{-}3318}$, $png^{12\text{-}158}$ and $png^{13\text{-}1058}$ were performed as described (Ashburner, 1989). *png* males were crossed to wild type Canton S females, and salivary glands dissected from heterozygous larvae. Microscopic examination did not reveal visible rearrangements in the region of 1F-2A.

Salivary gland chromosome in situs were performed in order to orient the chromosomal walk. Cosmids 1-2-1 and 4a-2b-1 were 80 kb apart and were biotinylated and used as probes for in situ hybridization (Ashburner, 1989). Cosmid 4a-2b-1 hybridized to bands 1F1-2, and 1-2-1 hybridized to bands 2A1-2.

Cosmids

20 cosmids representing contigs 1.6-1.10 and 2.1 were obtained from the Crete Drosophila genome project (Siden-Kiamos et al., 1990; Kafatos et al., 1991). Bacteria were grown in 2X YT supplemented with 50 μg/ml kanamycin, and cosmids were purified using Qiagen columns (Qiagen, Inc).

The *Drosophila melanogaster* iso-1 r2 genomic cosmid library was used for chromosomal walking (gift of J. Tamkun) (Tamkun et al., 1992). 30,000 recombinants were screened, by plating on Colony Plaque Screen filters (NEN), and replica filters were hybridized as described for Southern blots. Bacteria were grown in 1X LB supplemented with 40 μg/ml ampicillin, and cosmids were purified using Qiagen columns. Cosmids were restriction mapped with *Eco*RI, and comparison of restriction maps determined overlapping cosmids. Cosmids from one step that extended the furthest were then used as probes for the subsequent step. Cosmids to be used for P-element transformation were further purified by CsCl banding. Cosmid 8D8 from Crete contig 1.10 was used to initiate the chromosome walk.

Quantitative Southern Blots

Genomic DNA preps were performed as described in (Ashburner, 1989), and DNA from 20 to 40 flies was loaded per lane (2-3 μg). Agarose blots were run in 1X buffer (40 mM Tris, 36 mM KH_2PO_4, 1 mM EDTA, pH 7.6) and DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell) and UV cross-linked with a UV Stratalinker (Stratagene). Probes were labeled by random priming of linearized DNA or 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 200 μg/ml salmon sperm DNA (denatured with 0.1 volumes 1N NaOH for 5 min. at 37°C). Hybridization was performed overnight at 65°C in 6X SSC, 5X Denhardts, 1% SDS, and 100 μg/ml denatured salmon sperm DNA. Blots were washed at 65°C with the following; 2X for 15 min. (2X SSC, 1% SDS), 2X for 30 min. (0.2X SSC, 0.1% SDS), and rinsed in 0.2X SSC. Quantitation of bands was done using a Fuji Bio-image analyzer 2000 (Fuji, Inc.). Quantitation was performed

as described in Ch. II (see Methods). For most blots two *rosy* genomic bands were used as DNA loading controls.

Quantitative Southern blots were performed using DNA from duplication males or deficiency females. Both *Dp(1;f)101* and *Dp(1;3)E1* were in stocks carrying deficiencies, yet the deficiencies were both distal to 1E and were therefore outside the region being quantitated. DNA from wild-type and deficiency females was compared, and cosmids that had half the level of hybridization to deficiencies were therefore within the region defining *png*. Cosmids localized within *Dp(1;f)101* and *Dp(1;3)sta* showed a two-fold level of hybridization as compared to wild-type male DNA. The cosmids covering the *png* interval lay outside of *Dp(1;3)E1* and *Dp(1;f)RA*, and had equivalent levels of hybridization as compared to male DNA (Fig. 1).

Representative cosmids from the Crete contigs were labeled and used as probes. Contig 1.10 was located within the breakpoints defining *png*, whereas contigs 1.6 to 1.9 were contained within *Dp(1;3)E1* and contig 2.1 was past *Dp(1;f)101* (Fig. 2). Contig 1.10 contained 9 cosmids that were not characterized further. The location of contigs 1.6 through 1.9 enabled the ordering of *Dp(1;3)E1* and *Dp(1;f)RA* which both had cytological breakpoints at 1F. Contigs 1.6 to 1.9 were contained within *Dp(1;3)E1* yet were past *Dp(1;f)RA*, therefore *Dp(1;f)RA* contained a smaller duplicated region (Fig. 2). Similarly, quantitative Southern blots were performed using cosmids from every step of the Tamkun library chromosome walk.

Southern Blot Analysis of *png* alleles

DNA was isolated as described from homozygous *png* flies (see above). DNA was digested with the following enzymes and run on 1.5% agarose gels in order to separate small fragments. Double digests of *Eco*RI and the

following enzymes were done: *Sal*I, *Pst*I, *Bgl*II, *Bgl*I, *Bam*HI, *Hin*dIII, *Xho*I, *Xba*I, and *Cla*I. Alternatively, triple digests were done with *Eco*RI and the following pairs of enzymes: *Bam*HI + *Hin*dIII, *Sal*I + *Pst*I, *Bgl*I + *Bgl*II, *Xba*I + *Xho*I, *Cla*I + *Bcl*I, *Spe*I + *Nsi*I. Southern blots were made and cosmids from the Tamkun chromosome walk were linearized and used as probes.

P-element Transformation

P-element transformation experiments were performed with the assistance of J. Dines. Injections were performed as described in Ch. II (Methods). Cosmid transposons were coinjected with a helper plasmid containing a transposase gene (pIChsπΔ2-3), (gift of Ken Irvine). *y w* embryos were injected and resulting flies were crossed with *y w* flies to identify transformants. The cosmid vector, NotBamNot-CoSpeR, contained the mini *white* gene as a selectable marker (Tamkun et al., 1992). Cosmids were injected at 1 mg/ml and helper plasmid at 300 μg/ml. 14,112 embryos were injected and 37% of larvae hatched on average. Of those, 51% G_0 adults arose and 39% were sterile on average.

Transformants were crossed to *y w* flies to obtain a stock, and were then crossed to *y* $png^{13\text{-}1058}$ *w/FM0* virgins. For autosomal insertions, resulting *y* $png^{13\text{-}1058}$ *w/Y;* P[w^+] males were crossed to *y* $png^{13\text{-}1058}$ *w/FM0* virgins, and homozygous *png* females containing the w^+ transposon were mated and tested for rescue of maternal-effect lethality.

For the cosmid 3-3-1 transformant on the *X* chromosome, recombinants were made with *png* in order to test for rescue. First, y^+ was crossed onto the *y w* P[w^+] chromosome. Recombinants were easily obtained indicating that the insertion was proximal and not near *y* and *png*. y^+ *w* P[w^+]/*Y* males were crossed to *y* $png^{13\text{-}1058}$ *w/FM0* virgins and resulting

heterozygous, non-balancer virgins were crossed to *y png w/Y* males. Virgin female recombinants, *y w* P[w^+]*/y png w*, were mated and tested for rescue of maternal-effect lethality. Male recombinant progeny were also obtained and crossed to *y* $png^{13\text{-}1058}$ *w/FM0* virgins, and non-balancer females were tested for rescue. *y* is closely linked to *png* and most recombinants should therefore be homozygous for *png*. However, it was possible that a rare recombination event between *y* and *png* occurred. Therefore, several independent recombinants were tested. 4 female recombinants and 17 male recombinants were tested and all rescued the maternal-effect lethality of *png* homozygous females.

For the cosmid 3-1B-1 transformant on the X chromosome, recombinants between y^+ and P[w^+] were not obtained, indicating that the insertion was close to the *png* locus. Complementation tests revealed that the P-insertion did however complement the *png* mutation. New autosomal insertion sites were obtained by mobilizing the P-element as described in Ch. II (Methods), with the following exceptions; males that were w^+, Sb, and either CyO or Sp were crossed to *y w* virgins. Lines in which the transposon had mobilized off the *X* chromosome were detected by w^+ males in the next generation. 13 new independent insertion lines were obtained and all failed to rescue *png*. Quantitative Southern analysis was not performed however to determine whether rearrangements occurred.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gels were performed in the laboratory of D. Housman, with the help of V. Stanton. Genomic DNA was isolated from *png* homozygotes and control *y w* flies as described. Digests were performed with the following 4-bp recognition sequence restriction enzymes: *Alu*I, *Hae*III,

*Rsa*I, *Mbo*I, *Msp*I, *Hha*I, *Hin*fI, and *Dde*I. Digests were performed in 100 µl to ensure the complete digestion of DNA, and were precipitated using 20 µg glycogen. Samples were resuspended in 4 µl TE buffer, and 1 µl of 5X loading dye (80% glycerol, 10 mM Tris-HCl pH8, 25 mM EDTA, 0.25% bromphenol blue) was added.

Denaturing gradient gel electrophoresis (DGGE) was performed as described (Abrams and Stanton, 1992; Krolewski et al., 1992), with the following exceptions. 40% (w/v) acrylamide-bisacrylamide (37.5:1) was used to make the following stocks solutions. The 100% denaturant stock contained 7% acrylamide, 40% formamide, and 7M urea in TAE buffer, and the 0% denaturant stock contained 7% acrylamide in TAE buffer. Gradient gels were poured between glass plates (18 X 24 cm), and gels were 1 mm thick with 28 wells. All samples were run under the following three denaturant gradient gel conditions; 10-50%, 20-80%, and 35-85%. Gels were run at 60°C with recirculating buffer and run at 100 V for 15 hours (10-50%) or 20 hours (20-80% and 35-85%). Gels were electroblotted onto nylon membranes (Nytran, Schleicher & Schuell), and DNA was transferred at 1.5 A for 2 hrs (Hoefer Electro-transfer Unit). After denaturation and neutralization, the blot was UV crosslinked and hybridized, using probes no greater than 8 kb. The 3.9 kb *Xho*I doublet and 2.2 kb *Xba*I-*Xho*I fragments were subcloned into pBluescript KS$^-$ (Stratagene) and probes were made from fragments isolated in low-melting agarose gels. Alternatively, probes were made from fragments gel-isolated from restriction digests of cosmid 3-3-1.

Northern Blot Analysis

Ovaries were dissected from homozygous *png* females and stored at -80°. RNA was obtained from ovaries as described (Ashburner, 1989) and poly

A^+ RNA was isolated as described (Ausubel et al., 1987). Glyoxal RNA gels were run in 1% agarose (Ausubel et al., 1987), transferred to Hybond-N nylon filters (Amersham, Inc.), and UV crosslinked. Blots were deglyoxylated, prehybridized for 2 hours, and hybridized at 42°C, or 65°C for single-stranded riboprobes, overnight (0.25 M $NaPO_4$ pH 6.5, 7% SDS, 50% formamide). Blots were washed at 65°C 3X for 15 min. (0.2X SSC, 1% SDS). Single-stranded riboprobes were made from T7 and T3 transcription through the 2.2 kb *Xba*I-*Xho*I subcloned into pBluescript KS^-.

RESULTS

png is Localized to Salivary Gland Chromosome Bands 1F-2A

png was mapped at position 1 cM on the tip of the *X* chromosome between the markers *yellow* (*y*) and *crossveinless* (*cv*) (Mohler, 1977; Mohler and Carroll, 1984). *Df(1)A94* and *Df(1)S39* both failed to complement *png*, localizing *png* to salivary gland chromosome bands 1E3-4 to 2B5-6 (Fig. 1) (T. Orr-Weaver, personal communication). Four duplications within this region were used to further localize *png* to salivary bands 1F-2A2 (Fig. 1). *Dp(1;3)E1* and *Dp(1;f)RA* failed to rescue the maternal-effect lethality of *png* mutants, whereas *Dp(1;f)101* and *Dp(1;3)sta* rescued *png* homozygotes (see Methods). The breakpoints at salivary band 1F defined a minimal region for *png*, as these duplications might break within the *png* locus. Therefore, some portion of the *png* locus might be distal to the 1F breakpoint.

The region containing *png* was saturated for zygotic lethal complementation groups, and therefore *png* might have been a maternal allele of an existing lethal complementation group (Lefevre, 1981; Lefevre and Watkins, 1986; Perrimon et al., 1989). Complementation tests were performed with several lethal complementation groups in the region of salivary bands 1E to 2B. Maternal-effect alleles sometimes represent weak alleles of zygotic-lethal genes in which the homozygote contained enough functional product

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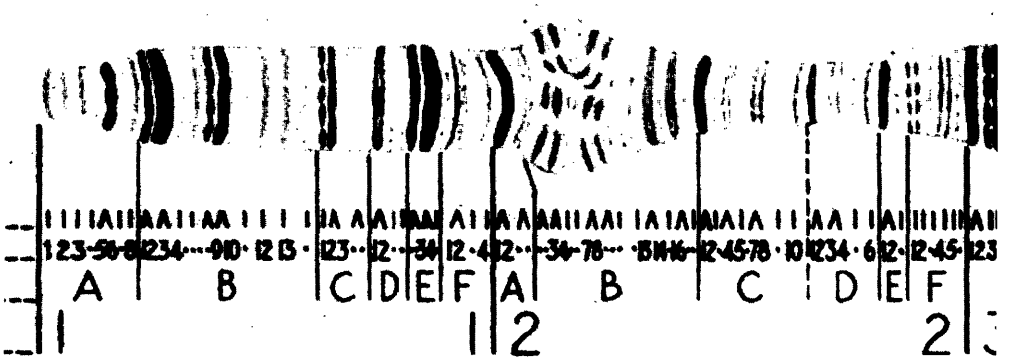
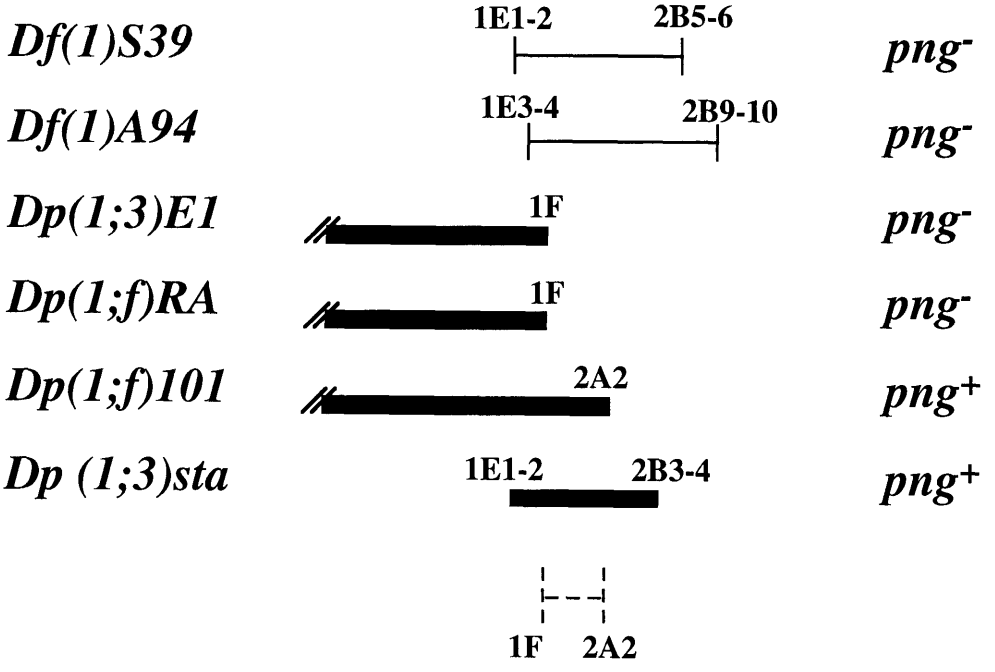
Fig. 1. png localizes to salivary chromosome bands 1F to 2A2. The deficiencies, *Df(1)S39* and *Df(1)A94*, localize *png* to bands 1E3-4 to 2B5-6. The regions missing in deficiencies are depicted by lines. The duplications shown further localize *png*. *Dp(1;3)E1* and *Dp(1;f)RA* fail to rescue *png*, whereas *Dp(1;f)101* and *Dp(1;3)sta* rescue the maternal-effect lethality of *png*. The 1F breakpoints define a minimal breakpoint for *png*, and 2A2 is a maximal breakpoint for *png*.


A
B
C
D
E
F
A
B
C
D
E
F
1
2
2


Df(1)S39
1E1-2
2B5-6
png-
Df(1)A94
1E3-4
2B9-10
png-
Dp(1;3)E1
1F
png-
Dp(1;f)RA
1F
png-
Dp(1;f)101
2A2
png+
Dp (1;3)sta
1E1-2
2B3-4
png+
1F
2A2
png locus

to survive to adulthood. A collection of 11 recessive lethal complementation groups, in the region covered by *Dp(1;3)sta*, were tested for complementation of *png* (see Methods). All lethals were fully viable in trans to *png* and complemented the maternal-effect lethality of *png* (Table I, Methods). Two recessive lethal alleles of *prickly pear* (*prp*), a gene interacting with the dorsal-ventral signaling pathway, also complemented *png*. The *prp* locus was positioned by the same duplication breakpoints that localized *png* (S. Misra, K. Anderson, personal communication). A third allele, prp^{75P}, failed to complement *png* and was later determined to be a large deletion in the region.

An allele of *png* that contained either a rearrangement or a P-element insertion might facilitate the molecular identification of the *png* locus. Alleles caused by rearrangements might result in an aberrant *png* transcript. All five *png* alleles were EMS induced and were not likely to contain visible rearrangements. EMS results in predominantly single base-pair mutations, although deletions are sometimes generated. Salivary gland chromosome squashes were examined from heterozygous *png* alleles, and cytologically visible rearrangements, such as inversions or deletions were not detected (see Methods). Known P-element insertions were also tested for complementation in order to identify any possible P-element alleles of *png*. Nine P-element insertions in the region of 1E to 2B all complemented *png* (Table I). An inversion, *In(1)GA120*, with a breakpoint in 1F also complemented *png* (Table I, Methods).

Smaller deficiencies in the region would also be helpful in delineating the *png* locus. *prp* and *png* were located within the same duplication breakpoints, and therefore deficiencies generated that removed *prp* might also remove *png*. The $prp^{15.236}$ P-allele was used to generate revertants

which might result in imprecise excisions or small deficiencies in the region (S. Misra, K. Anderson, personal communication). However, all 24 *prp* revertants examined complemented $png^{13\text{-}1920}$.

Table I. Complementation analysis with *png*

Lines Tested	Notes or synonyms	Complementation
l(1)EA48	$l(1)1Ea^1$	+
l(1)VA209	$l(1)1Eb^4$	+
l(1)A102	$l(1)1Ec^1$	+
l(1)C24	$l(1)1Ef^1$	+
l(1)VE676	$l(1)1Fc^6$	+
l(1)HC156	$l(1)1Fd^1$	+
l(1)VA92	$l(1)1Fe^1$	+
l(1)VA185	$l(1)2Aa^1$	+
l(1)EA97	$l(1)2Ab^1$	+
l(1)A70	$l(1)2Ac^1$	+
l(1)A60	$l(1)2Ad^1$	+
prp^{G9Fa2}	*prp* allele	+
prp^{115E}	*prp* allele	+
prp^{75P}	*prp* deletion allele	-
$prp^{15.236}$	*prp* P-allele	+
In(1)GA120	inversion, 1F-20	+
P407	P-element, 1F	+
P410	P-element, 1F	+
P793	P-element, 2B	+
P809	P-element, 2A	+
P1219	P-element, 1EF	+
P1325	P-element, 2A	+
P120	P-element, 1F	+
P592	P-element, 1E3	+

Molecular Characterization of *png*

In order to further characterize the *png* gene product, we decided to clone *png*. Twenty cosmids representing six contigs in the region of 1E to 2B were obtained from the Crete Drosophila genome project (Siden-Kiamos et al., 1990; Kafatos et al., 1991). In order to relate the known genetic map of *png* to the physical cosmid map of the region, we oriented the cosmids in relation to the deficiencies and duplications. This was done using quantitative Southern blots containing DNA from the two deficiencies and the four duplications that define *png*. Cosmids of interest would be within the deficiencies, yet would lie past the breakpoints of *Dp(1;f)RA* and *Dp(1;3)E1* and within *Dp(1;f)101* and *Dp(1;3)sta* (Fig. 1 and Methods). A single cosmid from each contig was used to probe quantitative Southern blots, and one contig was located within the same breakpoints defining *png*. The mapping of contigs also determined that *Dp(1;3)E1* extended beyond *Dp(1;f)RA*, thus defining the distal minimal boundary of *png* (Fig. 2 and Methods). *png* was therefore localized to a single contig containing 9 cosmids.

In order to perform future transformation rescue experiments to localize *png*, we wished to obtain cosmids contained within a vector suitable for transformation. This was necessary as the Crete cosmids were in a vector that did not contain P-element ends needed for transformation. One Crete cosmid from the region containing *png* was used to initiate a chromosome

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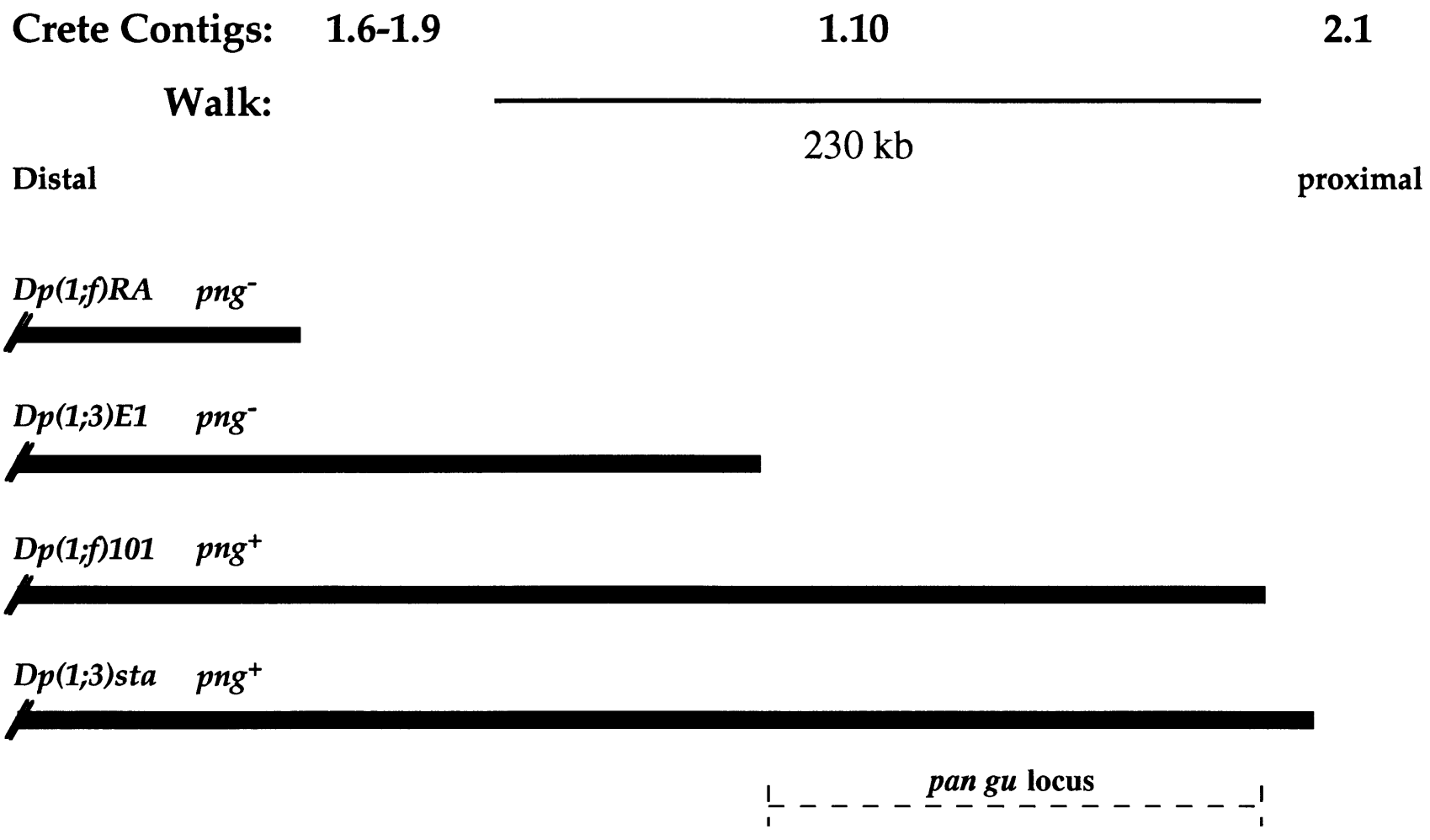
Fig. 2. Mapping duplication breakpoints to the Crete contigs and chromosome walk. Crete contigs 1.6-1.9 were proximal to the *Dp(1;f)RA* breakpoint, yet were contained within *Dp(1;3)E1*. A 230 kb cosmid walk was performed covering contig 1.10. Breakpoints of both *Dp(1;3)E1* and *Dp(1;f)101* were mapped to the 230 kb walk (Figs. 3 and 4). These breakpoints define the *png* locus.

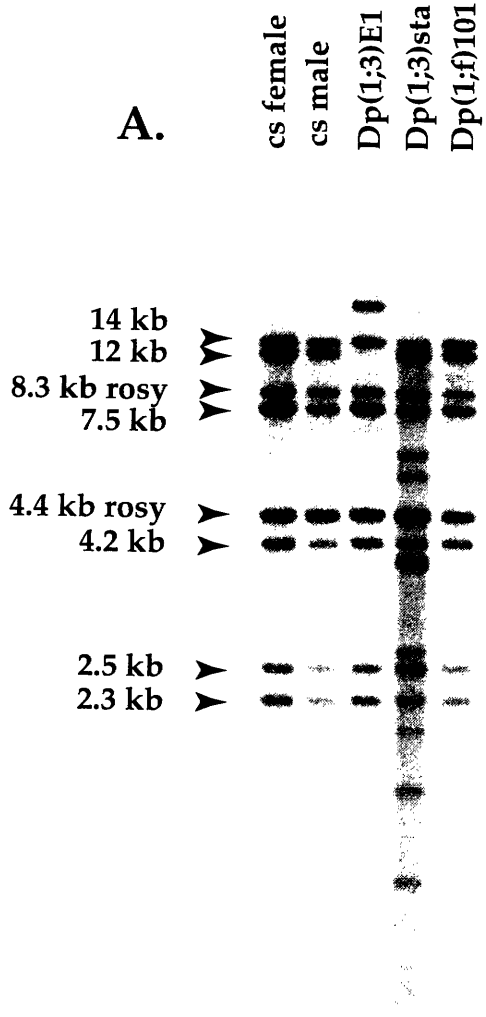

Crete Contigs: 1.6-1.9
1.10
2.1
Walk:
230 kb
Distal
proximal
Dp(1;f)RA png-
Dp(1;3)E1 png-
Dp(1;f)101 png+
Dp(1;3)sta png+
pan gu locus

walk using the Tamkun iso-1 r2 cosmid library (see Methods) (Tamkun et al., 1992). This library was cloned into the NotBamNot-CoSpeR vector which contained P-element ends and the mini *white* gene as a selectable marker for transformation (Tamkun et al., 1992). A genomic chromosome walk was performed, and cosmids covering 230 kb were obtained (Fig. 2). The cosmids were hybridized in situ to salivary gland chromosomes, and two cosmids localizing to bands 1F1-2 and 2A1-2 oriented the distal and proximal directions of the walk (see Methods). In order to determine the extent of the walk, quantitative Southern analysis was done using the duplications defining *png*. Breakpoints of both *Dp(1;3)E1* and *Dp(1;f)101* mapped to the chromosome walk, and 130 kb spanned the region between the breakpoints (Figs. 3, 4). The Dp(1;3)E1 breakpoint mapped to cosmid 3-3-1 and Crete cosmid 8D8, whereas the Dp(1;f)101 breakpoint was localized to cosmid 5-1-1.

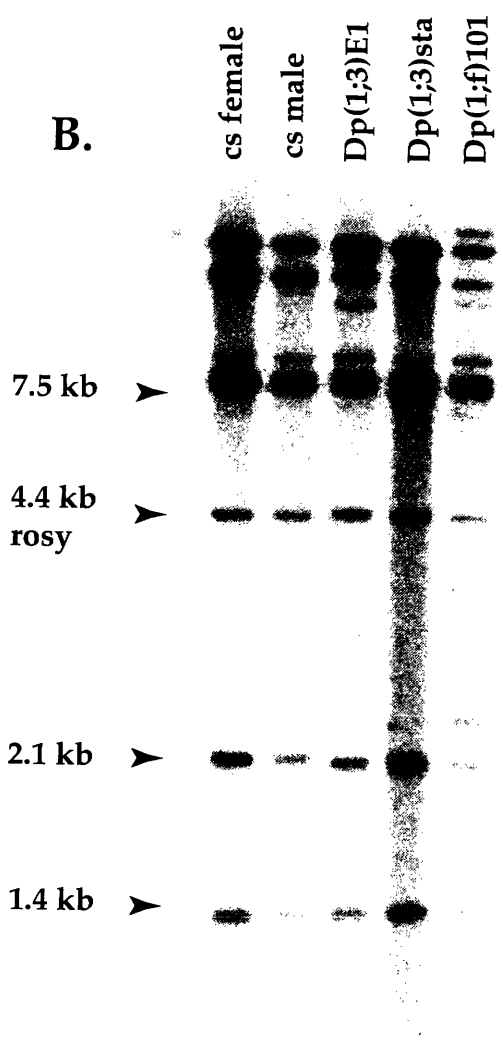
We wished to determine whether any DNA rearrangements were present in the *png* alleles within this 130 kb region. No cytologically visible rearrangements were present in *png* alleles, yet small deletions, inversions, or restriction fragment length polymorphisms (RFLPs) might have been associated with the mutations causing the *png* phenotype. Southern blot analysis of *png* alleles was performed, and several digests with either two or three enzymes were examined (see Methods). This was done in order to generate small fragments so that deletions on the order of 50 bp or larger might be detected. Probes were used throughout the entire chromosome walk with the exception of 10 kb containing repetitive DNA. No rearrangements were observed in Southern blots of the *png* alleles.

Fig. 3. Quantitative Southern blots showing the breakpoints of *Dp(1;3)E1* and *Dp(1;f)101*. Southern blots were performed containing Canton S (cs) wild-type female and male DNA, as well as DNA from males containing the three duplications shown. DNA was digested with *Eco*RI, and blots were probed with cosmids and *rosy* genomic sequences. Indicated bands were quantitated (see Methods) and compared to *rosy* standard bands. A. Crete cosmid 8D8 was used as a probe and contains the breakpoint for *Dp(1;3)E1*. The 12 kb band in *Dp(1;3)E1* is polymorphic and runs at 15-20 kb. This fragment and the adjacent 14 kb fragment are not contained within this duplication (Fig. 4). When compared to *rosy* standards, these two bands show equivalent levels of hybridization as wild-type male DNA. This indicates that only one copy of these fragments is present, and the fragments are not contained in duplicated sequences. In contrast all bands quantitated are contained within *Dp(1;f)101*, whereas bands were not analyzed from *Dp(1;3)sta* due to partial digestion. B. The breakpoint of *Dp(1;f)101* is located in cosmid 5-1-1. When compared to a *rosy* standard, the two lower bands quantitated show equivalent levels of hybridization as wild-type male DNA, whereas the 7.5 kb fragment shows levels equivalent to the female DNA. This indicates that *Dp(1;f)101* breaks within this cosmid. A novel fragment is formed (2.3 kb) presumably from the end fragment of the duplication. All fragments analyzed are past *Dp(1;3)E1* and within *Dp(1;3)sta*.

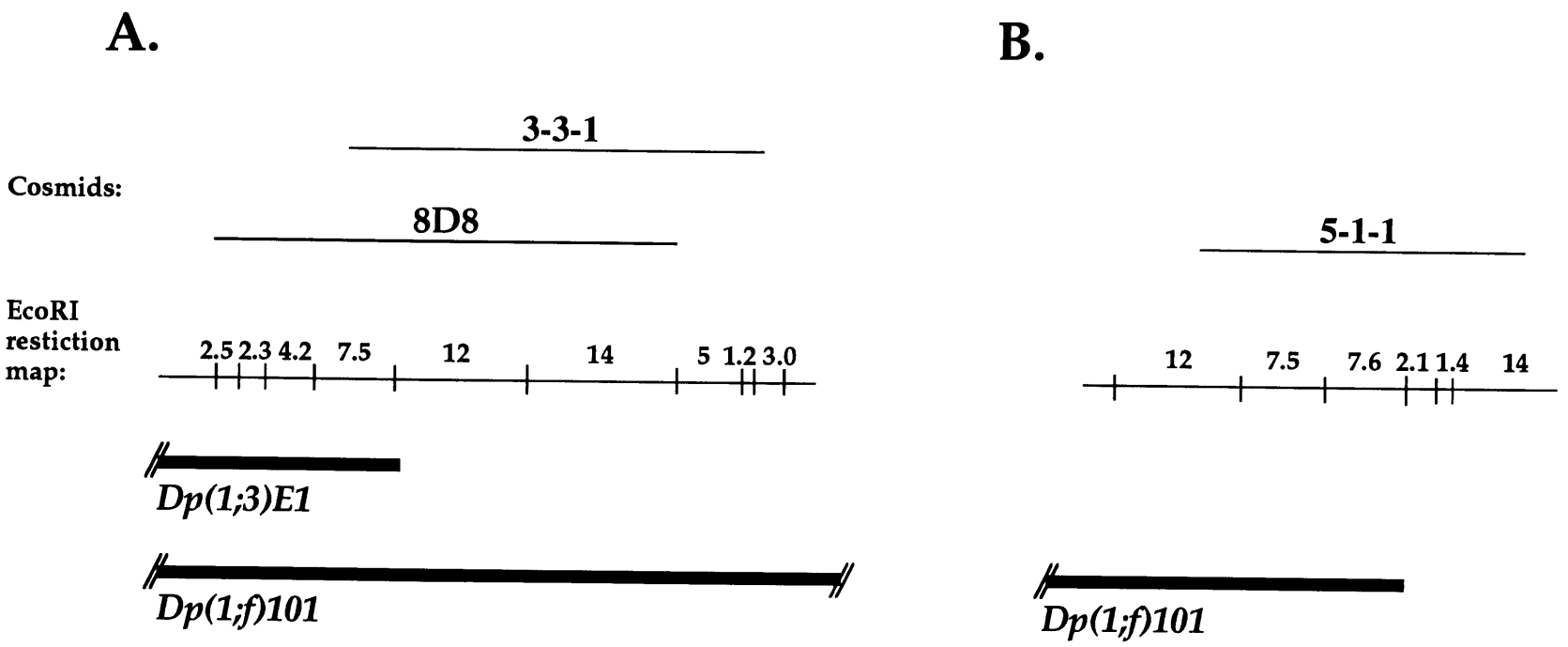
Fig. 4. Summary of quantitative Southern analysis as shown in Fig. 3. The *Eco*RI restriction map of the regions containing cosmids 8D8 and 5-1-1 is shown. A. An overlapping cosmid, 3-3-1, is indicated as a reference for the cosmid walk represented in Fig. 5. Crete cosmid 8D8 was used as a probe because it did not contain repetitive DNA present in cosmid 3-3-1. The *Dp(1;3)E1* breakpoint is located between the 7.5 kb and 12 kb *Eco*RI fragments, as shown by quantitative Southern analysis in Fig. 3. B. The *Dp(1;f)101* breakpoint is located between the 7.6 kb and 2.1 kb *Eco*RI fragments (Fig. 3).



Cosmid 8D8



Cosmid 5-1-1


A.
3-3-1
Cosmids:
8D8
EcoRI
restiction
map:
2.5 2.3 4.2 7.5 12 14 5 1.2 3.0
Dp(1;3)E1
Dp(1;f)101
B.
5-1-1
12 7.5 7.6 2.1 1.4 14
Dp(1;f)101

Transformation Rescue of *png*

In order to localize *png*, cosmids throughout the 130 kb region containing *png* were used for transformation rescue experiments. Cosmids chosen for injection contained on average 10 kb of overlap, and a cosmid spanning the *Dp(1;3)E1* breakpoint was also used to include 20 kb of DNA distal to the minimal *png* breakpoint (Fig. 5). Seven overlapping cosmids were injected into a *y w* stock and w^+ transformants were obtained (Table I and Methods). The expression of genes within a transformed region were subject to position effects, and therefore more than one independent transformant line was obtained for several cosmids. Quantitative Southern analysis determined the structure of independent transformants, as large cosmids often rearranged or deleted sequences upon transformation (see Methods). Only transformants containing an unrearranged insert are included in Table I.

A transformant containing cosmid 3-3-1 rescued the maternal-effect lethality of *png* homozygotes (Table I). This insertion was on the X chromosome, and therefore several independent recombinants with *png* were tested for rescue (see Methods). Cosmid 3-3-1 therefore contained all sequences necessary for *png* function. Cosmid 2-1-1 contained 8 kb of overlap with 3-3-1 yet did not rescue *png*, suggesting that *png* was distal to cosmid 2-1-1 (Fig. 5). Similarly, cosmid 3-1B-1 contained 18 kb of overlap and did not rescue *png*. However, transformants of 3-1B-1 were obtained upon mobilization of an insert on the X chromosome and may have contained rearrangements or deletions (see Methods).

Table I. Molecular Localization of *png* by Cosmid Transformation Rescue

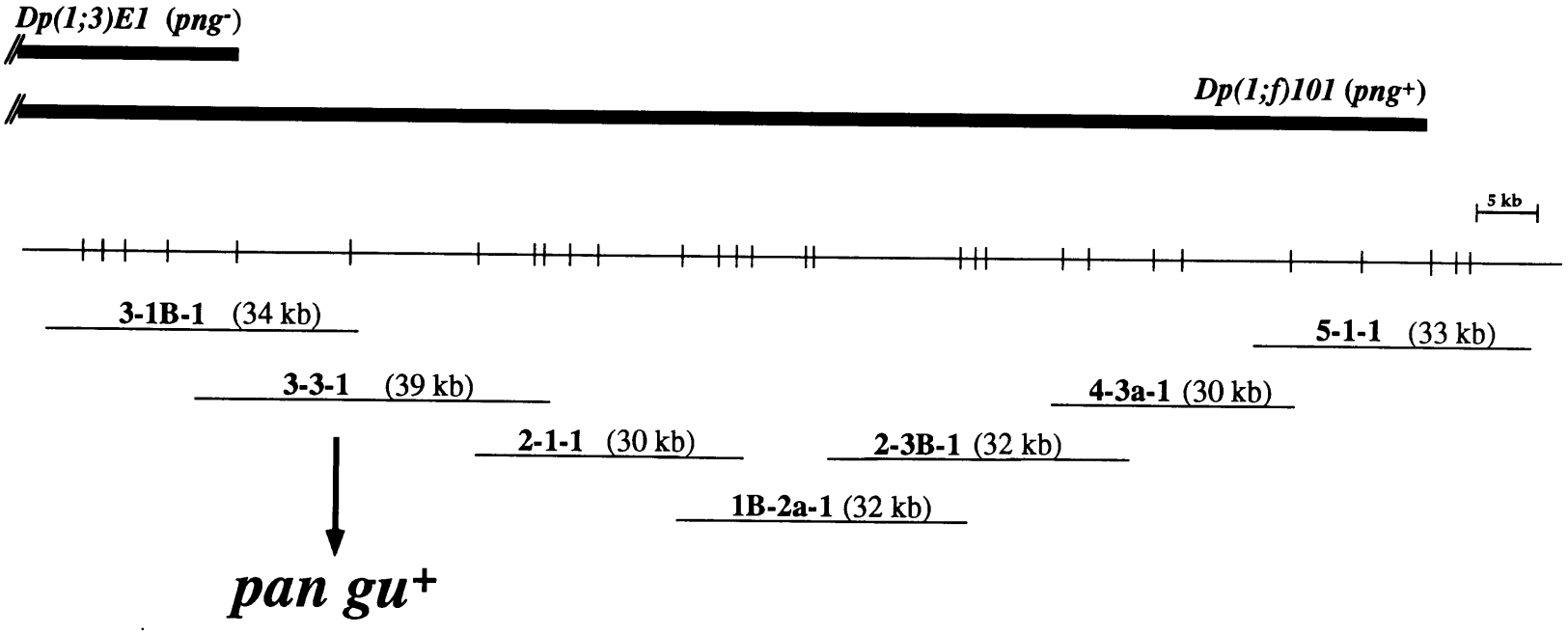
Cosmid:	No. Embryos Injected	No. of Transformants Obtained	Rescue of *png*
3-1B-1	2499	1[a]	-
3-3-1	1311	1[b]	+
2-1-1	1077	1	-
1B-2a-1	2150	2[c]	-
2-3b-1	1961	4[c]	-
4-3a-1	2015	1	-
5-1-1	3086	2[c]	-
(Total:)	(14,099)	(12)	

[a] The initial transformant was on the *X* chromosome near *png*, and 13 new insertion sites were obtained by mobilization of the P-element (see Methods).

[b] The transformant was on the *X* chromosome, and several recombinants with *png* were obtained and tested for rescue (see Methods).

[c] An unrearranged structure was not confirmed by Quantitative Southern analysis. One transformant for 1B-2a-1 did contain the correct structure.

Fig. 5. Cosmid 3-1-1 rescues the maternal-effect lethality of *png*.
Transformation rescue experiments were performed for seven overlapping cosmids. Cosmids were obtained by a chromosome walk between the two duplication breakpoints shown. Cosmids used for injection experiments contained on average 10 kb of overlap, and transformants were generated and tested for rescue of *png*. The *png* locus is contained within 39 kb of cosmid 3-1-1.


Dp(1;3)E1 (png-)
Dp(1;f)101 (png+)
5 kb
3-1B-1 (34 kb)
5-1-1 (33 kb)
3-3-1 (39 kb)
4-3a-1 (30 kb)
2-1-1 (30 kb)
2-3B-1 (32 kb)
1B-2a-1 (32 kb)
pan gu+

Molecular Analysis of the Genomic Region Containing *png*

png was localized to 39 kb within cosmid 3-3-1, and a more complete restriction map throughout this region was obtained with several enzymes (Fig. 8). The proximal 10 kb consisted of repetitive DNA that was not analyzed in subsequent Southern blot experiments. Larger rearrangements and deletions within the cosmid interval were not detected in *png* alleles, as previously described. Therefore, a more sensitive technique was used to identify mutations that might be associated with the *png* locus. All five *png* alleles were EMS induced which causes predominantly single base-pair mutations.

Single base-pair changes or small rearrangements could be detected by denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman, 1983). In this gel system, small fragments (200-700 bp) were electrophoresed through acrylamide gels containing a gradient of denaturants (formamide and urea), and gels were maintained at 60°C by recirculating buffer. Fragments usually melted in one or two domains, and as fragments migrated through higher concentrations of denaturant, the first melted domain would cause a severe retardation in mobility. A single base-pair change within the first melting domain might alter its melting characteristic and subsequent migration. Sequence changes located outside of the first melting domain would not affect the migration of a fragment, and therefore several digests were performed in order to increase the likelihood of detection. Several different ranges of denaturing gradients were also used to increase the detection rate.

In order to identify possible mutations, DGGE analysis was used to compare DNA from *png* alleles to each other and to a *y w* control stock. Eight separate restriction digests were examined, and DNA was run through gels containing three different denaturing gradient conditions (see Methods).

Following electrophoresis, gels were electroblotted onto nylon filters, and subsequent blots were probed with fragments from cosmid 3-3-1.

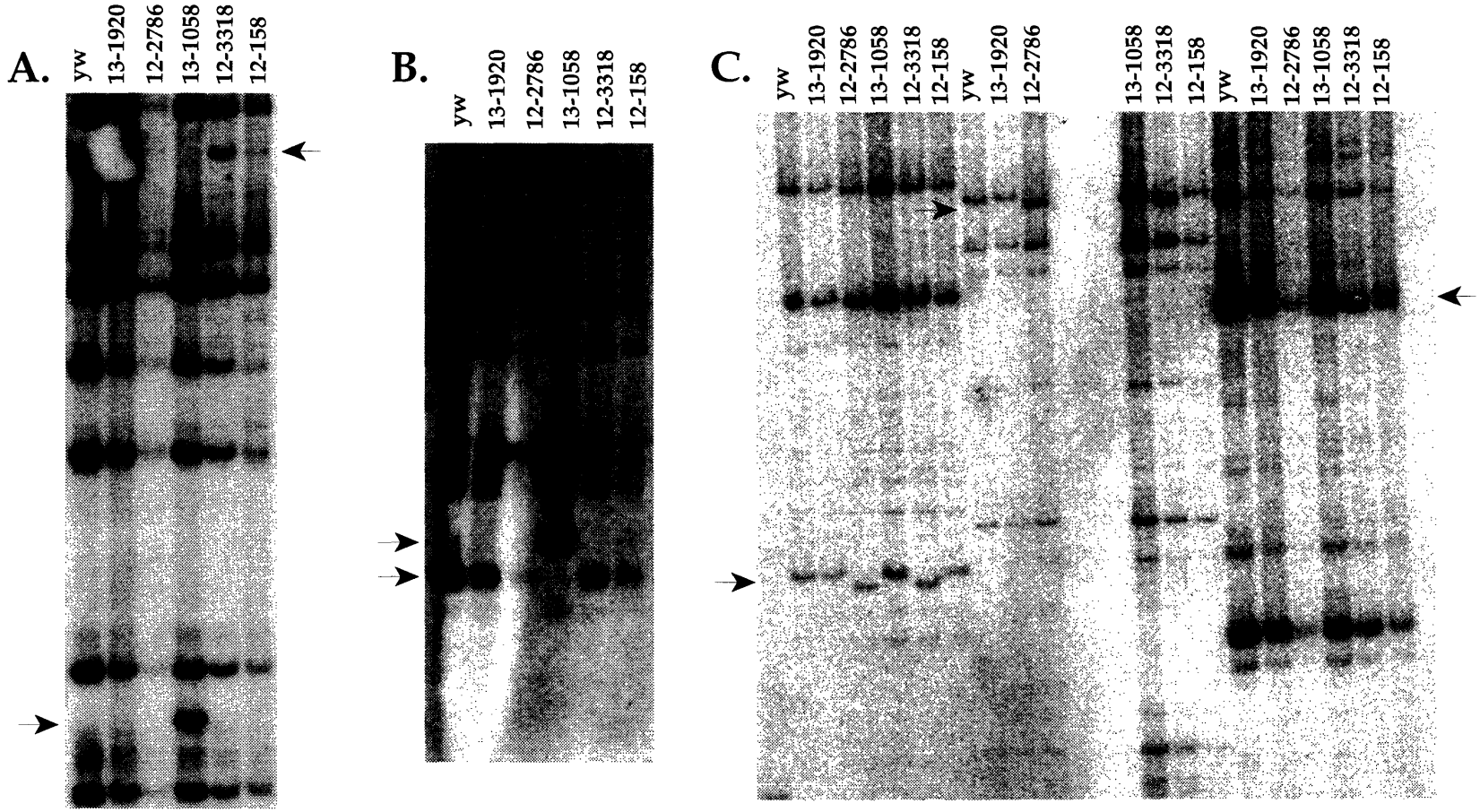
Several possible mutations within the distal portion of cosmid 3-3-1 were identified in *png* alleles (Fig. 6, 8). One mutation in each of the adjacent 3.9 kb *Xho*I fragments was identified in *Mbo*I digests of $png^{13\text{-}1058}$ (Fig. 6). The distal 3.9 kb *Xho*I fragment detected a band that was shifted up, whereas a dramatic downward-shifted band was detected by the proximal 3.9 kb fragment. It was possible that these two changes may have been caused by a single rearrangement covering both *Xho*I fragments. Band shifts were also detected in three other digests when probed with both 3.9 kb fragments. However, upon rehybridization with each subcloned fragment, many bands, including those in which the shifts occurred, were not detected, most likely due to the lowered sensitivity after multiple probings. These changes were all detected in $png^{13\text{-}1058}$, and may have been caused by a single rearrangement.

A mutation in the 2.2 kb *Xba*I-*Xho*I fragment was also detected in both $png^{12\text{-}2786}$ and $png^{12\text{-}3318}$ (Fig. 6). Band shifts were detected in all eight digests under various gel conditions, and in each case the same shift occurred with both alleles. This was most likely due to both alleles containing the same alteration. No DNA alterations were detected in DNA distal to the 2.2 *Xba*I-*Xho*I fragment, and DNA proximal to the 3.9 kb *Xho*I doublet was not analyzed for sequence alterations. With the fragments tested, no alterations were identified in $png^{13\text{-}1920}$ and $png^{12\text{-}158}$.

Identification of Candidate *png* Transcripts

The *png* gene product is required in the germ line of the egg and embryo (see Ch. IV), and is therefore likely to be transcribed during oogenesis. Whether *png* functions later in development is not known. Transcripts were

Fig. 6. Denaturing gradient gel electrophoresis (DGGE) detects DNA alterations in *png* alleles. DNA from *y w* flies and homozygous *png* alleles was prepared and digested with the indicated restriction enzymes. Denaturing gradient gels were performed as described (see Methods), and blots were probed using the indicated fragments from cosmid 3-3-1 (see Fig. 8). A. When probed with both 3.9 kb *Xho*I fragments, a band shift is detected in the *Mbo*I digest of $png^{13\text{-}1058}$. The upper band (arrowhead) is missing in $png^{13\text{-}1058}$ and is shifted to a much lower position in the gel (arrowhead). This gel contained a 10-50% denaturing gradient and was electrophoresed for 1500 Vhr. Upon reprobing with subcloned DNA of either 3.9 kb *Xho*I fragments, this shift is detected only using the proximal 3.9 kb probe. B. A second alteration is detected in the *Mbo*I digest of $png^{13\text{-}1058}$, when a 35-85% denaturing gel was electrophoresed at 2000 Vhr. The same 3.9 kb *Xho*I doublet is labeled as in A, and the lower arrowhead indicates a missing band from $png^{13\text{-}1058}$. The upper arrowhead indicates the position of the shifted band in $png^{13\text{-}1058}$. When reprobed with either subcloned 3.9 kb *Xho*I fragments, this shift is only detected with the distal 3.9 kb *Xho*I probe. C. Band shifts are seen in $png^{12\text{-}2786}$ and $png^{12\text{-}3318}$ in three separate digests, when probed with a 2.2 kb *Xba*I-*Xho*I fragment. Shifted bands are indicated by arrowheads. This 10-50% denaturing gel was electrophoresed at 1500 Vhr. Shifts were detected in all eight restriction digests in gels run under various conditions.



MboI
3.9 XhoI doublet probe

MboI
3.9 XhoI doublet probe

RsaI

AluI

MboI
2.2 XbaI-XhoI probe

therefore examined from dissected female ovaries in order to identify potential *png* transcripts. Northern blots containing poly A^+ RNA from ovaries of *png* alleles and wild type were probed with fragments within cosmid 3-3-1 (see Methods). Many transcripts were identified throughout the region (Fig. 8), and *png* alleles were examined for any alterations in transcript size or expression level. Repetitive DNA within this region was also used to probe Northern blots, and several transcripts were detected. Whether these transcripts were in this region or a homologous repetitive region was not determined.

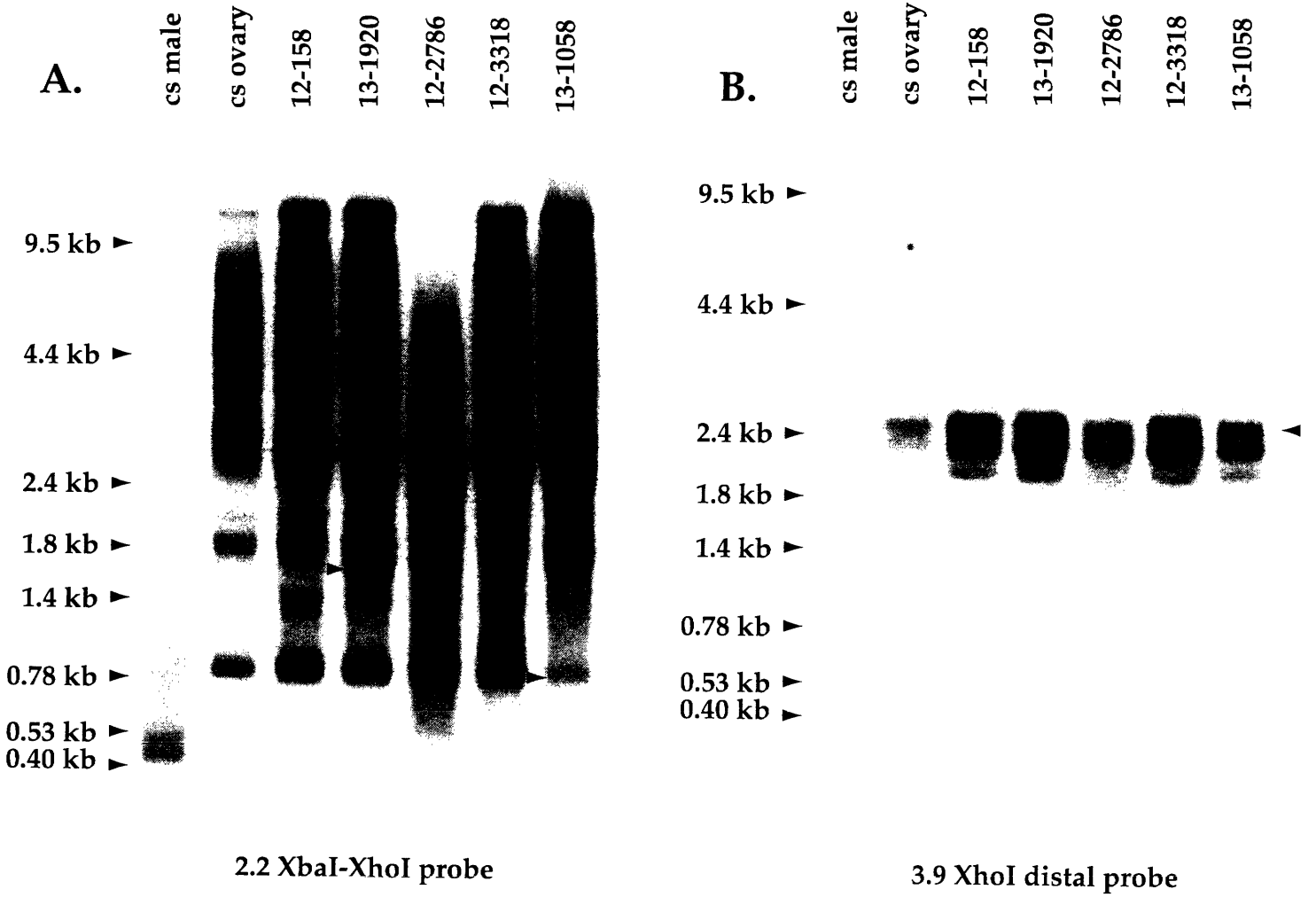
One candidate *png* transcript was identified in which the level of expression was decreased dramatically in $png^{13\text{-}1058}$ (Fig. 7A). A 2.2 kb *Xba*I-*Xho*I probe identified many transcripts, including a 0.8 kb transcript that was almost completely missing in $png^{13\text{-}1058}$. At least five other transcripts ranging in size from 10 kb to 1.3 kb were present in females, whereas males contained predominantly a 0.4 kb transcript. Another possible altered transcript in this region was detected in $png^{13\text{-}1920}$, in which a novel 1.5 kb transcript appeared (Fig. 7A). Using single-stranded probes from the 2.2 kb fragment, all transcripts were transcribed in the same direction, with the 5' end distal to the 3' end. Whether some of these transcripts are related is not known. Unprocessed transcripts might be detected, or complex transcription units might generate multiple transcripts within a region. The down regulation of the 0.8 kb transcript in $png^{13\text{-}1058}$ suggests that this transcript might encode the *png* gene product.

In the adjacent 3.9 kb *Xho*I fragment, four female-specific transcripts were detected from 1.8 kb to 2.5 kb (Fig. 7B). The 2.5 kb transcript was also down regulated in $png^{13\text{-}1058}$, although not as dramatically as the 0.8 kb transcript. However, it is possible that two overlapping 2.5 kb transcripts are

present, and one is completely absent in *png*$^{13\text{-}1058}$. This 2.5 kb transcript represents a second candidate *png* transcript.

Fig. 7. Candidate png transcripts. Northern blots were examined containing poly A$^+$ RNA from wild-type whole males, wild-type ovaries, and *png* homozygous ovaries for all five alleles. A. A 2.2 kb *Xba*I-*Xho*I fragment (see Fig. 8) was used to probe Northern blots, and at least 9 transcripts are detected. The 0.4 kb transcript is male specific, whereas all other transcripts appear female specific. A novel 1.5 kb transcript is present in *png*$^{13\text{-}1920}$, whereas the 0.8 kb transcript is down-regulated in *png*$^{13\text{-}1058}$. B. The adjacent 3.9 kb *Xho*I fragment (see Fig. 8) detected four female-specific transcripts. The 2.5 kb transcript also appears down-regulated in *png*$^{13\text{-}1058}$.

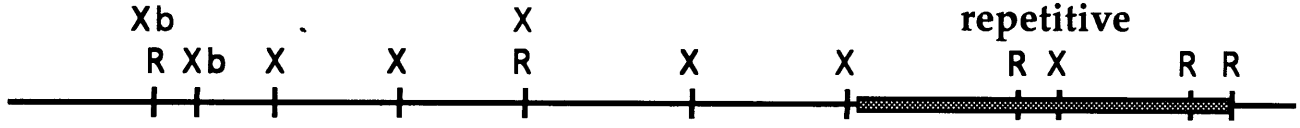
Fig. 8. Summary of DNA alterations and transcripts present in the region of cosmid 3-3-1. A restriction map of the 39 kb region is shown for the enzymes listed below. DNA alterations were detected by DGGE analysis, and transcripts were examined by Northern blot analysis. The 2.2 kb *Xba*I-*Xho*I fragment detects a DNA alteration in *png*$^{12\text{-}3318}$ and *png*$^{12\text{-}2786}$. In this same 2.2 kb fragment, a novel 1.5 kb transcript and a down-regulated 0.8 kb transcript are present in *png* $^{13\text{-}1920}$ and *png*$^{13\text{-}1058}$, respectively. The adjacent 3.9 kb *Xho*I fragment detects a DNA alteration in *png*$^{13\text{-}1058}$, and this same allele contains a down-regulated 2.5 kb transcript. A third DNA alteration is detected in the proximal 3.9 kb *Xho*I fragment. One transcript is present in this region. 25-30 transcripts are detected throughout the 39 kb region of cosmid 3-3-1. Repetitive DNA is indicated by a shaded box. Abbreviations used in the restriction map: Xb, *Xba*I; X, *Xho*I; R, *Eco*RI.



2.2 XbaI-XhoI probe

3.9 XhoI distal probe

Cosmid 3-3-1



kb:	4.5	1.3	2.2	3.9	3.9	5.0	4.5	5.0	1.7	4.4	1.2	1.4

DGGE alterations in *png* alleles: 3318, 2786 (2.2 kb); 1058 (3.9 kb); 1058 (3.9 kb)

5 kb

Transcripts: (kb)	4.4 1.1	10 2.6-10 2.6 1.8 1.6 1.5 1.3 0.8 0.4	2.5 2.3 2.0 1.8	2.5	4.0 3.8 2.9 2.4 1.9	No txs	6.2 5.8 4.5 2.0 1.8 1.6 1.2	4.5 2.0

DISCUSSION

Identification of *png* Sequence Alterations

The *png* locus is contained within a 39 kb region, as identified by transformation rescue experiments. DNA alterations in *png* alleles, and three altered transcripts in *png* alleles have been localized within this region. Whether the sequence alterations are polymorphisms in the stocks or actual *png* mutations is not yet known. Similarly, how the DNA alterations relate to the putative transcripts is unclear. All alterations lie in a 10 kb region also containing the putative *png* transcripts (Fig. 8).

DNA base-pair changes or small rearrangements are found in three of the five *png* alleles. Two altered bands in DNA from $png^{13\text{-}1058}$ were detected using two adjacent 3.9 kb *Xho*I probes (Fig. 8). Either two independent sequence alterations arose, or alternatively a rearrangement occurred spanning the two adjacent fragments. Perhaps a deletion or insertion occurred spanning the *Xho*I site. If this rearrangement created a novel *Mbo*I site, two separate band shifts would be detected by the two adjacent probes.

Mutations are also seen in the two multiple nuclei *png* alleles (Fig. 8). $png^{12\text{-}2786}$ and $png^{12\text{-}3318}$ contain a presumably identical mutation within a 2.2 kb *Xba*I-*Xho*I fragment. Identical mutations should create the same band shift in all instances, and these two mutations cause the exact same shift in eight different digests. There are two possibilities to explain the presence of the same mutation in both alleles. Both alleles might contain a polymorphism, which would not be associated with the mutation causing the *png* phenotype. Two independently isolated alleles are unlikely to have identical causative mutations. Alternatively, $png^{12\text{-}2786}$ and $png^{12\text{-}3318}$ may be independent isolates of the same premeiotic mutation. Mutagenesis can

cause premeiotic events during spermatogenesis resulting in several progeny carrying the same mutation. Mutagenized males can be removed following 3 days of mating to prevent the isolation of progeny from these premeiotic events, however this was not done in the screen that generated *png* alleles (Mohler, 1977). Both multiple nuclei alleles result in identical phenotypes and act similarly in genetic interactions, supporting this hypothesis (see Ch. IV). If both alleles are indeed reisolates of the same mutation, this alteration may be associated with *png*.

Identification of the *png* Transcription Unit

Between 25 to 30 transcripts are located throughout the 39 kb region (Fig. 8). One candidate *png* transcript is a female-specific 0.8 kb transcript located in a 2.2 kb *Xba*I-*Xho*I fragment. This transcript is down regulated in $png^{13\text{-}1058}$. Another negative regulator of replication, *plu*, encodes a small 0.7 kb female-specific transcript. *plu* and *png* act in a similar fashion and may encode similar gene products that regulate replication. In this same 2.2 kb fragment a novel 1.5 kb transcript is also seen in $png^{13\text{-}1920}$. How this transcript is related to the 0.8 kb transcript is not known. It might be that the 1.5 kb transcript appears due to a mutation causing an inefficient splicing event. Some 0.8 kb transcript is correctly spliced, yet a novel form of the transcript appears at 1.5 kb. A second candidate *png* transcript is in the adjacent 3.9 kb *Xho*I fragment, and is also down regulated in $png^{13\text{-}1058}$. This 2.5 kb transcript is also female-specific.

Whether the DNA alterations detected by DGGE analysis in $png^{13\text{-}1058}$ result in either altered $png^{13\text{-}1058}$ transcripts is not yet determined. The location of both sequence alterations and two altered transcripts in $png^{13\text{-}1058}$ within a 10 kb region strongly suggests that one of these transcripts encodes

the *png* gene product. The DNA alteration seen in $png^{13\text{-}1058}$ causes a large band-shift, which is often the case for deletions or insertions. Whether a rearrangement is present can be confirmed by the use of conventional acrylamide gels. The further delineation of this rearrangement would be useful in that smaller probes could then be used to detect transcripts. For example, it is of interest whether this possible rearrangement colocalizes with the region detecting the down regulated transcripts.

Given the transcriptional complexity of the 39 kb region as well as the highly transcribed 10 kb containing DNA alterations in *png* alleles, transformation rescue experiments of smaller constructs would be the most rapid method for confirming the identity of the *png* transcript. Smaller overlapping constructs on the order of 5-10 kb can be used to delineate the extent of the *png* locus. Being DNA alterations and altered transcripts in *png* alleles are seen throughout a 10 kb region, this construct and overlapping constructs would be the first to inject. Following transformation rescue of a smaller region, the identification of cDNAs and sequence comparison of wild-type and mutant alleles will be useful in identifying the open reading frame encoding the *png* transcription unit. Sequence information of *png* alleles might also be informative in understanding the molecular basis for both the single and multiple nuclei classes of *png* alleles.

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REFERENCES

Abrams, E. S. and V. P. Stanton. 1992. Use of denaturing gradient gel electrophoresis to study conformational transitions in nucleic acids. Methods In Enzymology *212*: 71-104.

Ashburner, M. 1989. Drosophila. A Laboratory Manual. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.).

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. 1987. Current Protocols In Molecular Biology. K. Janssen, (John Wiley & Sons, Inc., New York, N. Y.).

Axton, J. M., F. L. Shamanski, L. M. Young, D. S. Henderson, J. B. Boyd and T. L. Orr-Weaver. 1994. The inhibitor of DNA replication encoded by the Drosophila gene *plutonium* is a small, ankyrin repeat protein. Embo J. *13*: 462-70.

Cardoso, M. C., H. Leonhardt and B. Nadal-Ginard. 1993. Reversal of terminal differentiation and control of DNA replication: cyclin A and

Cdk2 specifically localize at subnuclear sites of DNA replication. Cell *74*: 979-92.

Duronio, R. J. and P. H. O'Farrell. 1994. Developmental control of a G1-S transcriptional program in Drosophila. Development *120*: 1503-15.

Dynlacht, B. D., O. Flores, J. A. Lees and E. Harlow. 1994. Differential regulation of E2F trans-activation by cyclin/cdk2 complexes. Genes Dev. *8*: 1772-86.

Fischer, S. G. and L. S. Lerman. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc. Natl. Acad. Sci. *80*: 1579-83.

Fisher, R. P. and D. O. Morgan. 1994. A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. Cell *78*: 713-24.

Freeman, M. and D. M. Glover. 1987. The *gnu* mutation of Drosophila causes inappropriate DNA synthesis in unfertilized and fertilized eggs. Genes Dev. *1*: 924-30.

Freeman, M., C. Nüsslein-Volhard and D. M. Glover. 1986. The dissociation of nuclear and centrosomal division in *gnu*, a mutation causing giant nuclei in Drosophila. Cell *46*: 457-68.

Heichman, K. A. and J. M. Roberts. 1994. Rules to replicate by. Cell *79*: 557-62.

Kafatos, F. C., C. Louis, C. Savakis, D. M. Glover, M. Ashburner, A. J. Link, I. Siden-Kiamos and R. D. C. Saunders. 1991. Integrated maps of the Drosophila genome: progress and prospects. Trends Genet. *7*: 155-61.

Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint and C. F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell *77*: 107-20.

Krek, W., M. E. Ewen, S. Shirodkar, Z. Arany, W. Kaelin Jr. and D. M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell *78*: 161-72.

Krolewski, A. S., B. Krolewski, M. Gray, V. Stanton, J. H. Warram and D. Housman. 1992. High-frequency DNA sequence polymorphisms in the insulin receptor gene detected by denaturing gradient gel blots. Genomics *12*: 705-9.

Lefevre, G. 1981. The distribution of randomly recovered X-ray induced sex-linked genetic effects in *Drosophila melanogaster*. Genetics *99*: 461-80.

Lefevre, G. and W. Watkins. 1986. The question of the total gene number in *Drosophila melanogaster*. Genetics *113*: 869-95.

Lindsley, D. L. and G. G. Zimm. 1992. The genome of *Drosophila melanogaster*. (Academic Press, Inc., New York, N. Y.).

Minshull, J. 1993. Cyclin synthesis: who needs it? Bioessays *15*: 149-55.

Mohler, J. D. 1977. Developmental genetics of the Drosophila egg. I. Identification of 59 sex-linked cistrons with maternal effects on embryonic development. Genetics *85*: 259-72.

Mohler, J. D. and A. Carroll. 1984. Sex-linked female-sterile mutations in the Iowa collection. Dros. Inf. Serv. *60*: 236-41.

Ohtsubo, M. and J. M. Roberts. 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science *259*: 1908-12.

Perrimon, N., L. Engstrom and A. P. Mahowald. 1989. Zygotic lethals with specific maternal effect phenotypes in *Drosophila melanogaster*. I. Loci on the *X* chromosome. Genetics *121*: 333-52.

Quelle, D. E., R. A. Ashmun, S. A. Shurtleff, J. Y. Kato, D. Bar-Sagi, M. F. Roussel and C. J. Sherr. 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. *7*: 1559-71.

Resnitzky, D., M. Gossen, H. Bujard and S. I. Reed. 1994. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol. Cell. Biol. *14*: 1669-79.

Richardson, H. E., L. V. O'Keefe, S. I. Reed and R. Saint. 1993. A Drosophila G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. Development *119*: 673-90.

Serrano, M., G. J. Hannon and D. Beach. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*: 704-7.

Shamanski, F. L. and T. L. Orr-Weaver. 1991. The Drosophila *plutonium* and *pan gu* genes regulate entry into S phase at fertilization. Cell *66*: 1289-300.

Sherr, C. J. 1994. G1 phase progression: cycling on cue. Cell *79*: 551-5.

Siden-Kiamos, I., R. D. C. Saunders, L. Spanos, T. Majerus, J. Treanear, C. Savakis, C. Louis, D. M. Glover, M. Ashburner and F. C. Kafatos. 1990. Towards a physical map of the *Drosophila melanogaster* genome: mapping of cosmid clones within defined genomic divisions. Nucleic Acids Res. *18*: 6261-70.

Tamkun, J. W., R. Deuring, M. P. Scott, M. Kissinger, A. M. Pattatucci, T. C. Kaufman and J. A. Kennison. 1992. *brahma*: A regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell *68*: 561-72.

AFTERWORD:
Future Considerations

The characterization of cis-acting elements involved in chorion gene amplification has identified several elements important for various aspects of amplification. ACE3 is sufficient to induce amplification with the correct temporal and tissue specificity, whereas the AER elements are needed for high levels of amplification, and AER-d contains a predominant amplification origin. One direction that would allow the further dissection of chorion amplification is the isolation of trans-acting factors involved in amplification.

Trans-acting amplification factors have been sought by both genetic and biochemical approaches. Several mutants have been isolated that affect chorion amplification and give rise to thin egg shells. Most of these mutants however, have lethal alleles or are lethal over a deficiency suggesting a role in general replication as opposed to a specific role in amplification (reviewed in Orr-Weaver, 1991). Biochemical approaches have identified proteins that bind to the ACE3 and AER-d sequences. Expression libraries of stage 10 egg chamber cDNAs were probed and two clones were isolated; one hybridizing only to ACE3, and the other binding both ACE3 and the origin region within AER-d (J. Tower and A. Spradling, personal communication). The characterization of these proteins will prove interesting in further dissecting the mechanism of chorion DNA amplification. This type of biochemical approach has also been successful in isolating trans-acting factors involved in chorion gene transcriptional control (Shea et al., 1990).

An efficient in vitro system would be useful in the biochemical purification of trans-acting factors involved in amplification. Extracts from

tissue culture cells or embryos would provide sufficient starting material for the biochemical purification of factors that interact with known cis-acting elements. It might be possible to further characterize replication in embryos as a system for assaying chorion replication. The initial results were encouraging in that replication appeared to be dependent on ACE3. With further experimentation, this in vitro system might become a reproducible and reliable system to study chorion replication. The important cis-acting elements could then be used as probes to identify trans-acting factors from embryos. The further characterization of any factors obtained would be necessary to test whether these proteins are involved in general replication or are specific for chorion amplification.

An alternative approach for the identification of trans-acting factors would be to perform a screen based on the two-hybrid system in *S. cerevisiae.* A one-hybrid screen has been used successfully to identify factors that interact with yeast replication origins. One of the ORC proteins, ORC6, was identified by such a screen (Li and Herskowitz, 1993). Another factor, Dbf4, that binds to yeast replication origins has recently been identified using this technique (Dowell et al., 1994). A similar screen could be used to identify factors that bind to ACE3, or the AER elements. In this type of screen, the cis-acting control element would be fused to a lacZ reporter construct, and a Drosophila cDNA library fused to the transcriptional activation domain of GAL4 could be transformed into yeast to identify clones that turn on lacZ expression. Preferably, an ovarian or egg chamber cDNA library would be used. Following identification of trans-acting factors, direct DNA binding assays, such as band shift and footprinting, would confirm a specific interaction with cis-acting elements. Further biochemical or genetic criteria would be necessary to establish a specific role in chorion replication or amplification.

The characterization of the maternal-effect gene, *png*, has identified a negative regulator of replication that acts both before fertilization, and following fertilization during the early cell cycles. Further experiments are needed to identify the *png* transcription unit. Both DNA alterations and altered transcripts have been identified in *png* alleles within a transcriptionally complex 10 kb region. Transformation rescue experiments using smaller and overlapping constructs are necessary to identify the *png* gene. Following the identification of a smaller rescuing construct, cDNAs would be isolated and characterized. Sequence information of cDNAs and the genomic region from both wild type and mutant alleles will be necessary to identify the *png* open reading frame. Sequence information might indicate homologies to known proteins, and the sequence of mutant alleles might point to regions important for protein function. The molecular basis for the single versus multiple class of alleles might also be evident from the location of mutations from both classes of alleles.

The generation of antibodies are necessary for future cytological and biochemical experiments. Both RNA and protein localization would provide information as to the time of action of *png* function. RNA in situs to embryos, and developmental Northern analysis would indicate at what times during development the transcript is present. Using Png antibodies, the protein localization of Png might suggest specific models of *png* function. For example, localization to either the nucleus or cytoplasm during different stages of the cell cycle might provide clues to the mechanism by which *png* regulates replication.

Following protein expression in vitro, several methods are available to determine the mode of action of *png*. *png* is a negative regulator of replication and one model for how *png* acts is that *png* directly inhibits DNA

replication. This might be tested using the in vitro Xenopus extract system that is able to support replication of added template DNA, either sperm nuclei or plasmid DNA. Mixing experiments with Png protein expressed in vitro, and Xenopus extracts might determine whether Png is able to directly repress DNA replication. Similarly, mixing experiments could be tested using the in vitro SV40 replication system. Recently, Drosophila extracts have been shown to replicate SV40 in vitro (Kamakaka et al., 1994), and similar mixing experiments with Png protein might show a direct repression of replication.

An alternative model for how *png* acts to negatively regulate replication is that *png* inhibits S phase regulators, such as cyclin kinases. This inhibition would prevent the entry into S phase. By looking at the expression levels or localization of these S phase regulators in *png* mutants, a role for *png* in regulating these genes might be determined. Preliminary evidence suggests that *png* does not affect cdc2c kinase levels. The level of cdc2c-associated histone kinase has been determined in *png* mutant embryos and does not appear derepressed as compared to wild type. (C. Lehner, personal communication) However, *png* might affect localization of S phase regulators, and altered localization in *png* mutants might cause inappropriate entry into S phase.

The future identification of interactors will also help to determine the mechanism of action of *png*. Biochemical experiments can be performed to identify proteins that interact with Png. Co-immunoprecipitation experiments using Png antibodies might reveal proteins that form complexes with Png. One hypothesis is that a specific interaction between Png and Plu occurs, and this can also be determined using co-immunoprecipitations. The yeast two-hybrid system can also be used to test interactions with specific proteins. For example, whether Png and Plu interact via a two-hybrid system

can be determined. Similarly, interactions between Png and S phase regulators can be examined either using the yeast two-hybrid system or biochemical co-immunoprecipitation experiments.

The future sequence analysis of the *png* gene product and the identification of gene products that interact with *png* will help to determine at what level *png* acts to negatively regulate DNA replication.

REFERENCES:

Dowell, S. J., P. Romanowski and J. F. Diffley. 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science *265*: 1243-6.

Kamakaka, R. T., P. D. Kaufman, B. Stillman, P. G. Mitsis and J. T. Kadonaga. 1994. Simian virus 40 origin- and T-antigen-dependent DNA replication with Drosophila factors in vitro. Mol. Cell. Biol. *14*: 5114-22.

Li, J. J. and I. Herskowitz. 1993. Isolation of *ORC6*, a component of the yeast origin recognition complex by a one-hybrid system. Science *262*: 1870-1874.

Orr-Weaver, T. L. 1991. Drosophila chorion genes: cracking the eggshell's secrets. Bioessays *13*: 97-105.

Shea, M. J., D. L. King, M. J. Conboy, B. D. Mariani and F. C. Kafatos. 1990. Proteins that bind to Drosophila chorion *cis*-regulatory elements: A new C_2H_2 zinc finger protein and a C_2C_2 steroid receptor-like component. Genes Dev. *4*: 1128-40.

Appendix I: Pan Gu

First there was the great cosmic egg. Inside the egg was chaos, and floating in chaos was P'an Gu, the Undeveloped, the divine Embryo. And P'an Gu burst out of the egg, four times larger than any man today and he fashioned the world.

Ancient Chinese Creation Myth