Genetic Control Of Maternal mRNA Degradation In The Early *Drosophila* Embryo

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Abstract

The maternal mRNA degradation pathway acts in the early *Drosophila* embryo to eliminate a subset of transcripts. This maternal pathway begins to function at or shortly after egg activation. Here it is shown that the wild-type activity of four maternal-effect loci, *png*, *plu*, *gnu*, and *temp* are involved in the positive regulation of maternal mRNA degradation in the early embryo of *Drosophila melanogaster*. *png*, *plu*, *gnu*, and *temp* also result in defects in the regulation of the S-M (DNA replication-Mitosis) transition at the end of meiosis and various processes which are known to occur during egg activation and early embryogenesis. My results suggest that maternal mRNA degradation may be associated with, and regulated, by the molecular genetic events which are known to occur during egg activation and early development.

Table Of Contents

CHAPTER 1

Introduction

The localization of mRNAs to specific cellular regions is an important developmental process which was first observed by Jeffery et al. in 1983, who demonstrated that actin mRNA is localized in the myoplasm of developing Ascidian eggs. Since then, many more maternally transcribed mRNAs have been found to be localized in the oocytes and early embryos of many species, such as *Xenopus*, *Drosophila*, Echinoderms, mammals and even the unicellular budding yeast, *Saccharomyces cerevisiae*.

Mechanisms Of mRNA Localization

Four general mechanisms utilized for the cytoplasmic localization of mRNAs during early development have been proposed (reviewed by Bashirullah et al., 1998; Hazelrigg 1998; St. Johnston, 1995). The first mechanism is achieved by vectorial nuclear transport. These transcripts are exported from one side of the nucleus to the cytoplasm, where they are trapped and anchored. The pair-rule transcripts, *hairy*, *runt*, *fushi tarazu*, and *even-skipped* were thought to be localized by this process (Davis and Ish-Horowicz, 1991; Edgar et al., 1987; Gergen and Butler, 1988). However, more recently it has been shown that these pair-rule transcripts are localized by active directed cytoplasmic transport (Lall et al., 1999). The exact mechanism for vectorial transport is still unknown as examples have yet to be discovered. The second mRNA localization mechanism involves the directed transportation of transcripts along the cytoskeleton. One example is *bicoid* transcripts, which are transported from the nurse cells to the anterior of the oocyte by the action of microtubules (Pokrywka and Stephenson, 1991). A third mechanism which has been proposed in the localization of mRNA is diffusion of transcripts in the cytoplasm followed by trapping and anchoring to localized binding sites. Microfilaments have been

proposed to be key components of the anchoring process as mutations in a microfilament-associated protein affect the localization of *oskar* transcripts at the posterior of the oocyte (Erdelyi et al., 1995; Tetzlaff et al., 1996). In addition, the posterior localization of Vasa, Staufen, and Oskar proteins are required for the anchoring and subsequent increase in concentration of specific transcripts at the posterior of the *Drosophila* oocyte (Jongens et al., 1992; Raff et al., 1990; Wang et al., 1994). These components of the polar plasm therefore may be involved in concentrating specific transcripts (*gcl*, cyclin B, and *nanos*) at the posterior.
The final mRNA localization mechanism involves generalized degradation and localized protection of transcripts. One such example is *Hsp83* RNA. This transcript is uniformly abundant in the early *Drosophila* embryo. However by 2.5 hours after fertilization the RNA is degraded throughout the embryo, apart from a small percentage at the posterior polar plasm / pole cells. The transcripts at the posterior have been shown to be protected from the mRNA degradation machinery (Ding et al., 1993a; Bashirullah et al., 1999)

Cis-acting Elements Involved In mRNA Localization

RNA localization requires the recognition of specific sequences located in the transcript itself which direct an RNA to it's final destination within a cell. Most cis-acting sequences that are involved in mRNA localization have been mapped to the 3'UTRs (**3' Untranslated Regions**) and are often found to be quite large possibly due to the fact that they are required to form specific complex secondary structures which the trans-acting factors recognize. It is also possible that there are multiple cis-acting localization sequences which bind distinct trans-acting factors.
The cis-acting localization sequences of several transcripts have been identified in *Drosophila*. These include anteriorly localized RNAs such as *bicoid* (Ferrandon et al., 1994; Ferrandon et al.,

1997; Macdonald and Struhl, 1988; Macdonald et al., 1993), posteriorly localized transcripts, such as *oskar* (Kim-Ha et al., 1993), *nanos* (Gavis et al., 1996), *Cyclin B* (Dalby and Glover, 1992), and *Hsp83* (Bashirullah et al., 1999), and apically localized transcripts, e.g., *fushi-tarazu* and *wingless* (Baker, 1988; Davis and Ish-Horowicz, 1991).

bicoid is an example of a localized mRNA which contains discrete cis-acting elements in its 3'UTR, each involved in distinct steps during the localization process. The BLE1 (***bicoid*** localization element **1**) is an approximately 50 nucleotide sequence which is necessary and sufficient to confer bicoid transport from the nurse cells to the oocyte and anterior localization during mid-oogenesis (Macdonald et al., 1993). Specific regions of the 3'UTR were also identified by linker scanning and point mutation analyses which are required for anterior localization of the transcript late in oogenesis and during early embryogenesis (Ferrandon et al., 1994; Ferrandon et al., 1997). These cis-acting elements bind and interact with trans-acting localization factors. In addition to these discrete localization sequences, dispersed localization elements also exist. For example, *nanos* possesses a 547 nucleotide region in its 3'UTR which is necessary and sufficient to direct localization. However, two regions which overlap are found to map within the 547 nucleotide sequence, both of which are capable of directing localization. These two regions cannot be subdivided without affecting localization (Gavis et al., 1996).

Trans-acting Factors Involved In RNA Localization

In addition to the localization sequences mentioned above, specific proteins that bind these sequences, and mediate the interaction with the cytoskeletal network during mRNA localization have been identified. Possibly the most studied and best understood example is the Staufen protein. This is a double-stranded RNA-binding protein (St. Johnston et al., 1992) which

colocalizes with *oskar* RNA at the posterior pole and with *bicoid* RNA at the anterior pole of the embryo, and is required for both the localization of the anterior RNA, *bicoid*, and the posteriorly localized RNAs, *nanos* and *oskar* (St. Johnston and Nusslein-Volhard, 1992; St Johnston et al., 1991). Mutations in the *staufen* gene result in delocalization of *bicoid* mRNA in the early embryo (Frohnhofer et al., 1987; St. Johnston et al., 1989). Also, in *staufen* mutants, *oskar* mRNA is restricted to the oocyte anterior and eventually becomes delocalized, and the normal posterior localization is lost (Ephrussi et al., 1991). Staufen is known to interact with the 3'UTRs of these localized RNAs. When the 3'UTR of *bicoid* mRNA is injected into *Drosophila* embryos, Staufen protein is found to interact with the *bicoid* element, and forms particles associated with microtubules suggesting that this trans-acting factor is involved in the microtubule-dependent localization of *bicoid* mRNA (Ferrandon et al., 1994). The formation of these particles requires the presence of the *bicoid* 3'UTR cis-acting elements (see earlier). More recently, Ferrandon et al. (1997) reported that specific single-stranded stem loop regions in the 3'UTR of bicoid mRNA interact with the Staufen protein. In addition to Staufen, many more trans-acting factors which are believed to function in mRNA localization have been discovered, though only a small fraction have been shown to interact directly with the localized RNAs so far.

Functions Of mRNA Localization

mRNA localization serves several roles during development. Firstly, intracellular mRNA localization is used to achieve high concentrations of specific proteins in particular regions of a cell where they are required. One such example is *bicoid* mRNA, which is localized to the anterior of the *Drosophila* embryo where it directs head and thorax formation (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988). Targeting of a protein for transport is a less efficient

method than localizing the mRNAs due to the fact that a single mRNA molecule can result in the translation of several proteins. Secondly, mRNA localization is needed to prevent the expression of the protein in a particular region of the cell where it's activity is deleterious. An example is *nanos* localization. If *nanos* is mislocalized to the anterior of the embryo, developmental defects occur due to the fact that the anterior cells adopt posterior fates (Gavis and Lehmann, 1992). A third role for mRNA localization is to serve structural roles in specific regions. For example, a non-coding mRNA, e.g., *Pgc1*, may be involved in forming the non-membrane bound organelles such as the polar granules in the *Drosophila* early embryo (Nakamura et al., 1996). Finally, RNA localization serves as a mechanism to segregate transcripts unequally during asymmetric cell division. This has been observed in the localization of *ASH1* mRNA during *Saccharomyces cerevisiae* budding, and *prospero* transcript localization during *Drosophila* neuroblast division (Li et al., 1997; Long et al., 1997; Takizawa et al., 1997).

Generalized mRNA Degradation And Localized Protection

As mentioned previously, one mechanism, which is utilized by *Drosophila* to localize transcripts to specific regions during development is general degradation throughout the embryo except at the site where the RNA has to be localized. This process was predicted a decade ago (Gottlieb et al., 1990), and has been shown to be used by *Drosophila* during early development (Ding et al., 1993). *Hsp83* represents the first, and so far only example of a transcript, which is localized exclusively by a degradation-protection mechanism (Ding et al., 1993a). In addition, the degradation machinery used to localize this transcript is also used to refine the localization of two additional maternally synthesized transcripts, *nanos* and *Pgc*, prior to the midblastula transition (MBT) when the control of development is passed from maternal transcripts to

zygotically expressed genes. The degradation complex of the localization mechanism also functions to eliminate maternal transcripts that are never localized, e.g., *string*. This may allow for the proper coordination of early development (Bashirullah et al., 1999).

Two RNA degradation pathways have been shown to function in the early *Drosophila* embryo (Bashirullah et al., 1999). One of these pathways is referred to as the "maternal " pathway. This begins to function at, or shortly after egg activation, and is independent of fertilization and zygotic gene transcription (Bashirullah et al., 1999). In unfertilized eggs, the maternal pathway is sufficient for degradation and localization of the abundant *Hsp83* transcript by approximately 4-5 hours after egg activation (Bashirullah et al., 1999). The "zygotic" degradation pathway requires fertilization and becomes active 1.5-2 hours after fertilization. It is twice as efficient as the maternal pathway as *Hsp83* transcripts are eliminated by the sole action of the zygotic machinery 2 hours after zygotic activation (Bashirullah et al., 1999). The zygotic pathway is required for the elimination of more abundant maternal transcripts by the MBT, such as *Hsp83* and the *Drosophila* homologue of the cell cycle regulator CDC25, *string* (Edgar and Datar, 1996; Bashirullah et al, 1999). Less abundant transcripts, such as *nanos* are mostly degraded by the sole action of the maternal degradation pathway (Bashirullah et al., 1999). Unlike previous work which suggested that degradation of specific transcripts at the MBT is regulated by zygotically expressed genes (Yasuda et al, 1991), it is now known that the combined action of both the maternal and zygotic degradation pathways is required for the timely destabilization of maternal transcripts (Bashirullah et al., 1999).

Cis-acting Sequences That Direct mRNA Localization By The Degradation-Protection Mechanism

Deletion of a specific 3'UTR cis-acting element from *Hsp83*, the HDE (***Hsp83*** **Degradation Element**) results in stabilization of this maternal transcript in unfertilized eggs. This cis-acting element was mapped to nucleotides 253 to 349 in the 3'UTR and is necessary for maternally encoded *Hsp83* RNA degradation (Bashirullah et al., 1999). Transcripts which had this element deleted were found to be stable for up to six hours in unfertilized eggs, which is two hours after endogenous transcripts have been fully degraded. However, all deletions of the *Hsp83* 3'UTR had no effect on the zygotic degradation pathway suggesting that either there are redundant zygotic degradation elements in the 3'UTR, or that the zygotic degradation elements are located in another region of the transcript independent of the 3'UTR (Bashirullah et al., 1999).

nanos RNA, which is normally localized to the posterior of the embryo where it is involved in directing posterior cell fates (Wharton and Struhl, 1991), also possesses a cis-acting localization element located in the first 186 nucleotides of its 3'UTR. This is known as the **Translational Control Element** (TCE), and is required for both translational regulation and RNA degradation (Dahanukar and Wharton, 1996; Smibert et al., 1996; Bashirullah et al., 1999). Transcripts that lack the TCE fail to degrade in unfertilized eggs 2-4 hours after egg activation (Bashirullah et al., 1999). By this time, endogenous *nanos* transcripts are eliminated by the maternal degradation machinery (Bashirullah et al., 1999).

nanos mRNA has also been shown to be degraded in the early *Drosophila* embryo. Unlocalized *nanos* transcripts are translationally repressed. This is quickly followed by degradation throughout the embryo except at the posterior where they are involved in posterior patterning (Dahanukar and Wharton, 1996; Lehmann and Nusslein-Volhard, 1991; Smibert et al.,

1996; Smibert et al., 1999; Wang and Lehmann, 1991). The novel trans-acting factor, Smaug, binds to SREs (Smaug Response Elements) located within a 3'UTR sequence in the transcript (TCE, Translational Control Element), and confers both translational regulation and smaug-dependent mRNA degradation (Smibert et al., 1996; Smibert et al., 1999).

The HDE and TCE are functionally interchangeable cis-acting elements; an *Hsp83* transgenic RNA , which lacks the HDE but possesses the *nanos* TCE, and a *nanos* transgenic RNA, which lacks the TCE but contains the *Hsp83* HDE are both degraded in unfertilized eggs by the maternal degradation machinery. These results suggest that specific cis-acting elements direct both *nanos* and *Hsp83* mRNAs to the same maternal degradation machinery (Bashirullah et al., 1999).

Maternal mRNA Protection

The maternal mRNA, *Hsp83* has been shown to be protected from the maternal degradation machinery in the posterior polar plasm and pole cells in unfertilized eggs and embryos respectively (Bashirullah et al., 1999). Candidate organelles that may be involved in masking this transcript from destabilization are the polar granules. These are non-membrane bound organelles which are found located at the posterior end of the oocyte and early embryo. Mutations that disrupt formation of the polar granules result in *Hsp83* RNA degradation in the polar plasm, while ectopic polar granule assembly at the anterior pole results in ectopic protection of *Hsp83* transcripts (Ding et al., 1993a).

Uniform Transcript Degradation In The Early *Drosophila* Embryo

Prior to the MBT (Midblastula Transition), a subset of maternal mRNAs is degraded. *string* encodes a homologue of the cell cycle regulator CDC25 (Edgar and Datar, 1996; Edgar and O'Farell, 1989; Edgar and O'Farell, 1990). Degradation of the maternal transcript is required for the proper progression from maternally controlled mitosis to zygotically controlled mitosis (Edgar and Datar, 1996). Precise timing of the degradation of *string* is required for this transition. This maternal mRNA is no longer detectable at stage 5, approximately 2.5 hours after fertilization (Myers et al., 1995; Bashirullah et al., 1999). Until recently it was reported that maternal *string* mRNA, like all maternal mRNAs, is degraded at the cellularization stage (Myers et al., 1995). However, it is now known that the degradation machinery is activated shortly after fertilization in *Drosophila*. By using northern blots to quantitatively measure RNA levels, it has been shown that *string* decreases throughout the pre-MBT stages of development, initiating within the first hour of embryogenesis (Bashirullah et al., 1999). 95% of maternal *string* transcripts are eliminated by the MBT (Bashirullah et al., 1999).

Transcript degradation was initially thought to be controlled by zygotically expressed proteins in the blastoderm embryo (Yasuda et al., 1991). Edgar and Datar (1996) using α-amanitin to inhibit zygotic transcription, showed that maternal *string* is stabilized in these embryos. This suggested that zygotic gene products promote maternal mRNA degradation. However, by looking at mutant embryos which lack individual chromosome arms, it had been shown that maternal mRNA degradation is unaffected. This suggested that there was redundancy in the mechanism or that the proteins required to activate the degradation of *string* mRNA in the early *Drosophila* embryo are encoded by maternally contributed transcripts (Myers et al., 1995). It is known now that the combined action of both the maternally encoded degradation machinery and

a newly discovered zygotically controlled pathway is necessary for elimination of the transcripts prior to the MBT (Bahirullah et al., 1999). The trans-acting factors and cis-acting degradation elements, which are necessary and/or sufficient to direct the degradation of maternal *string* have not been defined.

In addition, it has been reported that the nuclear:cytoplasmic ratio controls the degradation kinetics of maternal *string* transcripts (Yasuda et al., 1991). This can now be explained by the fact that two degradation pathways, the maternal and zygotic, are required for the correct elimination of *string* (see below). Altering this ratio probably affects the zygotic degradation machinery as this is controlled by zygotically expressed genes (Bashirullah et al., 1999).
One example of a stable mRNA in the early *Drosophila* embryo is *rpA1* (codes for an acidic ribosomal protein) (Kay and Jacobs-Lorena, 1985; Bashirullah et al., 1999). The maternal *rpA1* transcript is stable well beyond the MBT (Riedl and Jacobs-Lorena, 1996). However, whether specific cis-acting stabilization elements exist in non-degraded maternal transcripts remains unknown. *fushi tarazu* (*ftz*) is an example of a zygotically expressed gene, which is rapidly destabilized during embryogenesis (Edgar et al., 1986; Riedl and Jacobs-Lorena, 1996). This is a pair-rule gene essential for establishment of the *Drosophila* embryonic body plan. *ftz* mRNA contains at least two cis-acting destabilizing elements, one of which is located in the 5' region of the mRNA, the other is found in the 3'UTR, near the polyadenylation signal (Riedl and Jacobs-Lorena, 1996). Whenever, the *ftz* 3'UTR destabilizing element was inserted into the 3'UTR of the intact stable *rpA1* gene, the resulting *rpA1-ftz* transgenic mRNAs were destabilized. This result suggests that either instability elements act dominantly over proposed stability elements, or that transcript stability is the default state.

Mechanism Of RNA Degradation

Even though the importance of mRNA degradation in gene regulation has long been recognized, very little is still known about the mechanism and components (or trans-acting factors) involved in the maternal mRNA degradation pathways which act during early *Drosophila* development. This is due to the fact that very few ribonucleases in most eukaryotic organisms, with the exception of yeast, have been identified, cloned, or characterized. One such enzyme, *pacman*, has been identified and cloned in *Drosophila* (Till et al., 1998). This is a 5'-3' exoribonuclease (Till et al., 1998) and shows high structural and functional homology to the major 5'-3' ribonuclease of *S. cerevisiae*, Xrn1p (Heyer et al., 1995; Muhlrad et al., 1995). *pacman* may be a key component of the maternal mRNA degradation machinery in the early *Drosophila* embryo. *pacman* transcripts are maternally encoded, expressed at high levels in nurse cells and are abundant in 0-8 hour embryos (Till et al., 1998). In addition, *Flybase* predicts 16 regions of the genome which have recently been sequenced by the Drosophila Genome Project which code for 6 verified ribonucleases (*drosha*, *Rnase1*, *Rnase2*, *RnaseX25*, *rnh1*, and *Rpp30*) and 10 putative ribonucleases. Most of these have not been cloned or characterized and are based on sequence similarities.

mRNA degradation and its initiation is influenced by several different processes; some of these include cap-dependent and independent deadenylation of the poly(A) tail, decapping, polyadenylation, poly(A) binding proteins, AU-rich elements (AREs), and enzyme mediated cap-poly(A) tail interactions (Coller et al., 1998; Dehlin et al., 2000; Ford et al., 1997; Gagliardi et al., 1999; Gao et al., 2000; Jacobson and Peltz, 1996; Loflin et al., 1999; Myer et al., 1997; Wang et al., 1999). Three mRNAs in *Drosophila* which are involved in establishing embryonic asymmetry and are known to be translationally regulated by polyadenylation are *bicoid* (anterior

specification), *Toll* (dorsoventral specification), and *torso* (termini specification) (Salles et al., 1994). Mutations in *cortex* and *grauzone* disrupt cytoplasmic polyadenylation of these transcripts, which results in defective maternal mRNA translation in the early embryo and developmental arrest (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). In addition, maternal mRNAs such as *Hsp83*, *string* and *nanos* have been shown to be stabilized in these mutants well after a time point when degradation is normally achieved (Bashirullah et al., 1999). *In vitro* and *in vivo* polyadenylation has also been reported to promote degradation of transcripts in diverse organisms, such *as E. coli* and sunflowers (Blum et al., 1999; Gagliardi and Leaver, 1999). In contrast, poly(A) tail shortening is often considered to be a prerequisite for mRNA degradation. Poly(A) deadenylation has been reported to be the initiating factor in mRNA degradation in yeast and somatic metazoan cells, as well as during oogenesis and early embryogenesis (Richter, 1996). The presence of the poly(A) tail has been shown to inhibit mRNA degradation *in vitro*, by inhibiting the assembly of the exonuclease (Ford et al., 1997). The stabilization of the transcripts is thought to be achieved by the interaction of poly(A) binding proteins with the poly(A) tail (Ford et al., 1997). However it has also been reported that mRNA stabilization is independent of the poly(A) tail but instead requires translation (Coller et al., 1998). In this case the function of the poly(A) tail during mRNA stabilization is to bring the poly(A) binding protein to the transcript (Coller et al., 1998). ELAV proteins have been implicated in mRNA stabilization in mammals (Ford et al., 1999). These AU-rich element binding proteins may be involved in protecting mRNAs from degradation by nucleases, or they may play a role in displacing components that are involved in degradation (Ford et al., 1999).

The 5' cap structure also plays a key role in mRNA degradation. One such mechanism involves deadenylation-dependent decapping, which, in turn allows ribonucleases to initiate 5'-

3' degradation of transcripts (Jacobson and Peltz, 1996). Sm-like proteins in yeast have been implicated in mRNA decapping and degradation, as mutations in seven yeast Lsm proteins result in inhibition of mRNA decapping. These Lsm proteins were found to co-immunoprecipitate with an mRNA decapping enzyme (Dcp1), a decapping activator, and with mRNA (Tharun et al., 2000). Deadenylation in HeLa cell extracts by the action of a mammalian poly(A)-specific exoribonuclease, (PARN), is promoted by the presence of the m^7-guanosine cap (Dehlin et al., 2000). In addition, it has been shown that the DAN (Deadenylating Nuclease) deadenylase directly and specifically interacts with the 5' cap in mRNA substrates (Gao et al., 2000). This novel interaction is increased by the presence of a poly(A) tail (Gao et al., 2000).

Although a lot of work has been carried out on mRNA degradation in mammals and yeast, very little is known about the mechanism and regulation of this process in *Drosophila*. However, the facile genetics and molecular biology of *Drosophila* are beginning to be employed in order to begin to provide insights into this important gene regulatory process.

Egg Activation

Egg activation is a biological event which occurs in a variety of organisms. In *Drosophila*, egg activation occurs as the egg passes from the ovary to the uterus (Doane, 1960; Mahowald et al., 1984). The purpose of this process is to mature the egg and prepare it for subsequent fertilization. Studies in egg activation/maturation began as early as 1924 in *Drosophila melanogaster* (Huettner, 1924), however very little is understood about the molecular genetic events which are triggered by the process, as it has been widely neglected in flies. Some of the events which are known to occur during *Drosophila* egg activation have been well documented (Doane, 1960; Lieberfarb et al., 1996; Mahowald et al., 1984; Page and Orr-Weaver, 1996; Page and Orr-

Weaver, 1997; Bashirullah et al., 1999). As the mature oocyte passes from the ovary to the uterus, activation stimulates the completion of meiosis, chromosome condensation, vitelline membrane crosslinking, mRNA polyadenylation and translation, cytoskeletal reorganization, S-M transition regulation, and maternal mRNA degradation. However, studies on the 'egg activation pathway' and its genetic regulation have largely been ignored in *Drosophila*. Many organisms arrest in meiosis during early development, to allow for proper timed coordination of meiotic completion, maturation, and fertilization (Sagata, 1996). *Drosophila* oocytes arrest at metaphase I during meiosis. However, once activated, the eggs (both unfertilized and fertilized) are stimulated to complete meiosis. Three genes important for this aspect of egg activation are *twine*, *cortex* and *grauzone* (Alphey et al., 1992; Courtot et al., 1992; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). *twine* is required for the arrest at metaphase I, whereas *cortex* and *grauzone* are required for completion of meiosis. More recently, a novel technique for activating *Drosophila* oocytes *in vitro* has been developed (Page and Orr-Weaver, 1997). By this method, many mature oocytes can be activated to complete meiosis. Meiosis in these *in vitro* activated eggs has been shown to be similar to *in vivo* activated eggs (Page and Orr-Weaver, 1997). This system should prove useful for studying egg activation, and important developmental processes which are triggered by activation, such as meiosis in *Drosophila*. *In vitro* activation of sea urchin eggs has also been studied (Miller and Epel, 1999). An increase in pH in unfertilized eggs stimulated the activation of pronuclear movements and entry into mitosis (Miller and Epel, 1999). However, increasing NAD(P)H alone had no effect on egg activation.

Most work on egg activation has been performed in *Xenopus* oocytes and has shed light on several important biochemical aspects of egg activation and maturation in this organism. For example, the protein kinase p90 Rsk has been identified as an essential mediator of cytostatic

factor activity in Xenopus oocytes. This cytostatic factor has been identified as the proto-oncogene, Mos (Sagata, 1997). Rsk is required for cytostatic factor arrest, which is responsible for preventing the activation of unfertilized eggs (Bhatt and Ferrell, 1999). These Rsk proteins are critical targets of p42 MAPK in regulating cell cycle progression and the development of fertilizable eggs (Bhatt and Ferrell, 1999). Due to the fact that the fly genome has been sequenced, searches for homologues will prove interesting in trying to gain a better insight into the molecular components that regulate egg activation in *Drosophila*. Several genes which show high sequence similarity and which have a similar predicted function to Rsk have been identified in *Drosophila*: *bin4*, *CG3105*, *lok*, *SgkII*, and *SAK*. Another well studied aspect of egg activation in many species, including *Xenopus*, is that of calcium signaling (reviewed by Stricker, 1999). During fertilization and egg activation in many animals, intracellular calcium signals are produced in the oocyte. This is required for egg activation and early development to proceed normally (Hogben et al., 1998; Ozil, 1998; Stricker, 1999). However this aspect of egg activation has not been studied in *Drosophila*.

Recently, it was reported that maternal mRNA degradation in the early *Drosophila* embryo commences at, or shortly after egg activation (Bashirullah et al., 1999) suggesting that transcript stability is also regulated by this early developmental process. This novel finding allows for the phenotypic analyses of both egg activation mutants and maternal mRNA degradation mutants to try to unravel the regulatory relationship between egg activation and mRNA degradation in the early *Drosophila* embryo.

CHAPTER 2

Abstract

Two RNA degradation pathways act in the early *Drosophila* embryo to localize and eliminate a subset of maternal transcripts. The first "maternal" pathway is maternally encoded, independent of fertilization and begins to function at, or shortly after egg activation. The second "zygotic" pathway begins to function two hours after fertilization. Here it is shown that maternal mRNA degradation fails in four maternal-effect mutants, *pan gu*, *plutonium*, *giant nuclei*, and the novel mutation, *temprano*. Unlike two previously identified RNA degradation mutants , *cortex* and *grauzone*, all four mutants complete meiosis and activate the maternal translational pathway. However, all four mutants are defective in other aspects of egg activation and early development. *pan gu*, *plutonium*, *giant nuclei* and *temprano* mutants fail to regulate the S-M transition which occurs upon completion of meiosis. In addition, *pan gu* mutants fail to depolymerize the cortical microtubules upon egg activation suggesting that *pan gu* may have pleiotropic functions.

Introduction

Early embryonic development in *Drosophila* is controlled by maternally synthesized RNAs and proteins which are deposited into the egg during oogenesis (Reviewed by Bashirullah et al., 1998; St Johnston and Nüsslein-Volhard, 1992). In addition, specific maternal transcripts must be degraded in the early embryo, likely to allow the control of development to be switched over from the maternal machinery to zygotic control (Bashirullah et al., 1999; Edgar and Datar, 1996; Merrill et al., 1988). Two RNA degradation pathways act in the early *Drosophila* embryo to eliminate a subset of maternal transcripts (Bashirullah et al., 1999). The first 'maternal' pathway begins to function at or shortly after egg activation. This pathway has been shown to be entirely independent of both fertilization and zygotic gene transcription as maternal transcripts such as *Hsp83*, *string*, *nanos* and *Pgc* have all been shown to be degraded in activated unfertilized eggs (Bashirullah et al., 1999). The maternal degradation pathway is targeted to the transcripts by specific cis-acting RNA degradation elements. Bashirullah et al. (1999) reported that a deletion in the 3'UTR of *Hsp83* RNA (*Hsp83* RNA Degradation Element, or HDE) and a deletion in the 3' UTR of *nanos* (Translational Control Element, or TCE) resulted in stabilization of both maternal *Hsp83* RNA and maternal *nanos* RNA respectively, due to failure of the maternally encoded RNA degradation machinery to act on these transcripts in unfertilized eggs. The 'zygotic' pathway begins to function 2 hours after fertilization and does not require the *Hsp83* HDE. While either pathway alone is sufficient to degrade maternal transcripts, the combined action of both pathways is required for the elimination of abundant maternal transcripts prior to the midblastula transition (MBT) when there is a transition from maternal to zygotic control of development.

Specific transcripts such as *Hsp83*, *nanos* and *Pgc* fail to degrade in the posterior polar plasm and pole cells (Ding et al., 1993a; Nakamura et al., 1996; Wang and Lehmann, 1991). Recently, it has been shown that the maternally encoded degradation machinery is present throughout the egg and embryo (Bashirullah et al., 1999); therefore, these transcripts must be protected from the maternal RNA degradation machinery in these regions of the egg or embryo. In the case of *Hsp83* protection, the necessary protective element (*Hsp83* Protection Element, or HPE) also maps to the 3'UTR (Bashirullah et al., 1999).

The maternal RNA degradation pathway begins to function at or shortly after egg activation (Bashirullah et al., 1999). Egg activation is a process which occurs independent of, but usually concomitant with fertilization in *Drosophila*. This occurs as the egg passes through the oviduct into the uterus before fertilization occurs (Mahowald et al., 1983) and is essential for maturation and for preparing the egg for the rapid program of early development. Upon activation, the egg becomes swollen due to rehydration and the vitelline membrane becomes cross-linked and impermeable (Mahowald et al., 1983). Class I *nudel (ndl)* mutants are defective in this aspect of egg activation (Hong and Hashimoto, 1996). Females homozygous for this maternal-effect mutation lay fragile eggs that are difficult to dechorionate and fix and are readily destroyed by bleach treatment. *nudel* has been cloned and is known to encode an extracellular matrix protein with a serine protease catalytic domain. This is essential for dorsoventral polarity and is secreted by follicle cells into the future perivitelline space during mid-oogenesis where it associates with the oocyte surface (LeMosy et al., 1998).

It is also known that egg activation results in mRNA polyadenylation and translation of maternal transcripts (Driever and Nusslein-Volhard, 1988; Mahowald et al., 1983). It has been suggested that new protein synthesis may be required at a very early stage during egg activation

in *Drosophila* as it may be needed for the release from metaphase I arrest and the completion of meiosis (Lieberfarb et al., 1996). In agreement, it had been shown in several other animal oocytes, such as *Xenopus* and starfish, that translation is required for the meiotic divisions (Gerhart et al., 1984 ; Picard et al., 1985) . However, Page and Orr-Weaver (1997) reported that *Drosophila* oocytes do not require new protein synthesis to complete meiosis or to maintain the metaphase I arrest. New protein synthesis is required for proper chromatin recondensation following meiosis (Page and Orr-Weaver, 1997). In addition, egg activation also results in reorganization of the cytoskeleton. Theurkauf et al. (1992) have shown that long fibrous microtubules, visible by confocal microscopy, which are present in the cortex of non-activated oocytes are depolymerized upon egg activation so that in both mitotically dividing embryos and in unfertilized eggs, these cortical microtubules have cleared from the cytoplasm. The eggs then complete the two meiotic divisions independently of fertilization, the polar body nuclei fuse forming a rosette structure in the anterior-dorsal quadrant of the egg, and if fertilized, the fused pronuclei go on to begin syncytial mitotic divisions. The timing of these events is rapid. It has been shown that both meiosis and the first mitotic division are completed 17 minutes after egg activation is initiated (reviewed by Foe et al., 1993).

Two maternal effect mutations (*cortex* and *grauzone*) identified in a previous screen were shown to be non-degraders of maternal transcripts (Bashirullah et al., 1999). The cause of the defects, which results in stability of RNA is not known. However, these two mutations are known to affect several aspects of egg activation (Lieberfarb et al., 1996: Orr-Weaver, 1996). In particular, mutations in *cortex* and *grauzone* cause abnormal arrest in *Drosophila* female meiosis, defects in translation of maternal transcripts, and cytoskeletal reorganization (Lieberfarb et al., 1996; Page and Orr-Wever 1996). *grauzone* has recently been cloned, and is known to

encode a C2H2-type zinc finger transcription finger (with weak homology to cdc20 family members) which activates *cortex* transcription. *cortex* in turn, is required for the proper completion of meiosis (Chen et al., 1998; Chen et al., 2000).

Little is known about the regulation of maternal RNA degradation in *Drosophila*. Therefore, the major objective of my thesis work was to identify mutants defective in maternal transcript degradation in the early embryo.

Here I show that the four maternal-effect genes, *png*, *plu*, *gnu*, and the novel mutation, *temp*, are all required for maternal mRNA degradation in the early *Drosophila* embryo. *png*, *plu*, *gnu*, and *temp* embryos complete meiosis but are defective in the regulation of the S-M transition at the end of meiosis (Elfring et al., 1997; Fenger et al., 2000; Freeman and Glover, 1987; Freeman et al., 1986; Shamanski and Orr-Weaver, 1991;). This correlation between a DNA over-replication phenotype and failure to degrade maternal RNA represents a possible control point for maternal RNA degradation.

Materials And Methods

Drosophila Strains

Fly strains were supplied by the following sources : (1) 68 EMS-induced X chromosome maternal-effect mutations were generated in the laboratory of John Lucchesi and supplied by Beat Suter (McGill University). Mutant chromosomes contained *y w* and were balanced by FM6. Female homozygotes were identified as having white eyes. To collect embryos which were homozygous for the maternal-effect mutations, these white eyed females were mated to white eyed sterile males from the same line. From this X chromosome collection, four alleles of *png gu*, png^{172}, png^{246}, png^{50}, and png^{48}, and 7 alleles of *temprano*, $temp^{181}$, $temp^{248}$, $temp^{187}$, $temp^{39}$, $temp^{249}$, $temp^{231}$, and $temp^{40}$ were isolated.

The *giant nuclei* mutation (gnu^{305}) was isolated by Freeman et al. (1986) in a screen for third chromosome maternal-effect lethals, and was supplied by Terry Orr-Weaver (MIT). This was balanced over TM3. Only one allele of *gnu* has been isolated. The deletion $Df(3L)fz^{m21}$ / TM6(70D2-3;71E4-5) removes the region containing the wild-type *gnu* gene. This was obtained from the Bloomington stock centre. The *plutonium* mutations (plu^2, plu^3, and plu^4) were isolated and supplied by Terry Orr-Weaver (MIT). These three mutations were isolated in an EMS screen for plu^1 non-complementers. All three *plu* alleles were balanced over CyO chromosomes. The original plu^1 mutation was isolated from a maternal-effect mutant collection of Trudi Schupbach (1989).

The *cortex* (*cort*) allele, *QW55* and the *grauzone* (*grau*) allele *QQ36* were generated by Schupbach and Wieschaus (1989) in their EMS genetic screen for female sterile mutants on the second chromosome. These two lines were supplied by Trudi Schupbach . Homozygous females (white eyed due to the presence of *cn* and *bw* markers on the mutant chromosome) were

collected and used to set up cages to collect homozygous maternal-effect mutant embryos by mating these to sterile *cortex* and *grauzone* males.

The null *mei-41* (*D3*) allele was obtained from the Umea Drosophila Stock Centre (Umea, Sweden). The stock carrying this allele has the following genotype, C(1)DX, y[1] f[1] / w[1] mei-41 [D3].

The wild-type strains used were homozygous for mutations in the *yellow* (*y*) and *white* (*w*) genes.

Embryo Fixation And DNA Analyses

Fertilized wild-type and mutant embryos were collected and dechorionated in 50% bleach. For DNA analyses, *in situ* hybridization and immunostaining, embryos were fixed in formaldehyde or methanol, devitellinized, and rehydrated (Ding et al., 1993a; Ding et al., 1993b; Tautz and Pfeifle, 1989). Fixed embryos were stored in 100% methanol at 4°C. DNA was analyzed by staining embryos with DAPI (4', 6-diamidino-2-phenylindole) or Pico Green. DAPI (Boehringer Mannheim) was used at a concentration of 1µg/ml in 1 x PBS for 5 minutes in the dark. The embryos were then washed at least twice for 5-10 minutes in 1 x PTW (1XPBS, 0.1% Tween-20) and mounted in 50% glycerol in 1xPBS.

For Pico Green analyses, a stock concentration (Molecular probes, Oregon USA) was diluted 1/4000 in 1xPTW. Embryos were stained for 5 minutes in the dark. The stained embryos were then washed 3 X 15 minutes with 1 X PTW. The embryos were then cleared with 70% glycerol + 2.5% DABCO (anti-bleaching agent) and mounted.

Determination Of Defective Meiosis

Embryos were considered to have completed meiosis if the three (fertilized), or four (in the case of unfertilized eggs) unused meiotic products / polar bodies were condensed into the rosette structure which is observed after the completion of normal meiosis in wild-type eggs and embryos. This rosette structure is found on the anterior-dorsal surface of the embryo. In wild-type embryos, this structure persists in this location until the first few mitotic cell cycles are completed.

Maternal-Effect Screen For The Isolation Of Maternal RNA Degradation Mutants / Whole Mount RNA *in situ* Hybridization

To define components that regulate maternal RNA degradation and protection, a maternal effect screen was carried out. In this genetic screen, homozygous maternal-effect embryos that were 0-5 hours old were collected, fixed and probed for *Hsp83* RNA using whole mount RNA *in situ* hybridization. (These were EMS-induced X chromosome maternal-effect mutations generated in the laboratory of John Lucchesi and supplied by Beat Suter (McGill University)). This allowed for the identification of maternal effect mutants that were defective in maternal RNA degradation as this maternal transcript is normally degraded throughout the somatic region of the embryo by approximately 2.5 hours (1% of the RNA is protected and tightly localized to the posterior plasm / pole cells) (Bashirullah et al., 1999). Three categories of mutant phenotypes were deemed possible: (a) maternal-effect mutants which fail in *Hsp83* RNA degradation; (b) maternal-effect mutants which fail in *Hsp83* protection at the posterior plasm / pole cells (degradation throughout the egg or embryo) and (c) those mutants which show an abnormal RNA localization pattern. 49 maternal-effect lethal mutations on the X chromosome

were screened (see '*Drosophila* strains') for defects in maternal *Hsp83* mRNA degradation by whole mount *in situ* hybridization.

Digoxigenen-labeled antisense *Hsp83* RNA probe was synthesized using the Megascript RNA transcription kit (Ambion Inc.) and included digoxigenen-labeled UTP (Boehringer Mannheim). Digoxigenen probes were labeled by random priming of DNA synthesis according to instructions from the manufacturer (Boehringer Mannheim). The RNA probe was then hydrolyzed and precipitated (Bashirullah et al., 1999).

RNA *in situ* hybridization was based on the method of Tautz and pfeifle (1989) and Ding et al., (1993a, 1993b). Following hybridization, embryos were cleared in 50% glycerol (in 1 X PBS) followed by 70% glycerol (30% PBS), which was also used for mounting. Images were analyzed using a Zeiss Axioplan microscope and then photographed using a Spot cooled-CCD camera (Diagnostic Instruments Inc.).

Immunohistochemistry

For analyses of cortical microtubules, embryos (which were 0-3 hours old) were rehydrated with 1:1 Methanol / PT (1 X PBS + 0.1% Triton X-100), followed by two 10 minute washes with 100% PT. Embryos were then blocked with 10% BSA (Bovine Serum Albumin) (Sigma) for at least two hours at room temperature followed by incubation with a monoclonal mouse anti-α-tubulin antibody (NEN) at a dilution of 1/50 (diluted in a solution of 1% BSA in PT) in 2ml eppendorf tubes overnight at 4^0C. After the overnight incubation, embryos were washed 3 x 25 minutes on a nutator using 100% PT and then rinsed twice with PT. 300µl of non-preadsorbed goat anti-mouse secondary antibodies conjugated to Rhodamine-red (Jackson) were incubated with the embryos for at least two hours (preferably overnight incubation) at room temperature in

a solution containing 270µl PT, 30µl Normal Goat Serum (NGS) (Jackson Labs), and 1µl of the secondary antibody. After 4x20 minute washes with 100% PT, the embryos were cleared in 70% glycerol with 2.5% DABCO (1,4-Diazabicyclo[2, 2, 2] Octane) (SIGMA). If α-tubulin and DNA double staining were required, the embryos were incubated with a 1/4000 dilution of Pico Green (in PT) for 10 minutes directly after these four PT washes. Following PicoGreen incubation, embryos were washed 2 x 30 minutes in 100% PT. Embryos were mounted using DAKO mounting medium (DAKO Corporation, CA.) and analyzed using confocal microscopy.

For Bicoid immunostaining, the above protocol was used with the following modifications: the primary antibody was a rabbit anti-Bicoid polyclonal antibody (Driever and Nusslein-Volhard, 1988). This was used on formaldehyde fixed embryos at a 1/3 dilution. The secondary antibody was a goat-anti-rabbit antibody (Jackson) conjugated to HRP (Horse Radish Peroxidase). This was pre-absorbed by incubating the antibody with a 6hr-overnight collection of wild-type embryos at 4^0C and was subsequently used at a 1/300 dilution. Following the PT washes, Bicoid protein was detected using HRP staining reaction: 135µl of PT and 5µl DAB (3,3'-Diaminobenzidine) (SIGMA) were added to watch-glass wells containing the embryos. 0.5µl 3% H_20_2 was added to each well to begin the enzymatic reaction. Embryos were stained for the same amount of time and the reaction was closely monitored. The HRP staining reaction was terminated by the addition of PT to each of the wells. The embryos were then washed 3 x 10 minutes in PT, followed by one 10 minute wash in PBS. The embryos were then cleared in 1:1 Glycerol:PBS and mounted in 70% Glycerol in PBS.

Confocal Microscopy

For confocal analyses, a Leica TCS 4D confocal microscope was used. For double Pico green and tubulin analyses (Rhodamine red), identical images were scanned, captured and then processed as red (tubulin) and green (DNA) in Adobe Photoshop. This distinguished both images and allowed for the tubulin and DNA images to be superimposed.

Complementation Tests

Approximately ten 0-5 hour collections of fertilized line 181 embryos were collected and stained with DAPI . Each collection consisted of approximately 50 embryos. Most of the embryos had a giant nuclei phenotype similar to that of *pan gu*, *plu*, and *gnu*. Therefore, to test if this line harbored another allele of *pan gu*, maternal-effect line 181 heterozygous virgin females (181 / *FM6*) were crossed to the strong allele *pan gu*^{172} to test for sterility. However, 181 / *pan gu* ^{172} was fertile and thus not a *png* allele. Therefore, 181 is probably a novel mutant which may interact with and regulate the same processes as *pan gu*, *plu* and *gnu* as it also is defective in RNA degradation, early development, and early cell cycle regulation (various aspects of egg activation). Line 181 was subsequently placed into a complementation group of 8 alleles by Arash Bashirullah which he gave the name *temprano.*

To determine whether line 181 was an allele of *mei-41*, a complementation test between line 181 and a strong allele of *mei-41* (D3) was carried out. Heterozygous 181 virgin females (181 / *FM6*) were crossed to heterozygous *mei-41* males (white eyed due to the presence of *cn bw*), the *mei41*/181 progeny were collected (Bar^{+}, normal shaped white eyes) and tested to see if RNA degradation was defective in the embryos. All embryos in the 0-5 hour collection showed normal

degradation of *Hsp83* RNA. Therefore, line 181 and *mei41* complement, hence line 181 is not a *mei-41* allele.

Results

Identification Of Maternal - Effect Mutations Affecting RNA Degradation In Early Embryogenesis

By approximately 2.5-3.0 hours after fertilization, more than 96% of maternal *Hsp83* transcripts are degraded by the joint action of the maternal and zygotic RNA degradation pathways (Bashirullah et al., 1999). *Hsp83* RNA present after this time represents protected transcripts in the pole cells and zygotically expressed *Hsp83* RNA in the anterior of the embryo (Fig. 1a), which serves as an excellent internal control.

To identify genes required for maternal RNA degradation in the early embryo, X chromosome EMS generated maternal-effect mutants were screened for failure of *Hsp83* RNA degradation in 0-5 hour embryos (See "Materials and Methods"). Forty-nine maternal-effect lines were screened, yielding eleven mutants defective in maternal RNA degradation (lines 172, 246, 50, 48, 181, 39, 40, 187, 231, 248, and 249) (Fig. 1b-1k). All embryos in 0-5 hour collections failed to degrade *Hsp83* RNA throughout the entire embryo. This result suggests that not only is the maternal RNA degradation machinery inactive, but also that the zygotic degradation machinery may be inactive. Bashirullah et al.(1999) showed that the zygotically synthesized or activated degradation machinery becomes active 2 hours after fertilization and is sufficient for degradation of maternal transcripts by 4 hours after fertilization. It is possible that the same degradation machinery comprises both the maternal and zygotic pathways. Alternatively, the zygotic degradation machinery may never be activated because development may stall prior to the stage at which it is activated. These results from the maternal-effect screen clearly demonstrate that not only have several mutations been identified that prevent maternal RNA

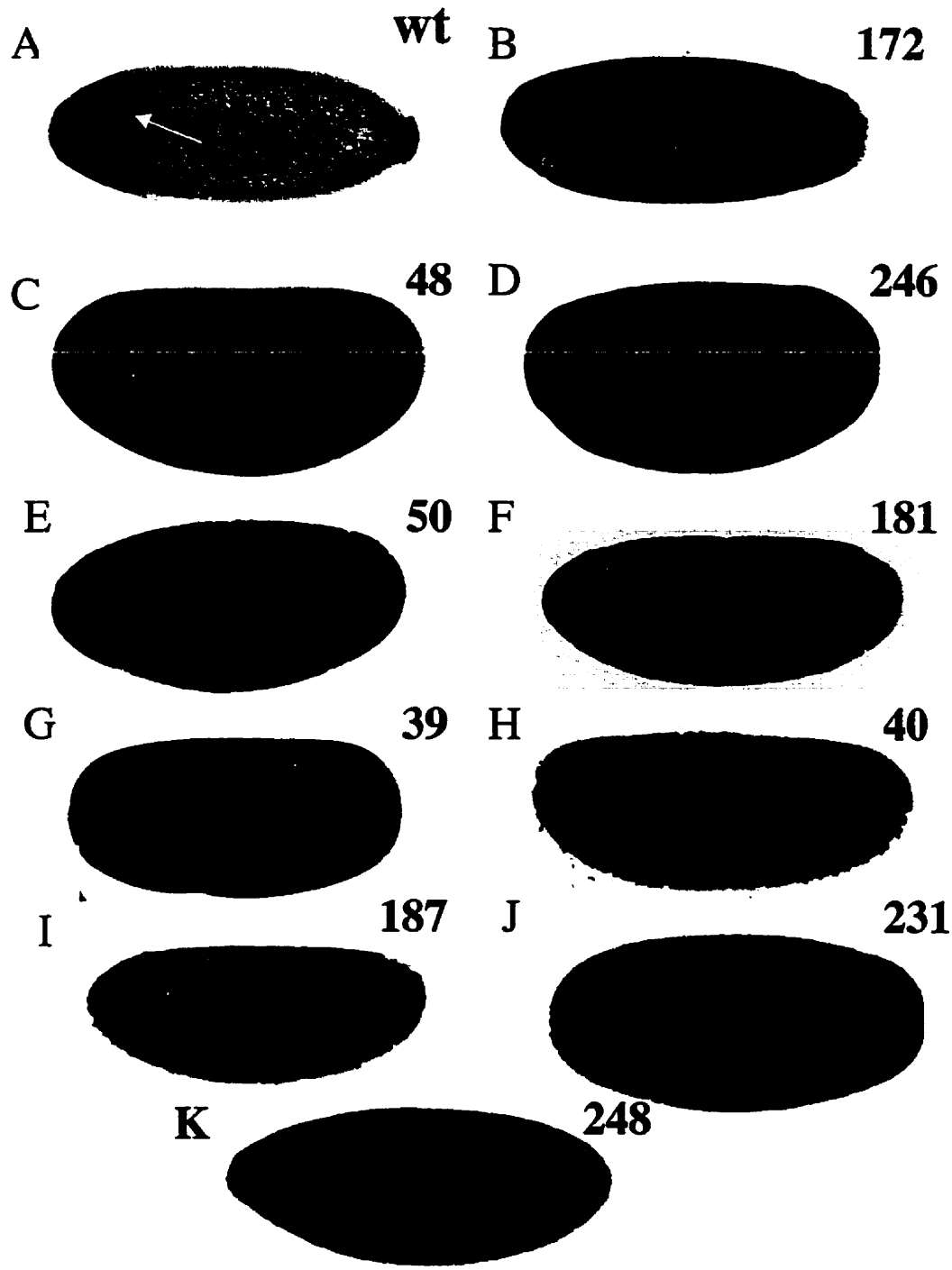


Fig 1. In stage 5 embryos (2.10-2.50 hours after fertilization), 96% of maternal *Hsp83* RNA is degraded (1A). *Hsp83* RNA present after this time represents protected maternal transcripts in the posterior plasm/pole cells (black arrow) and zygotically expressed RNA (yellow arrow). B-K represent X chromosome maternal-effect mutants defective in maternal mRNA degradation in the early embryo. Line 249 also failed to degrade maternal *Hsp83* RNA (data not shown). Anterior is to the left, dorsal is on top.

degradation, but also that the maternal-effect screen is an effective means to identify genes that may be involved in the process of maternal RNA degradation in the early *Drosophila* embryo.

Identification Of Complementation Groups

Lines 172, 246, 50, and 48 were identified by DNA analyses as phenotypically resembling the previously identified mutation ***pan gu*** (***png***) (Shamanski and Orr-Weaver, 1991)(see result section "Defective S-M transition"). Lines 172, 246, and 50 were confirmed to be ***png*** by Terry Orr-Weaver (M.I.T.; Personal communication), all of which failed to show any degradation of maternal mRNA as detected by whole mount *in situ* hybridization. Complementation analyses revealed that line 48 also belonged to the *png* complementation group.

Line 181 also produced a phenotype, when analyzed by DAPI, similar to that of *png* (see later). Therefore, in order to determine whether line 181 was an allele of *png* or a novel mutation also on the X chromosome, line 181 was crossed to both png^{172} and an X chromosome maternal-effect candidate, *mei-41*, which is required for proper mitosis and meiosis. Heterozygous 181 virgin females were crossed to heterozygous *mei-41* males from a strong allele of *mei-41* (*D3*). However, all embryos produced by a 0-5 hour collection of *mei-41*/line181 flies showed normal degradation of *Hsp83* RNA (Fig. 2). When line 181 was crossed to *png*, the cross produced fertile progeny as indicated by the presence of larvae in egg collections. These complementation tests confirmed that line 181 was neither *png* or *mei-41*. Therefore, the maternal RNA degradation mutant, line 181 may be a previously uncharacterized and novel mutation that affects maternal RNA degradation. Later it was confirmed that line 181 belongs to a

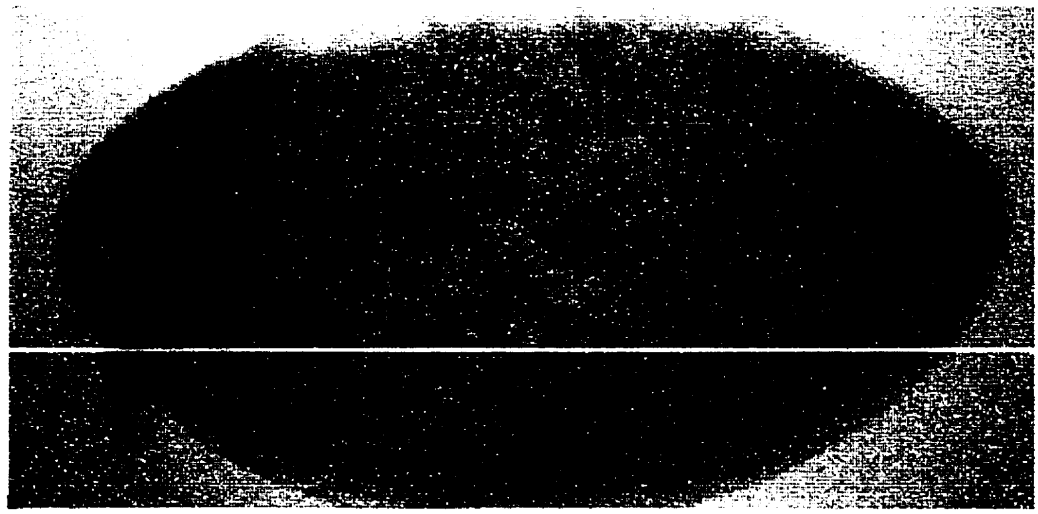


Figure 2. Line 181 is not an allele of *mei-41*.

Line 181 was crossed to *mei-41*. RNA *in situ* hybridization was performed on the progeny's embryos. Anterior is to the left, dorsal is on top.

All embryos in 0-5 hour collections showed normal *Hsp83* RNA degradation.

complementation group consisting of lines 181, 39, 40, 187, 231, 248, and 249. All seven alleles are defective in maternal RNA degradation by the maternal effect screen. Subsequently, the complementation group containing line181was named ***temprano*** (***temp***) (see table below).

Complementation Group	Lines in Complementation Group
Group 1/ *pan gu* (*png*)	48 (new allele), 50,172, 246
Group 2/ *temprano* (*temp*)	39, 40, 181, 187, 232, 248, 249

RNA Degradation Mutants Are Defective In Egg Activation And Early Development

Previously, the only two mutants known to stabilize maternal RNA, *cortex* and *grauzone* (Bashirullah et al., 1999), had also been shown to be defective in several aspects of egg activation, and as a result to stall in early development (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). Therefore the next goal was to confirm whether or not the eleven maternal mRNA degradation alleles identified here were also defective in early development (stalled at or prior to cellularization), or whether these mutants were able to cellularize normally. Cellularization occurs at about 2.5 hours after fertilization, at which stage the rapid S-M cell cycles have produced approximately 5000 nuclei (Foe and Alberts, 1983). Hence, any defect in early development, from the first mitotic division to cellularization, should be conspicuous from DNA analyses (Fig. 3). Therefore, embryos which were 0-5 hours old were collected from homozygous mutant females from the 11 maternal-effect lines that failed to degrade RNA. These were formaldehyde fixed, stained with the DNA markers DAPI and/or Pico Green, and analyzed as whole mounts.

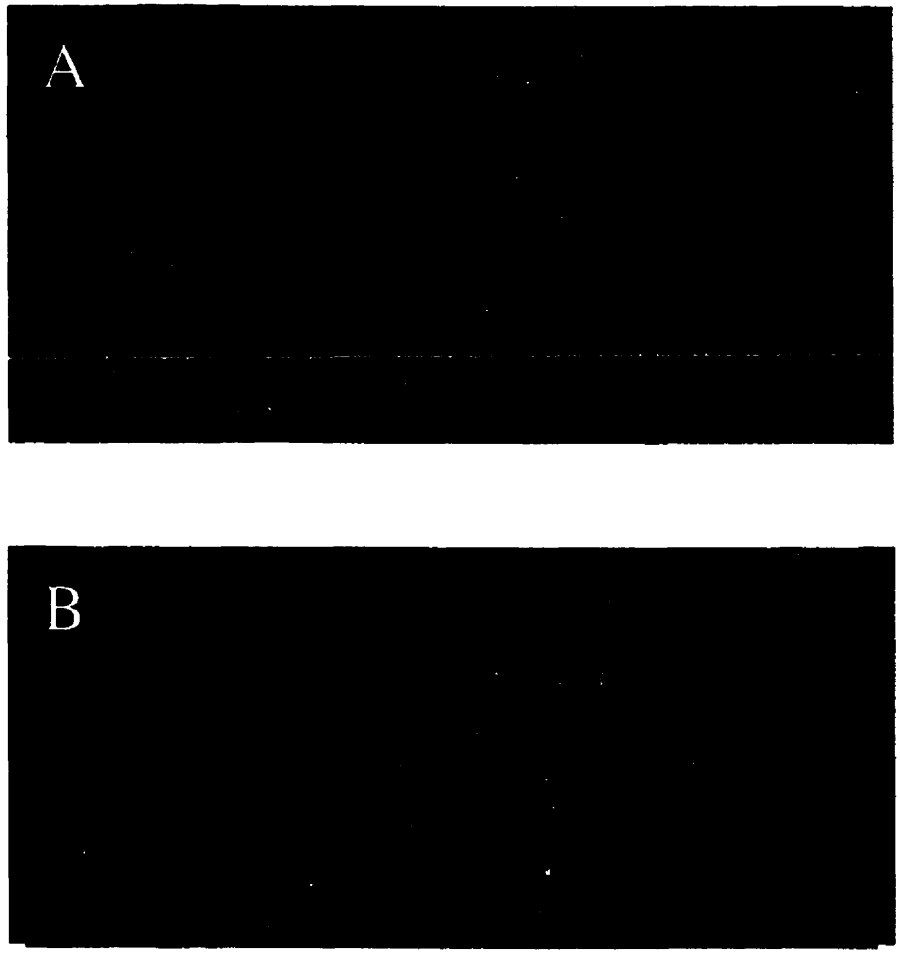


Figure 3. Nuclei of wild-type embryos.

Embryos were stained with DAPI. Anterior is shown to the left.

Once the S-M cell cycles are initiated approximately 3000 nuclei are produced by stage 13 which divide to produce 6000 nuclei by stage 14 (fig B).
Figure A shows a mitotically dividing embryo. This is an earlier stage embryo than B as seen from the lesser number of nuclei throughout the cytoplasm.

All eleven mutants found to be defective in maternal RNA degradation were also found to be defective in early embryonic development. Embryos from lines 172, 246, 50, and 48 often exhibited a phenotype in which the embryos were not as fully lengthened as wild-type embryos. Embryos were more rounded at the ends, diagnostic of a defect in egg activation. All of the RNA degradation mutants stalled very early in development, as they often appeared to enter, but arrest during the very early mitotic divisions. In some embryos, meiosis was not completed (See Later Results).

It was previously reported that ***png*** , and two other maternal-effect genes, ***plutonium*** (***plu***) (2^{nd} chromosome) which codes for a small ankyrin repeat protein (Axton et al., 1994) and ***giant nuclei(gnu)*** (3^{rd} chromosome), interact genetically and regulate the same processes. All three genes had been shown to have identical phenotypes (Shamanski and Orr-Weaver, 1991) (see Results S-M transition section). All *plu* and *gnu* alleles also fail to develop further than the first few mitotic divisions during the rapid S-M cell cycles during early embryogenesis (Freeman and Glover, 1987; Freeman et al., 1986 ; Shamanski and Orr-Weaver, 1991). Therefore, both mutants were tested for their ability to degrade maternal RNA using the same maternal-effect screen approach that had been used previously in the successful isolation and identification of *png* and *temp*. Like *png*, all the embryos from *plu*, and *gnu* in 0-5 hour collections failed to degrade *Hsp83* RNA (Fig. 4a and 4b). They are also both defective in early development and never progressed past the early few mitotic divisions (Freeman et al., 1986 ; Shamanski and Orr-Weaver, 1991). In addition, both strong and weak alleles of all RNA degradation mutants tested laid embryos that were defective in early development and displayed stabilization of maternal RNA in the early embryo as compared to wild-type embryos (Fig 4c). In summary, four loci

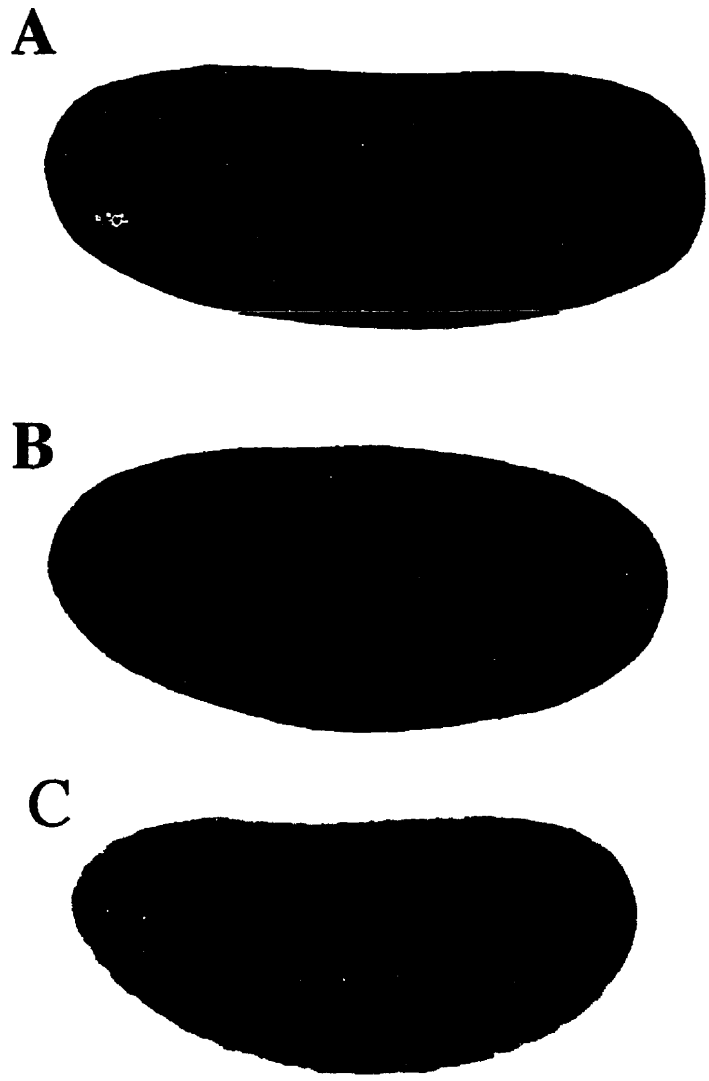


Figure 4. *Hsp83* RNA is not degraded in *giant nuclei* (*gnu*), *plutonium* (*plu*), and png^{50} embryos.

These embryos were stained with an *Hsp83* RNA probe. Anterior is shown to the left, dorsal is on top.
A/ In a 0-5 hour collection of *gnu* embryos, no degradation was observed.
B/ In a 0-5 hour collection of *plu* embryos, no degradation was observed.
C/ *Hsp83* RNA fails to degrade in png^{50} embryos in a 0-5 hr. collection.

have been identified that are necessary for maternal mRNA degradation , ***png***, ***plu***, ***gnu***, and ***temp***. All four are also defective in egg activation / early embryogenesis.

Failure To Degrade Maternal RNA Does Not Correlate With Depolymerization Of The Cortical Microtubules

Phenotypic characterization of each of the four RNA degradation mutants identified from the maternal-effect screen was carried out to look for various aspects of egg activation and early development that could be associated with the defect in maternal RNA degradation.

As mentioned, egg activation results in reorganization of the cytoskeleton. Theurkauf et al. (1992) reported that in non-activated oocytes (stage 14 oocytes) long fibrous microtubules, which are usually found to be copolymers of tubulin isotypes (Baker et al., 1990) located in the cortex of the egg, are depolymerized once the egg is activated irrespective of whether the egg has been fertilized or not. This results in clearing of the cortical microtubules from the cytoplasm. Hence, these microtubules can no longer be detected in wild-type eggs and embryos by immunostaining once they are laid (Page and Orr-Weaver, 1996; Theurkauf et al., 1992).

In the egg activation and RNA degradation mutants, *cortex* and *grauzone*, the cortical microtubule network fails to disassemble, and therefore laid fertilized and unfertilized eggs both resemble a non-activated stage 14 oocyte (Lieberfarb et al., 1996 ; Page and Orr-Weaver, 1996).

To determine possible microtubule reorganization defects in the four RNA degradation mutants *png*, *plu*, *gnu*, and *temp*, homozygous embryos from 0-3 hour collections of all 4 mutants were stained with anti α-tubulin antibody. A 0-3 hour collection of wild-type (yw) embryos were stained in conjunction with the RNA degradation mutants. These were then analyzed using confocal microscopy. As expected, in the wild-type embryos, no cortical microtubules could be

detected in any embryo from a 0-3 hour collection due to the successful activation of the egg as it passed from the ovary to the uterus, resulting in depolymerization of the dense cytoskeletal array (Fig. 5a). The fact that spindles, and the sperm tail could be clearly seen in these wild-type embryos was evidence that the immunostaining was successful (Fig. 5b). *plu*, *gnu*, and *temp* all behave like wild-type in that they all clear the cortical microtubules whenever the egg is laid suggesting that these three RNA degradation mutants are all normal in this aspect of egg activation (Fig. 6). However, *png* is clearly defective in this aspect of egg activation; the cortical region is full of microtubules in 0-3 hour embryo collections (Fig. 7) due to the failure of microtubule depolymerization in response to egg activation.

The anti α–tubulin results for *png*, *plu*, *gnu*, and *temp* together with those reported for *cortex* and *grauzone* (Lieberfarb et al., 1996 ; Page and Orr-Weaver, 1996) demonstrate that a failure to clear the cortical microtubules during egg activation does not necessarily correlate with stabilization of maternal RNAs in the early embryo. In three cases (*plu*, *gnu*, and *temp*) depolymerization is successful while in three (*png*, *cort*, and *grau*) depolymerization fails.

Defective Maternal RNA Degradation Does Not Correlate With Failure To Activate Translation Of Maternal mRNAs

Once egg activation occurs, translation of maternal transcripts is initiated (Mahowald et al., 1983). *bicoid* mRNA is an example of a maternal mRNA that is translationally silent following synthesis during oogenesis. However, *bicoid* translation is activated during early development when it participates in early embryogenesis. The protein normally begins to be produced during the first hour of embryogenesis from an anteriorly localized mRNA, generating

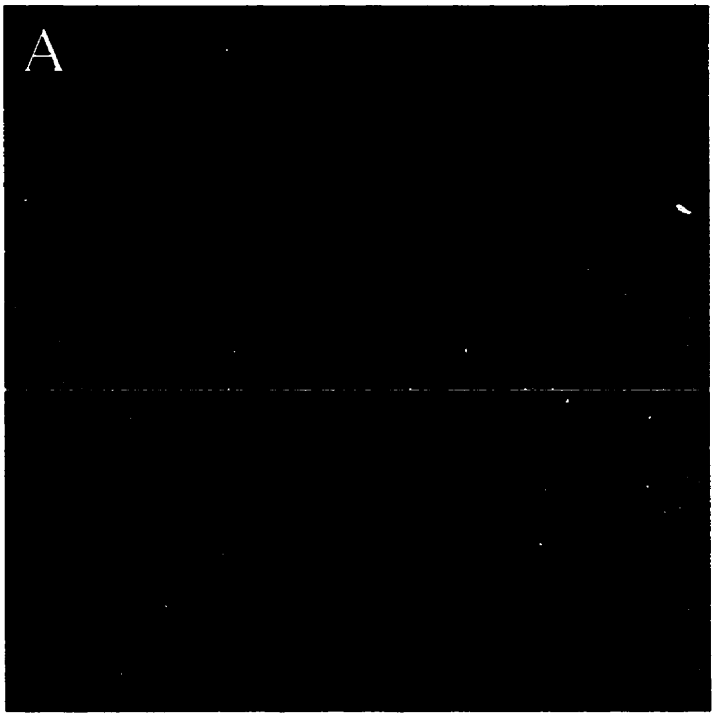
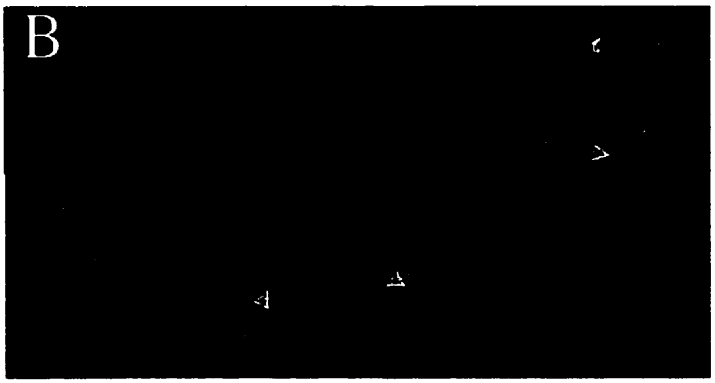


Figure 5. Wild-type anti-tubulin antibody staining. No cortical microtubules could be detected by immuno-staining in 0-3 hour collections of wild-type embryos (A). Cortical microtubules are cleared once the embryo is activated.



High magnification (B) of the cortical microtubules in an unactivated egg (yellow arrows).

Spindles were clearly evident in wild-type embryos (C) indicating that the antibody staining had been successful.

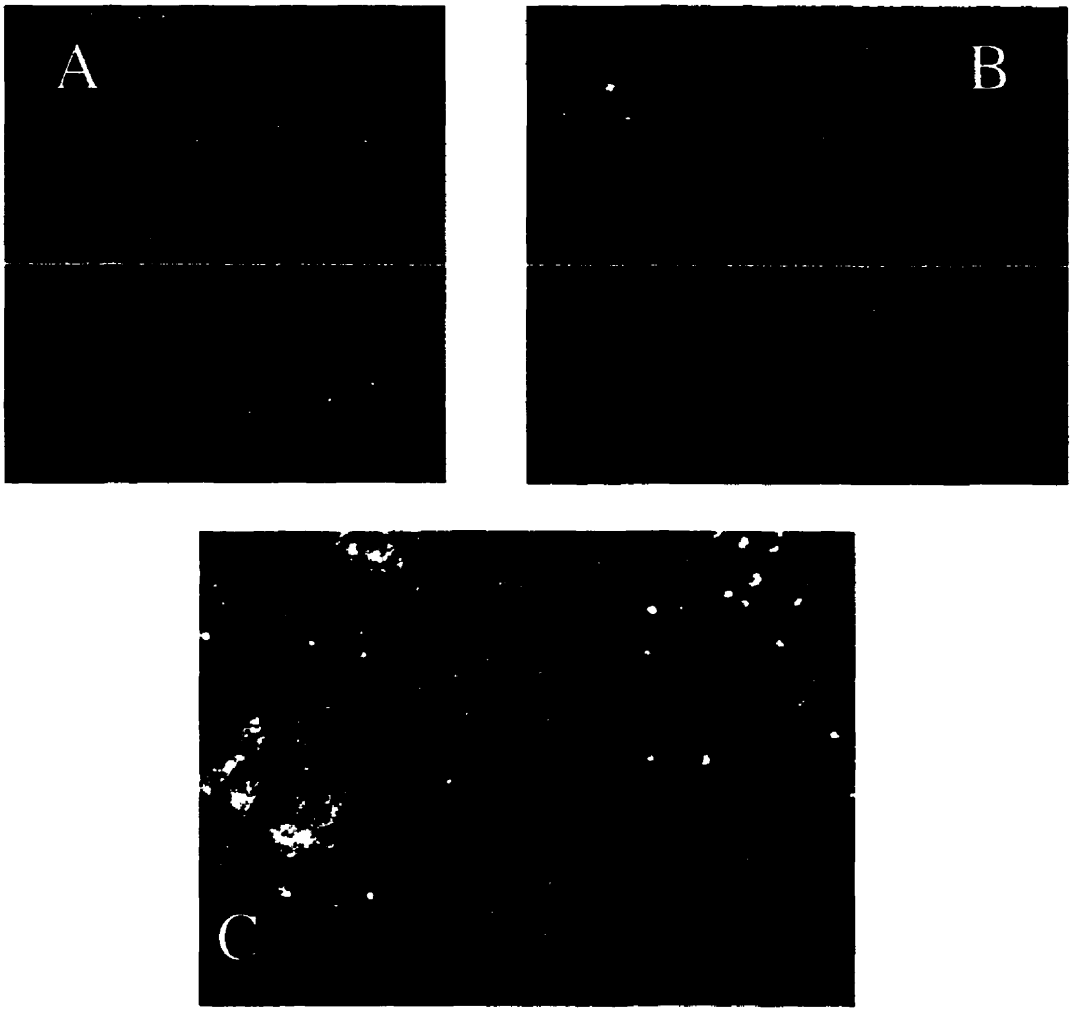


Figure 6. *temp*, *gnu*, and *plu* depolymerize the cortical microtubules upon egg activation. These embryos were stained using anti-α-tubulin antibodies. Homozygous *temp* embryos (6A), *gnu* embryos (6B), and *plu* embryos (6C) do not possess the dense cytoskeletal array characteristic of unactivated eggs.

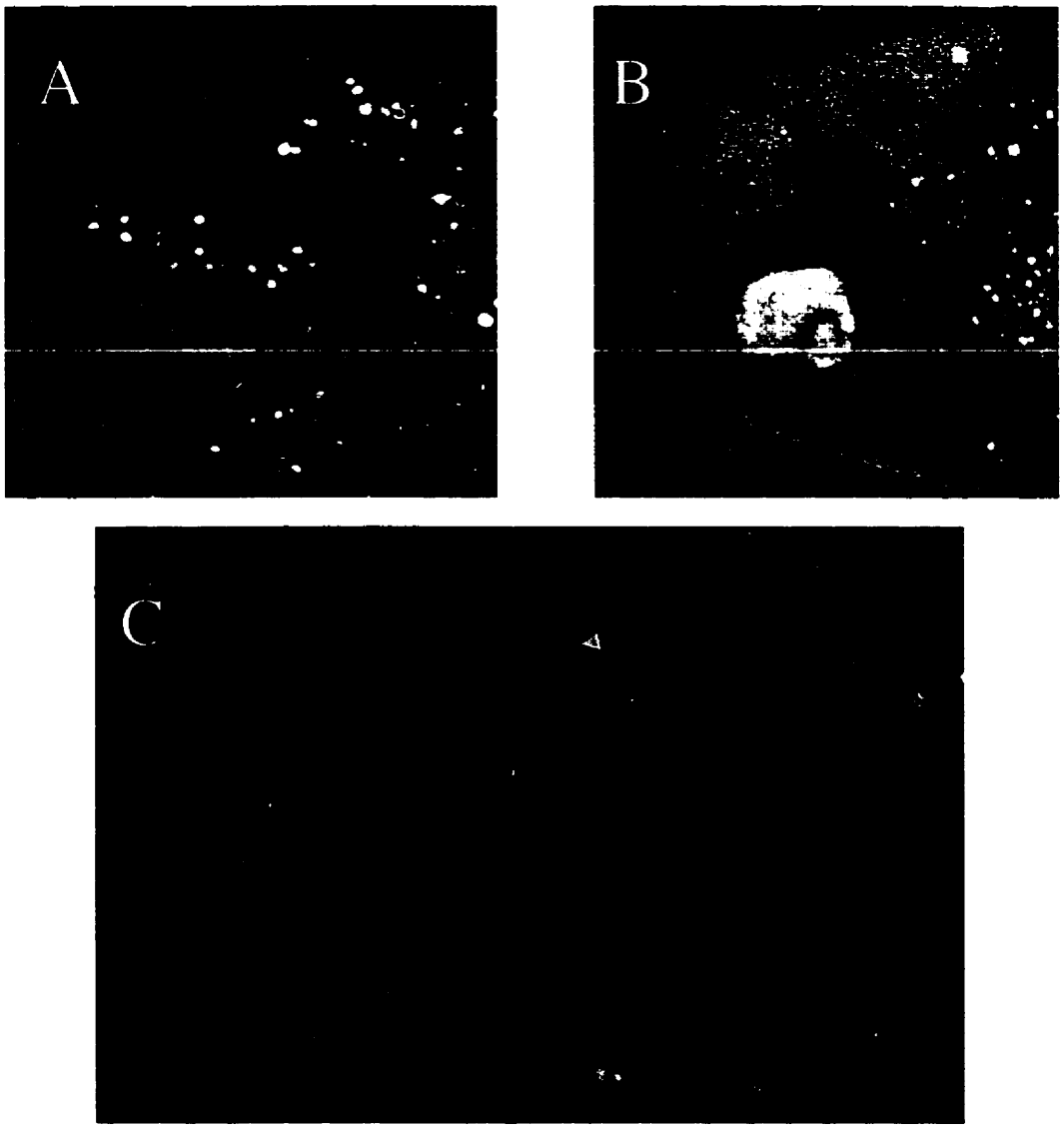


Figure 7. *png*^{172} fails to depolymerize the cortical microtubules upon egg activation. These embryos were stained with anti-α-tubulin antibodies. Anti-α-tubulin staining resulted in the detection of the dense cytoskeletal array characteristic of unactivated eggs / embryos. In figures 7A and 7B, the giant nuclei are clearly visible. The arrow in figure 7C shows the persistence of cortical microtubules after the embryo has been laid.

an anterior-to-poserior concentration gradient that guides head and thorax formation (Driever and Nusslein-Volhard, 1988). Bicoid protein thus serves as an excellent marker for the activation of maternal mRNA translational pathways during early embryogenesis.

All four RNA degradation mutants isolated by the maternal-effect screen were analyzed for Bicoid expression using 0-3 hour collections of mutant embryos that were homozygous for strong alleles of each mutation (with the possible exception of *temp*^{181}; strength of mutation undetermined). These were analyzed in conjunction with a wild-type positive control, and the two negative controls, *cortex* and *grauzone*. As expected, wild-type expressed a clear anterior-to-posterior gradient of Bicoid protein (Fig. 8a). *cortex* and *grauzone* were found to express no Bicoid protein (Fig. 8b and 8c) ; no staining above background levels was observed. This is in agreement with the results of Page and Orr-Weaver (1996). Unlike *cortex* and *grauzone*, *png*, *plu*, *gnu*, and *temp* all express Bicoid protein; anterior-to-posterior gradients were observed in all four mutants during the first three hours of embryogenesis (Fig. 9a-9d).

These results show that failure to activate the maternal mRNA translational machinery during egg activation does not correlate with the defect in maternal mRNA degradation; two mutants fail to activate translation (*cort* and *grau*), while four successfully activate translation (*png*, *plu*, *gnu*, and *temp*).

Failure To Complete Meiosis Does Not Correlate With Failure Of Maternal mRNA Degradation

Another important aspect of egg activation, failure of which could result in failure of degradation of maternal transcripts, is the completion of meiosis. Meiosis in *Drosophila* is a developmentally regulated process in which two rounds of chromosome segregation occur

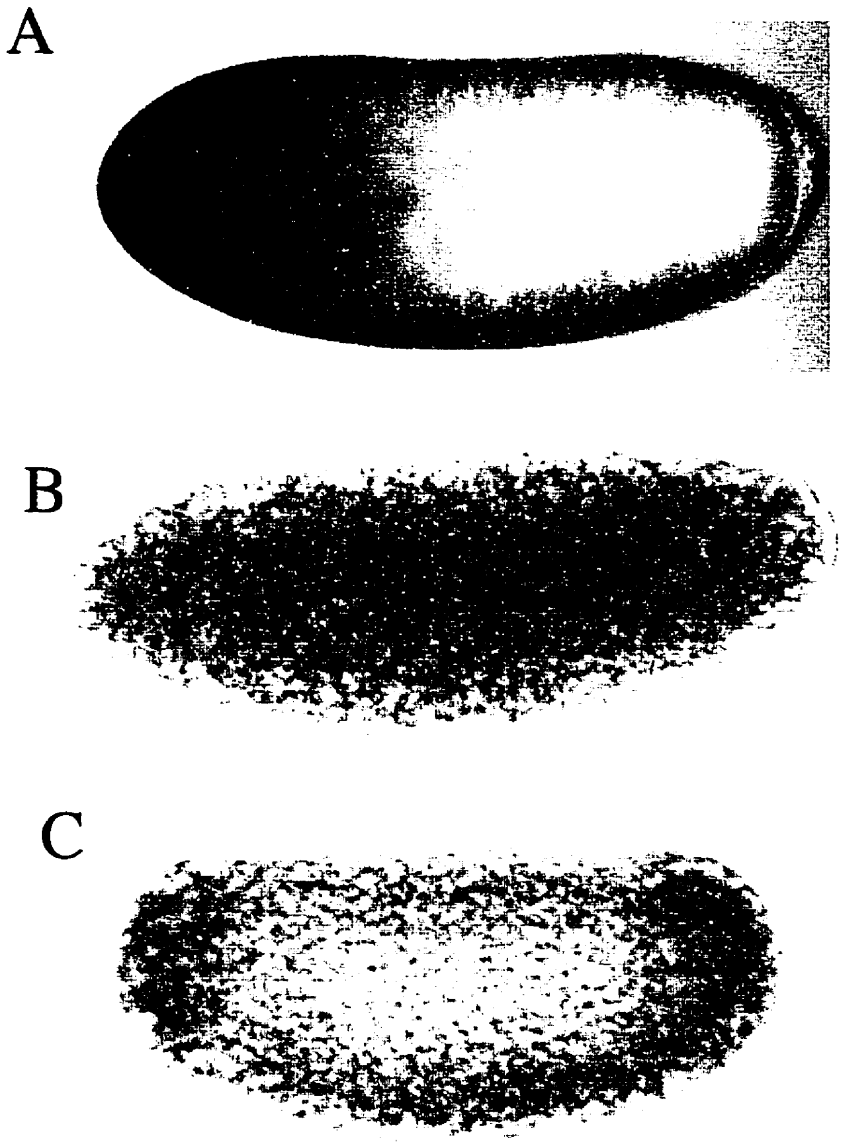


Figure 8. Bicoid protein is expressed in a clear anterior-to-posterior gradient in 0-3 hour wild-type embryos (8A).
Homozygous *cort*^{QW55} embryos (8B) and homozygous *grau*^{QE70} (8C) embryos show no Bicoid gradient. No staining above background levels was observed.

These embryos were stained with anti-Bicoid antibodies. Anterior is shown to the left, dorsal to the top.

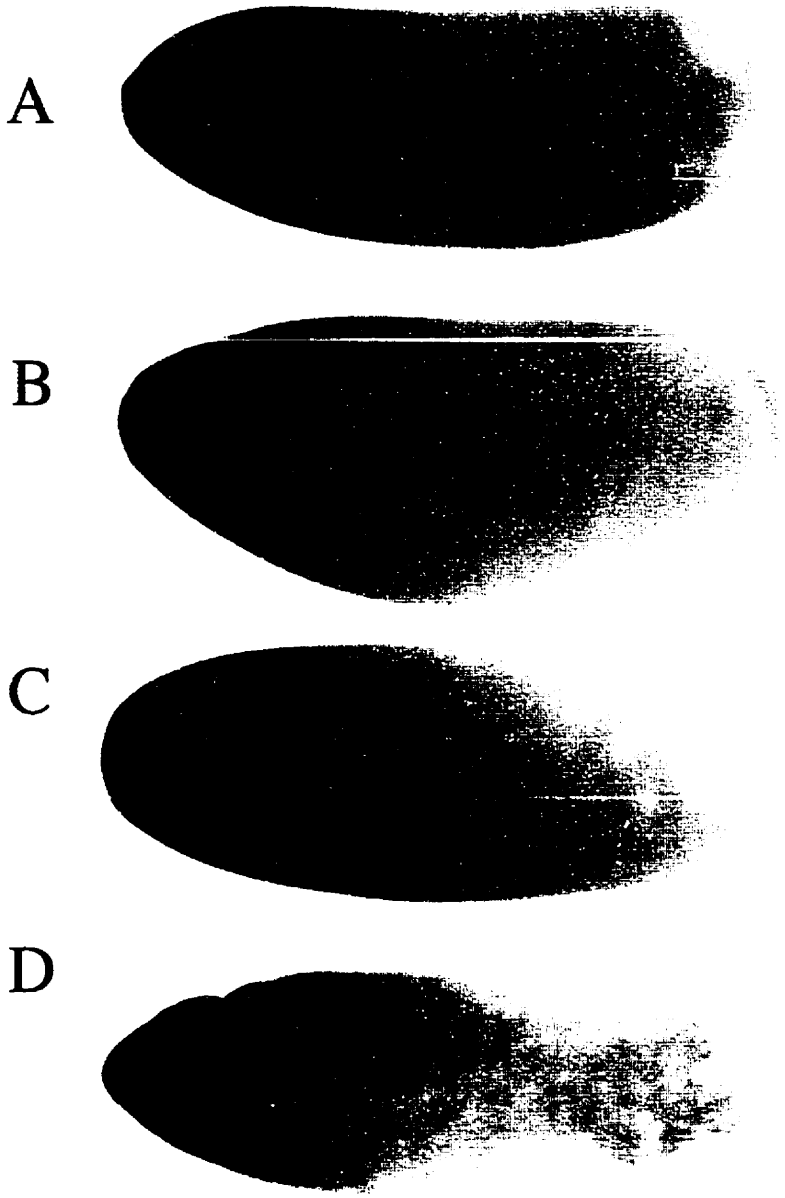


Figure 9. Bicoid protein is expressed in homozygous *png*, *plu*, *gnu* and *temp* embryos.
These embryos were stained with anti-Bicoid antibodies. Anterior is shown to the left , dorsal to the top.
Embryos from *pan gu* (A), *plu* (B), *gnu* (C), and *temp* (D) show obvious Bicoid protein expression in 0-3 hour collections.

without DNA replication. To determine whether completion of meiosis is required for the positive regulation of maternal RNA degradation during early embryogenesis, timed collections of homozygous embryos from each RNA degradation mutant were stained using the DNA markers DAPI and Pico Green and analyzed as whole mounts for defects in this early egg activation process.

The mature *Drosophila* oocyte is arrested in metaphase I of meiosis; this arrest is released by egg activation (Doane, 1960 ; Mahowald et al., 1983). There then follows a post-meiotic arrest where the three polar bodies, or if the egg is unfertilized, four unused meiotic products (polar bodies) condense in the anterior dorsal quadrant of the egg. These condensed chromosomes then form a rosette structure that persists in this region of the egg during the first several S-M cell cycles in the *Drosophila* syncytium. Therefore, the presence of the three (fertilized) or four (unfertilized) visible polar bodies is evidence that meiosis has been completed.

Previously it had been shown by Page and Orr-Weaver (1996) and Lieberfarb et al. (1996) that the two egg activation mutants *cortex* and *grauzone* (which are defective in maternal RNA degradation (Bashirullah et al., 1999)) were defective in female meiosis. They found that in *cortex* and *grauzone* eggs, chromosome segregation during the first meiotic division is unequal and meiosis was also found to arrest during meiotic metaphase II. Some embryos were found to enter into a defective anaphase II, however these embryos were never capable of completing meiosis.

Both DAPI and Pico Green analyses revealed that meiosis was completed in *pan gu* , *plutonium*, and *giant nuclei* (data not shown). This is in agreement with findings reported by Shamanski and Orr-Weaver (1991) and Freeman et al., (1986).

temprano differed from *png*, *plu*, and *gnu* in that completion of meiosis varies depending on the strength of the allele. Eggs/embryos from *temp*^{181} complete meiosis; a small fraction of embryos (approximately 10%) display the "*png* overreplication phenotype" (see below) (Fig. 10a and 10b). The majority of *temp*^{181} embryos (~90%) fail to complete meiosis (Fig. 10c and 10d). *temp*^{248} always stalls during meiosis as the three polar bodies in fertilized embryos (or the four polar bodies in the unfertilized egg) were never seen. Trans-heterozygote analyses of *temp*$^{181/248}$ resulted in a phenotype more similar to *temp*^{248} in that most embryos appeared to stall during meiosis as the three meiotic products / polar bodies were not seen in early stage embryos (Fig. 11). However, completion of meiosis may have occurred in a small percentage of the embryos (<10%) as signs of DNA over-replication were evident (similar to *png* phenotype) (Fig. 12). These results are consistent with the possibility that *temp*^{181} is a weaker allele than *temp*^{248}. These results, in conjunction with the previous findings for *png*, *plu*, *gnu*, *cortex* and *grauzone* (Freeman et al., 1986; Page and Orr-Weaver, 1996; Shamanski and Orr-Weaver, 1991) suggest that failure to complete meiosis during egg activation does not correlate with failure to degrade maternal mRNA; three mutants fail to complete meiosis (*cort*, *grau*, and *temp*), while the other three mutants complete meiosis (*png*, *plu*, and *gnu*).

Failure Of The S-M Transitions At The End Of Meiosis Correlates With Failure To Degrade Maternal RNA

In the activated oocyte, the four meiotic products decondense their chromosomes in telophase II. If fertilization is not achieved in wild-type eggs, then the four polar bodies recondense their chromosomes (Doane, 1960), and they remain in this condensed state. If, on the other hand,

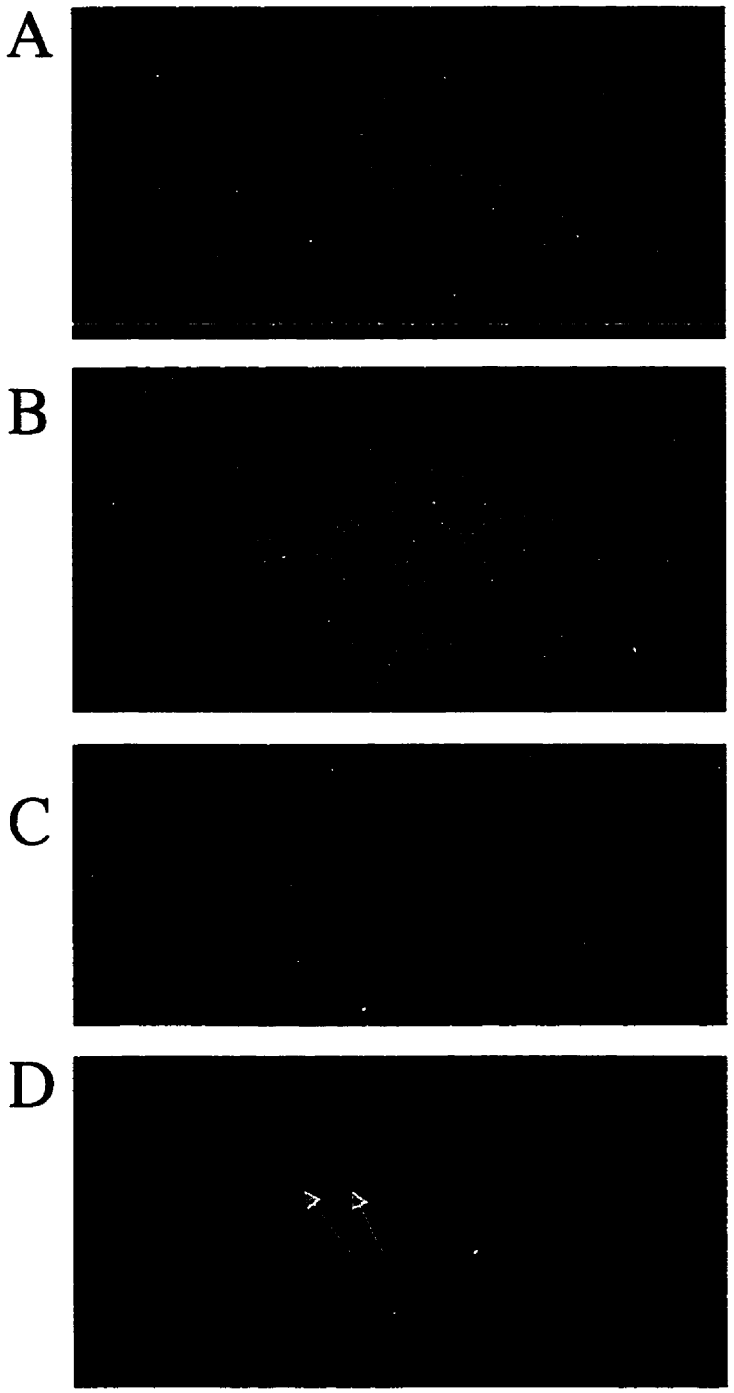


Figure 10. DNA staining of *temp*^{181} and *temp*^{248}.
temp^{181} embryos sometimes possess giant nuclei similar to *png*, *plu*, and *gnu* (A and B). However, the majority of *temp*^{181} fail to complete meiosis (D) resulting in a fragmented chromosomal phenotype (yellow arrows), in which the chromosomes are not condensed to form the meiotic rosette. *temp*^{248} always stalls during meiosis (C).

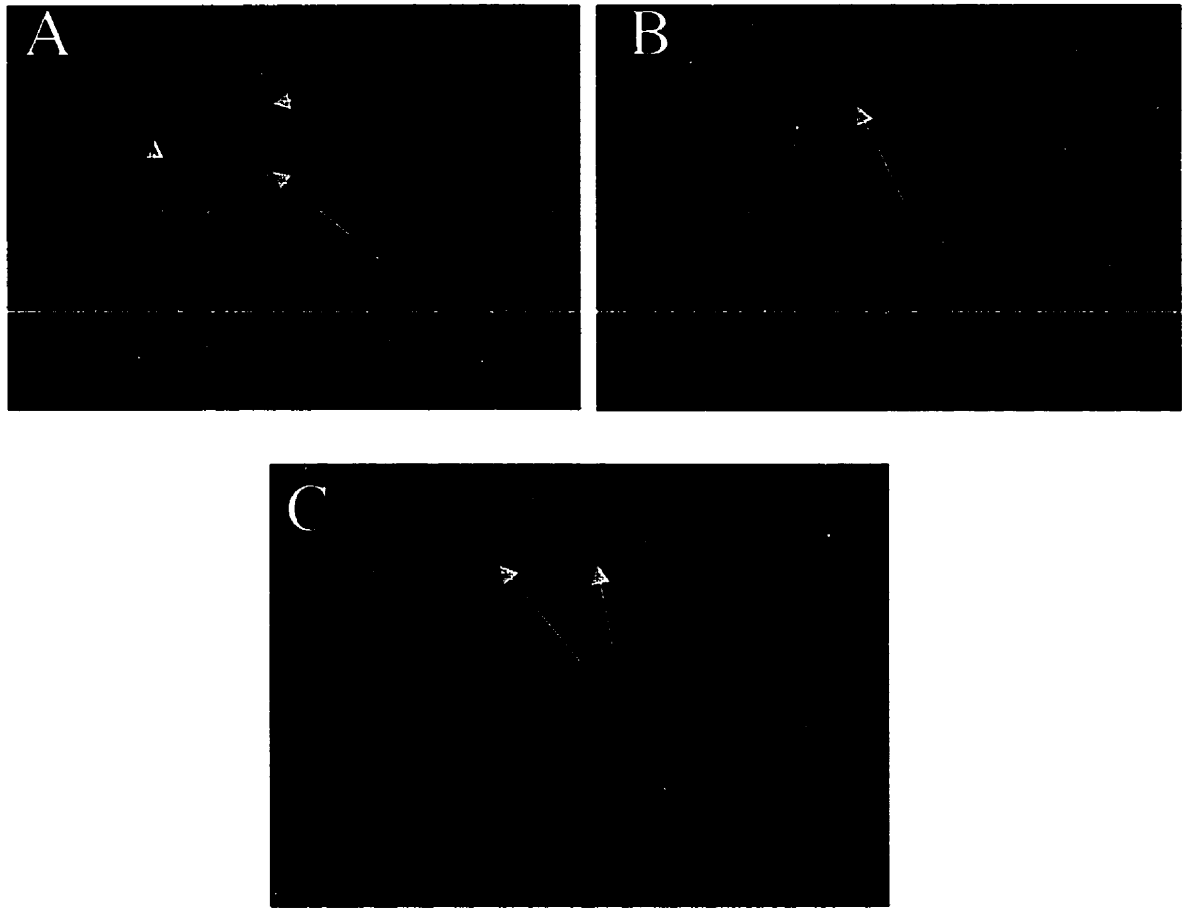


Figure 11. Meiosis is not completed in the majority of *temp*$^{181/248}$ trans-heterozygotes.

These embryos were stained with DAPI. Anterior is shown , with dorsal to the top.

Three examples of *temp*$^{181/248}$ embryos (A,B,C), stalled at different stages of meiosis.
This phenotype is very similar to *temp*^{248} in that most embryos stalled during meiosis as the three meiotic products/polar bodies are never seen to condense into the meiotic rosette structure characteristic of successful meiotic completion. Chromosomes appear fragmented (yellow arrows).

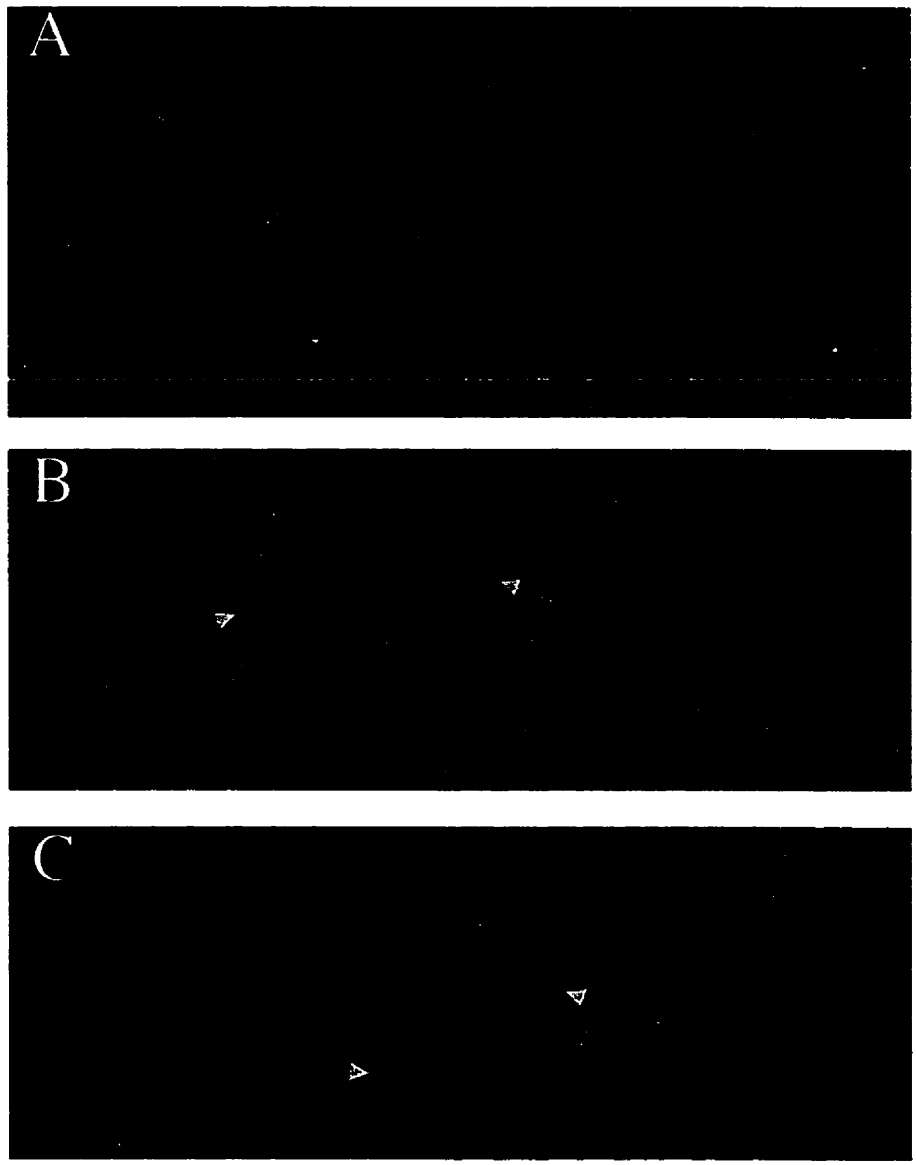


Figure 12. Giant nuclei are observed in a fraction of *temp*$^{181/248}$ trans-heterozygotes.

Embryos were stained with DAPI. Anterior is to the left , and dorsal to the top.

Completion of meiosis may have occurred in a small percentage (<10%) of embryos as signs of DNA replication were evident (similar to *png*). In both B and C, diffused DNA staining can be seen (yellow arrows) which may be either giant nuclei due to defective regulation of the S-M transitions / failure in chromosome condensation.
In A, DNA replication and mitosis may not be properly coordinated.

fertilization occurs, the four meiotic products enter interphase, and the male pronucleus also decondenses. The three polar bodies then recondense their chromosomes, whereas the male and female pronuclei undergo mitosis and DNA replication begins (Foe and Alberts, 1983). Failure to undergo chromosome condensation (S-M transitions) results in unregulated over-replication of the DNA in the activated oocytes and early embryos (Shamanski and Orr-Weaver, 1991). To determine whether there is a correlation between failure to undergo the S-M transitions and failure of RNA degradation, homozygous embryos were collected from *png*, *plu*, *gnu*, and *temp* lines, fixed, and analyzed using the DNA markers DAPI and Pico Green.

It was observed that all alleles of *png*, *plu*, and *gnu* never possessed the multiple nuclei typical of the syncytial divisions (or S-M cell cycles), instead embryos appeared to possess 1-5 giant, possibly polyploid nuclei (Fig. 13a-13c). $temp^{181}$ also showed a similar giant nuclei phenotype when stained with DAPI. However, unlike *png*, *plu*, and *gnu* , only approximately 10% of $temp^{181}$ embryos possessed the giant nuclei phenotype in a 0-5 hour collection (Fig. 13d).
To confirm the non-degradation phenotype of each of the identified mutants, trans-heterozygote analyses using the same *in situ* hybridization protocol was performed. png^{172}/png^{50} (strong allele/weak allele) and png^{246}/png^{172} (strong allele/strong allele) trans-heterozygotes were both sterile and produced embryos that failed to degrade *Hsp83* RNA in 0-5 hour embryo collections. plu^{3}/plu^{2} (strong allele/strong allele), $gnu^{305}/Df(3L)fz^{m21}$(70D2-3;71E4-5) (strong/strong), and $temp^{181}$/ $temp^{248}$ (weak/strong - see above) also were sterile and failed to degrade maternal RNA in 0-5 hour collections (Figs. 14 and 15). These confirm that the defective RNA degradation phenotype in all 4 mutants identified from the maternal-effect screen is due to the mutations at each of the loci, ruling out the possibility that failure to degrade RNA is due to genetic background. To confirm the correlation between the giant nuclei phenotype and maternal

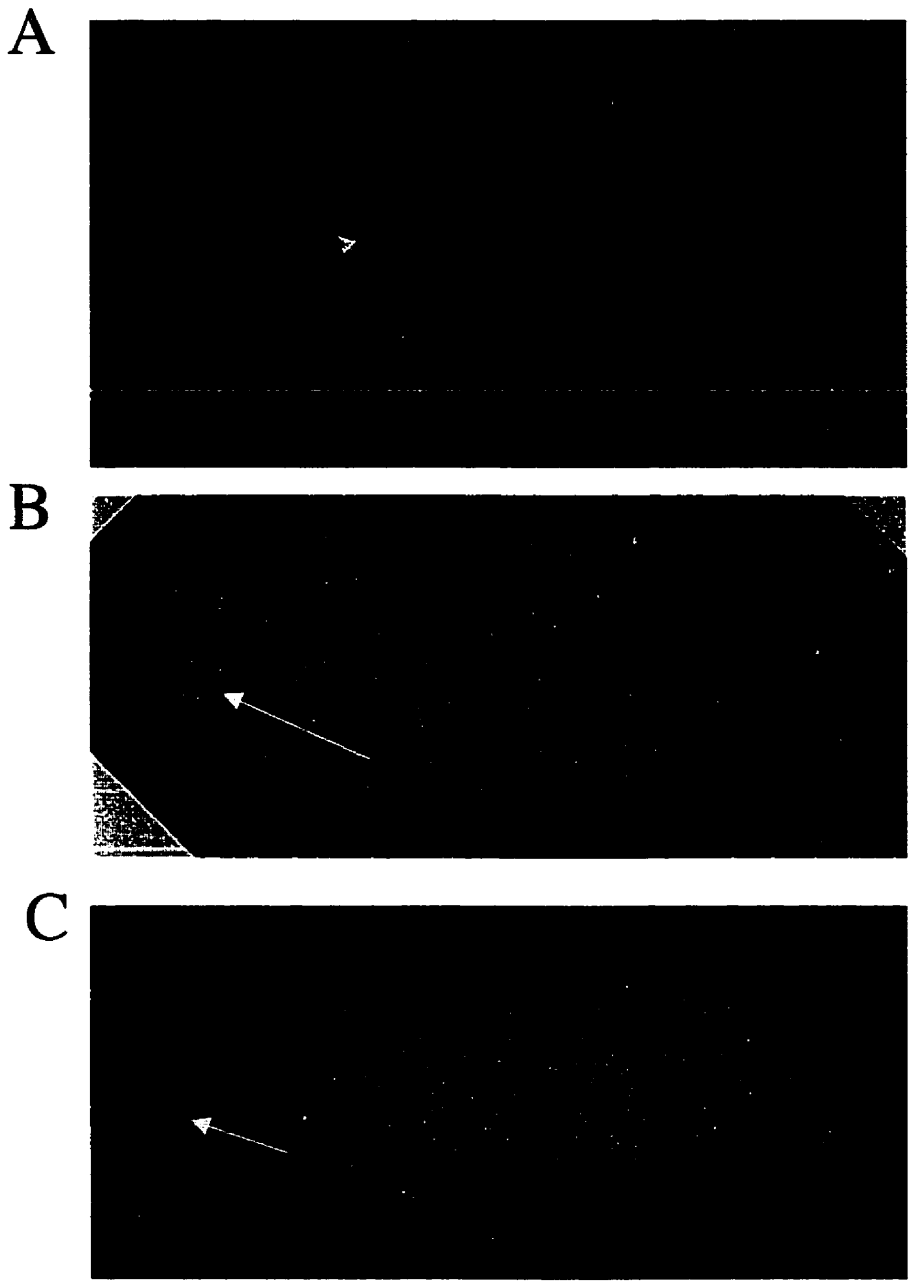


Figure 13A-C. *png*, *plu*, and *gnu* embryos possess 1-16 giant polyploid nuclei.
These embryos were stained with the DNA marker , DAPI. Anterior is to the left , dorsal to the top.
A/ *png* embryos possess giant nuclei , due to inappropriate regulation of the early embryo S-M transitions (yellow arrow).
B/ *plu* embryos possess giant nuclei (yellow arrow).
C/ *gnu* embryos show the same rare phenotype of giant polyploid nuclei resulting from defective S-M cell cycle control (yellow arrow).

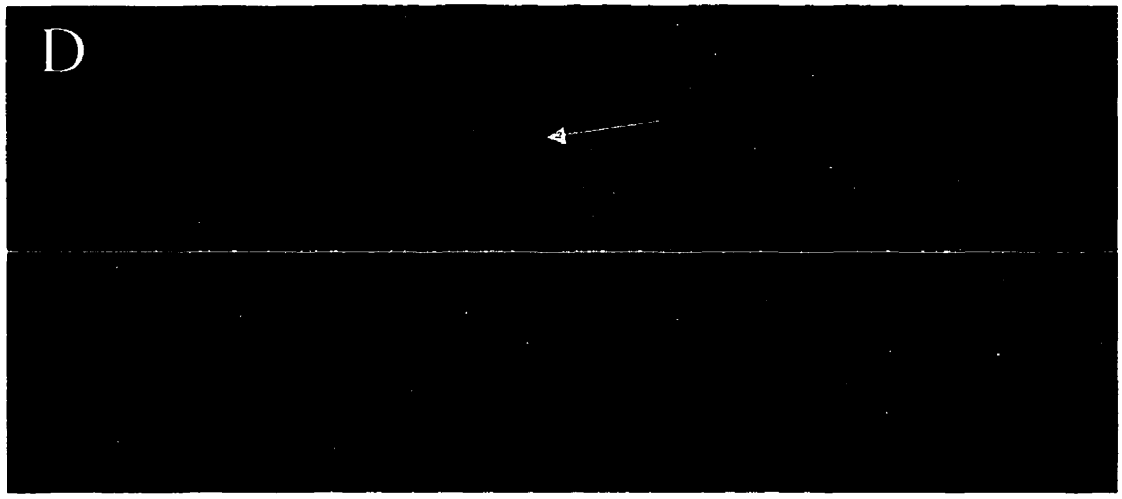


Figure 13D. *temp*^{181} embryos often show the giant nuclei phenotype.

These embryos were stained with the DNA marker DAPI. Anterior is to the left , and dorsal to the top.
temp^{181} also displayed the giant nuclei phenotype (yellow arrow). However, unlike *png*, *plu*, *and gnu* embryos, this was only observed in a minority of embryos.

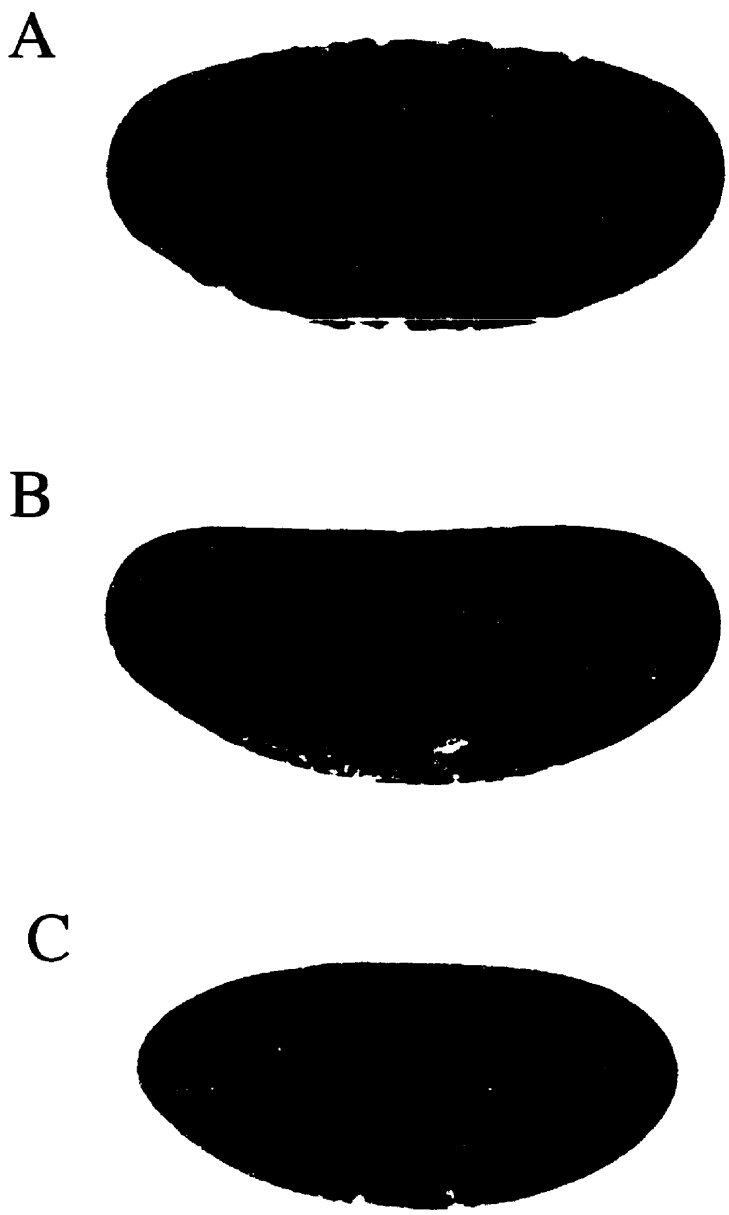


Figure 14. *Hsp83* RNA fails to degrade in *png* and *plu* trans-heterozygotes.
Embryos were probed with *Hsp83* RNA probe. Anterior is to the left, and dorsal to the top.
A/ png^{172}/png^{50} fails to degrade maternal RNA.
B/ png^{172}/png^{246} fails to degrade maternal *Hsp83* RNA.
C/ plu^{3}/plu^{2} fails to degrade maternal *Hsp83* RNA.
This data confirms that defective maternal mRNA degradation is due to the mutations at each of the loci identified from the screen.

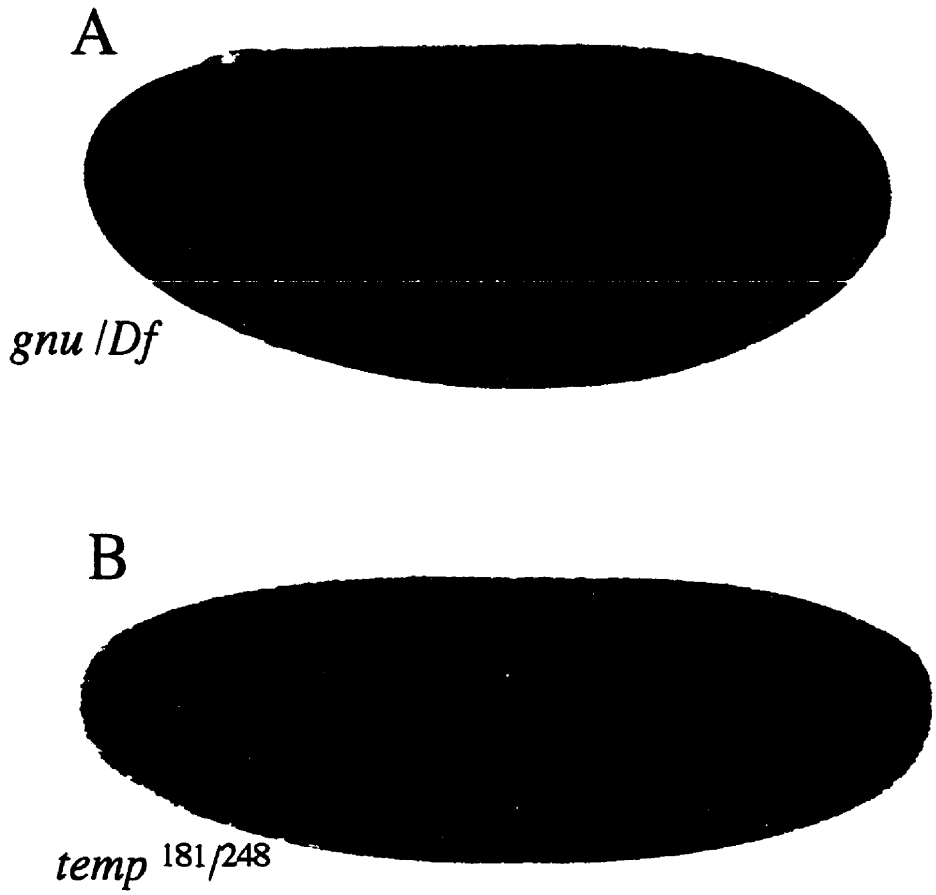


Figure 15. Maternal mRNA degradation is defective in *gnu* and *temp* trans-heterozygotes.

RNA *in situ* hybridization was performed on each of the trans-heterozygotes, using an *Hsp83* RNA probe. Anterior is to the left , and dorsal is to the top.

A/ *gnu*^{305}/deficiency results in stabilization of maternal *Hsp83* mRNA in the early *Drosophila* embryo.
B/ *temp* $^{181/248}$ trans-heterozygotes fail to degrade maternal *Hsp83* mRNA.

RNA degradation, the various transheterozygotes were also analyzed for the presence of the giant nuclei phenotypes. *png*^{172}/*png*^{50} (strong allele/weak allele), *png*^{246}/*png*^{172} (strong allele/strong allele), *plu*^{3}/*plu*^{2} (strong allele/strong allele), *gnu*^{305}/*Df(3L)fz*^{m21}, and *temp*^{181}/*temp*^{248} (weaker allele/stronger allele) all were shown to possess the giant nuclei phenotype (Figs. 16 and 17) as previously reported for the corresponding homozygotes (Freeman and Glover, 1987; Freeman et al., 1986 ; Shamanski and Orr-Weaver, 1991).

In conclusion, all four mutants identified from the maternal-effect screen that failed to degrade maternal RNA also show the giant nuclei overreplication phenotype suggesting a strong correlation between the S-M cell transition at the end of meiosis and the initiation of maternal RNA degradation (see Table 1 below). Embryos from *cort* and *grau* females could not be assayed for the S-M transition/over-replication defect since they do not complete meiosis.

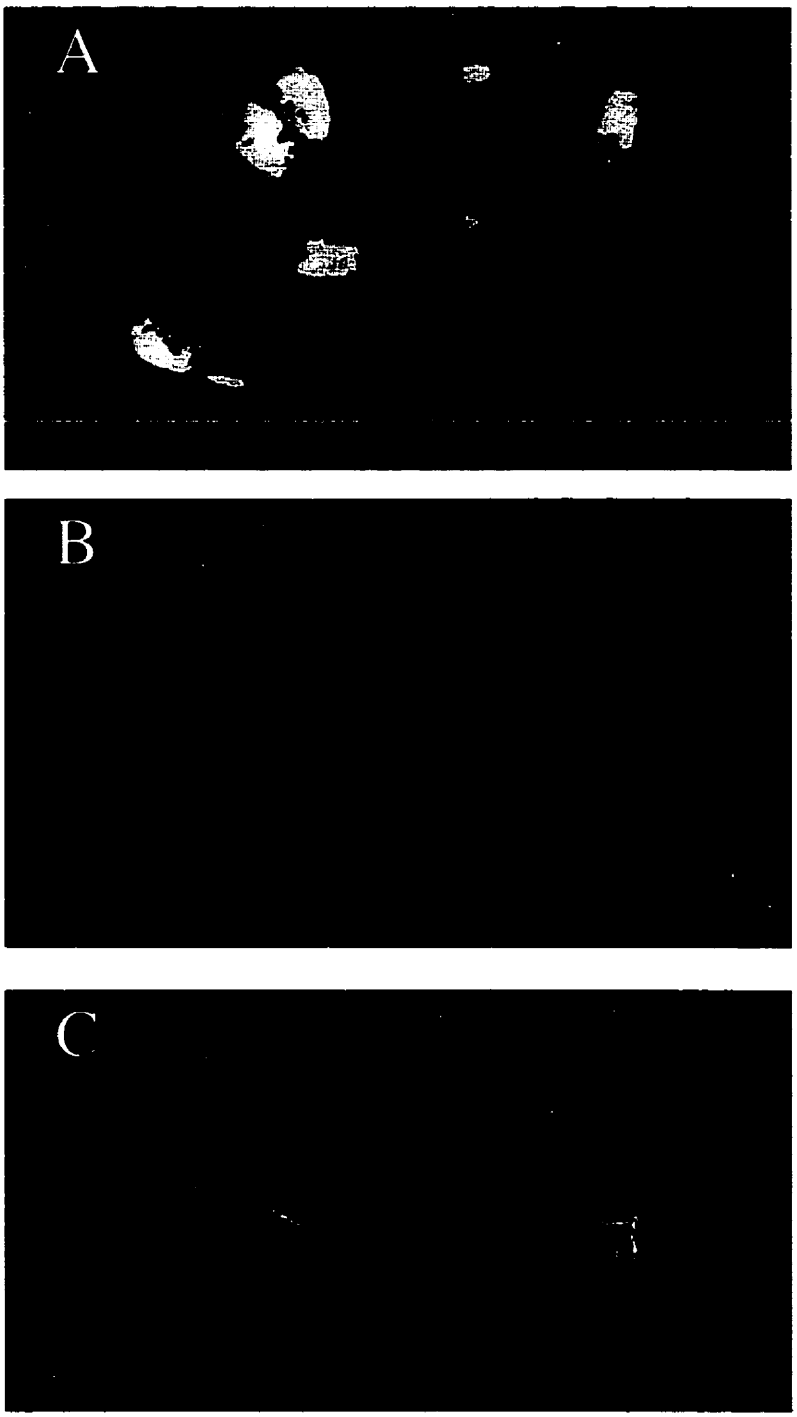


Figure 16. Confirmation of the giant nuclei phenotype in the various *png* and *plu* trans-heterozygotes.
0-5 hour collections of trans-heterozygote embryos were stained using DAPI. Anterior is to the left , and dorsal is on top. png^{172}/png^{50}(A), png^{172}/png^{246}(B), and plu^{3}/plu^{2}(C) embryos possess the giant polyploid nuclei phenotype as reported for the corresponding homozygotes.

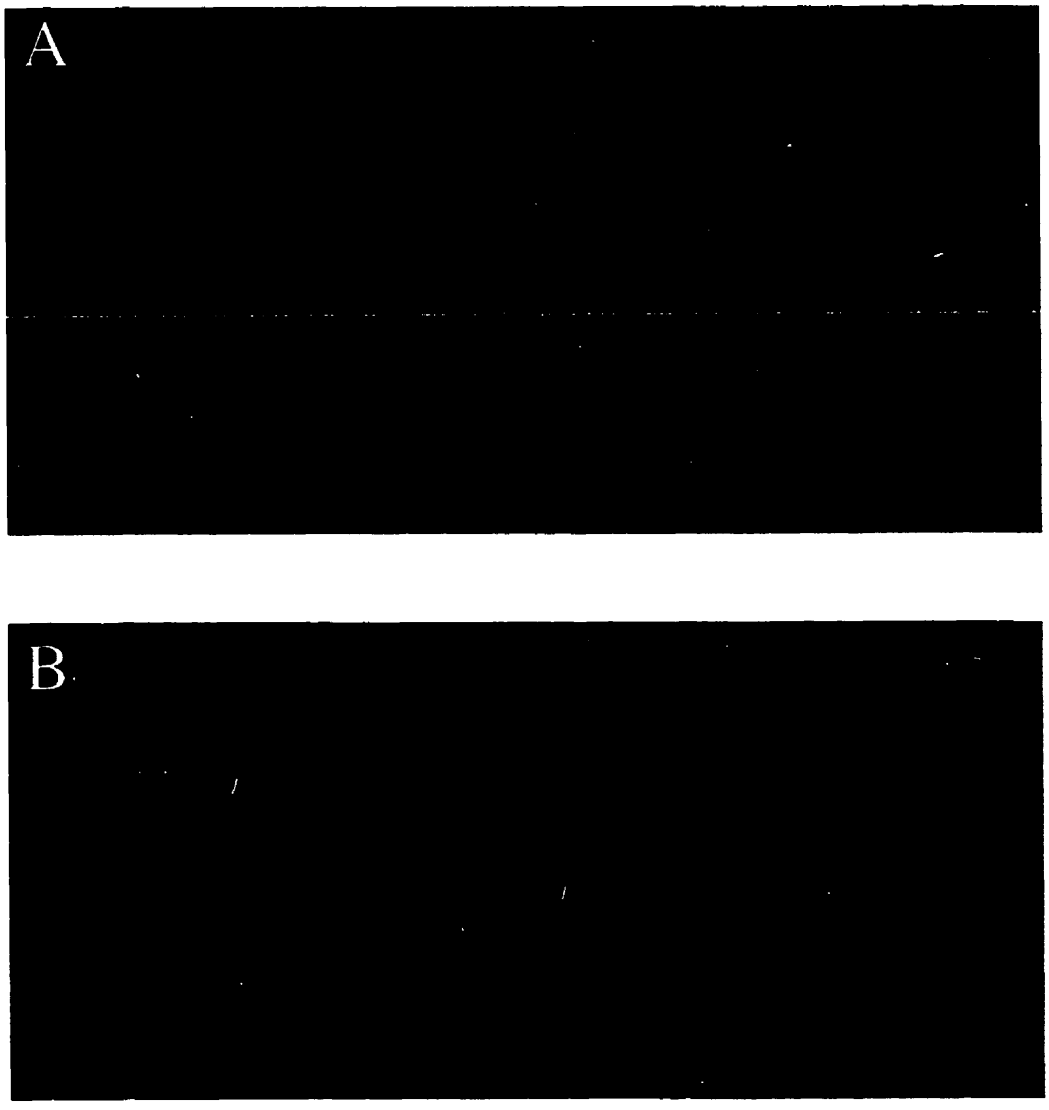
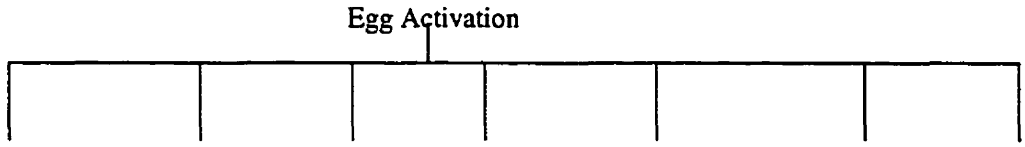


Figure 17. Confirmation of the defective regulation of the S-M cell cycles in the early *Drosophila* embryo of *gnu* and *temp* trans-heterozygotes.
Embryos were stained with DAPI. Anterior is to the left, and dorsal is at top.
gnu^{305}/*df* (A) fails to properly regulate the S-M transitions.
temp^{181}/*temp*^{248} (B) shows a similar phenotype to *temp*^{181} in that the minority of embryos appear to over-replicate DNA as seen above. However, whether these are actual giant nuclei or decondensed diffused chromatin remains to be determined.



	Rehydration And Cross-Linking of Vitelline Membrane	Meiosis Completion	poly(A) Status	Translation of maternal Transcripts	Depolymerization Of Cortical Microtubules	S-M Cell Cycle	Maternal mRNA Degradation
cort	+	-	-	-	-	?	-
grau	+	-	-	-	-	?	-
png	+	+	ND	+	-	-	-
plu	+	+	ND	+	+	-	-
gnu	+	+	ND	+	+	-	-
temp	+	-	ND	+	+	-	-
in vitro	+	+	ND	+	+	+	-

Table 1. All four maternal mRNA degradation mutants, *png*, *plu*, *gnu*, and *temp*, identified from the maternal-effect screen failed to properly regulate the S-M cell cycle. Embryos from *cort* and *grau* could not be assayed for the S-M transition defect because they fail to complete meiosis. *In vitro* activated eggs failed to degrade maternal mRNAs but showed normal S-M transition regulation (Ramona Cooperstock, unpublished Result).

Discussion

Four maternal-effect loci were identified, *png*, *plu*, *gnu*, and *temp*, which are necessary for maternal mRNA degradation in the early *Drosophila* embryo.

Both unfertilized eggs and embryos from homozygous females of all alleles tested, failed to degrade maternal transcripts. Similar to two previously identified egg activation mutants, *cortex* and *grauzone* (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996) which are also known to be defective in maternal mRNA degradation (Bashirullah et al., 1999), these four maternal mRNA degradation mutants (*png*, *plu*, *gnu*, and *temp*) also result in defects in various processes that are known to occur during egg activation and early embryogenesis. This suggests that maternal mRNA degradation in the early *Drosophila* embryo may be associated with or regulated by molecular genetic events known to occur during egg activation and early development.

Genes Involved In The Regulation Of Maternal mRNA Degradation In The Early Drosophila Embryo

Four loci, *png*, *plu*, *gnu*, and the novel mutation *temprano* were identified that were defective in maternal mRNA degradation in the early *Drosophila* embryo. While all embryos from the various RNA degradation mutants fail to show a normal reduction in RNA levels as assayed by *in situ* hybridization, it will be necessary to use Northern analyses to determine whether any RNA is actually degraded and whether the strength of each allele determines the amount of degradation.

After two hours it is known that the zygotic degradation machinery begins to function (Bashirullah et al., 1999), therefore a total failure to degrade RNA would indicate that

both the maternal and zygotic degradation machinery is defective in these mutants. Alternatively, this failure in degradation in 'post-two hour embryos' could be due to the fact that development stalls before the zygotic degradation pathway is activated. All four mutants are defective in aspects of egg activation and early development suggesting that the mutant gene products are not components of the degradation machinery *per se*. Rather, they are likely to be useful in the investigation of the relationship between specific aspects of egg activation and the regulation of maternal mRNA degradation.

Mutations In png, plu, gnu, And temp Disrupt Various Aspects Of Egg Activation

The vitelline membrane of activated eggs is cross-linked as the egg passes from the ovary to the uterus, and as a result, it is resistant to 50% bleach treatment (Mahowald et al., 1983). Eggs and embryos from *png*, *plu*, *gnu*, and *temprano* mutant eggs are all resistant to bleach treatment. Therefore, this early aspect of egg activation is normal in the four RNA degradation mutants.

In *Drosophila*, the egg is arrested at metaphase I prior to fertilization. This meiosis I arrest is released by egg activation. Following rehydration of the activated oocyte and crosslinking of the vitelline membrane, the meiotic divisions are completed in about 20 minutes after activation is initiated and are usually complete before the egg or embryo is laid (Riparbelli and Callaini, 1996). Shamanski and Orr-weaver (1991) and Freeman et al. (1986) showed by DAPI analyses that *png*, *plu*, and *gnu* all possess four separate small polyploid nuclei on the dorsal side of the egg at the position of polar bodies. This result is interpreted as evidence of meiosis being completed. This was confirmed here by DAPI analyses and Pico Green staining. Hence, the three RNA degradation mutants identified

from the maternal-effect screen develop further than the two previously identified RNA degradation mutants, *cortex* and *grauzone* that are known to stall early in egg activation during meiosis (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Bashirullah et al., 1999). As for *temprano*, weaker alleles such as $temp^{181}$ seem to complete meiosis whereas possible stronger alleles such as $temp^{248}$ do not. However, more detailed analyses using shorter time windows and more alleles (including the many alleles of *temprano* which still need to be looked at) are required to determine whether or not the polar bodies are condensed and actually maintained in the normal meiotic rosette structure prior to DNA replication in the weaker alleles. If meiosis is completed in the weaker alleles, then *temp* will also be useful for further analyses of the control of RNA degradation as the defect that results in RNA stabilization must occur downstream of the extremely early events that occur during egg activation and development. This will allow for examination of the more direct regulatory components in maternal RNA degradation.

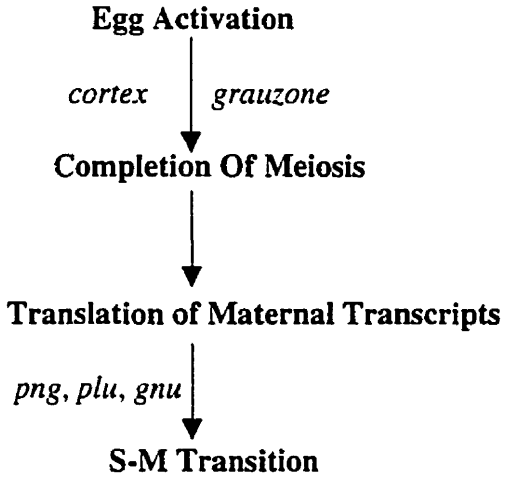
Egg activation also results in initiation of the maternal translational pathways that are required for establishing proper embryonic asymmetry (St Johnston and Nusslein-Volhard, 1992). Here translational activation has been assayed by immunostaining for Bicoid protein which is normally translated during the first hour of embryogenesis from an anteriorly localized mRNA, generating an anterior-to-posterior concentration gradient that guides head and thorax formation (Driever and Nusslein-Volhard, 1988). It is also known that for *bicoid* mRNA to be translated, poly(A) tail elongation is crucial (Salles et al., 1994). Previously, it had been reported that in 0-3 hour collections of *cortex* and *grauzone* embryos, Bicoid protein expression is reduced as a consequence of these mutants being defective in several aspects of early development including egg activation,

which adversely affects the translational pathway of certain maternal transcripts (Lieberfarb et al., 1996 ; Page and Orr-Weaver, 1996). However certain discrepancies arise in data from this previous work. Lieberfarb et al. (1996) found that in both homozygous and trans-heterozygous *cortex* embryos, 90% of embryos failed to express any detectable levels of Bicoid protein by immunostaining. The remaining 10% appeared to express Bicoid protein at levels that were estimated to be slightly higher than background levels. They also report that *grauzone* embryos show lower levels of Bicoid expression than wild-type, but slightly higher levels than *cortex* embryos. In contrast, Page and Orr-Weaver (1996) found that the Bicoid protein gradient was not detectable in both *cortex* and *grauzone* embryos. In addition, both sets of authors looked at the expression of Toll protein levels by Western blots in *cortex* and *grauzone* embryos, which is also a translationally regulated protein during early embryogenesis. *Toll* mRNA is cytoplasmically polyadenylated concomitant with protein production (Salles et al., 1994). Lieberfarb *et al.* (1996) reported that Toll levels are reduced compared to wild-type whereas Page and Orr-Weaver (1996) found that Toll levels are unaffected in four grauzone mutants and both cortex mutants. Finally, Page and Orr-Weaver (1996), in collaboration with B. Edgar (Fred Hutchinson Cancer Center), reported that the cell cycle regulator, String (which is translated *de novo* after egg activation) is translated at normal levels in both *cortex* and *grauzone* mutants. This finding argues that in these egg activation mutants, there is not a generalized defect in the activation of maternal translational pathways.

From the present analyses, Bicoid translation in *png*, *plu*, *gnu*, and *temp* was clearly evident. All four RNA degradation mutants expressed Bicoid protein at levels which

were comparable to wild-type. In agreement with Page and Orr-Weaver (1996), *cort* and *grau* failed to express detectable levels of Bicoid protein in the early embryo. One explanation is that the translational activation of maternal transcripts is not involved in directly regulating RNA degradation as there is no correlation between the two processes in the six maternal RNA degradation mutants (*png*, *plu*, *gnu*, *temp*, *cort*, and *grau*). However, given the fact that both *toll* and *bicoid* mRNA polyadenylation is a prerequisite to translation, and that translationally regulated transcripts are not translated in *cortex* and *grauzone* (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996) whereas they are in the four RNA degradation mutants, it may be concluded that polyadenylation rather than general maternal mRNA translation is not involved in the direct regulation of maternal mRNA degradation. These results predict that *png*, *plu*, *gnu*, and *temp* all carry out normal cytoplasmic polyadenylation unlike *cortex* and *grauzone* which are known to both be defective in the polyadenylation of a subset of maternal transcripts (Lieberfarb et al., 1996). This would agree with *cort* and *grau* being higher up than the four RNA degradation mutants in the egg activation pathway and its control of maternal mRNA degradation. However, Page and Orr-Weaver (1996) reported that *cort* and *grau* show normal Toll translation, which argues against a generalized defect in polyadenylation and translation of transcripts in the early *Drosophila* embryo. Thus, Bicoid translation may not be representative of all maternal mRNA translation in the egg and early embryo as this represents only one specific category of maternal transcripts; those that are translationally regulated by polyadenylation and that are involved in establishing embryonic asymmetry. This is in agreement with the finding that *cortex* and *grauzone* mutations do not block poly(A)-independent translational activation of nanos mRNA

(Lieberfarb et al., 1996). An alternative explanation which could explain these differences is that translational activation of maternal transcripts occurs upstream of *png*, *plu*, *gnu*, and *temp* but downstream of *cort* and *grau* in the same linear pathway. This would result in defective translation in *cort* and *grau*, but normal translation in *png*, *plu*, *gnu*, and *temp* as these act downstream of translation (see below).



The fact that *png*, *plu*, *gnu*, and *temp* translate *bicoid* does not rule out the possibility that maternal RNA translation may still have an indirect (or even direct) role in the regulation of maternal RNA degradation during the egg activation pathway, but does suggest that there is no correlation between translation of polyadenylated patterning transcripts and a failure to degrade maternal mRNAs. More detailed analyses of the activation of maternal translation in the early embryo will be required by investigating the expression of additional maternal transcripts, including those that are not translationally regulated by poly(A) tail elongation. This can be done by the combined use of immunostaining and more quantitative analyses using Westerns, as minor

differences in the amount of proteins such as Bicoid, may be difficult to detect by immunostaining alone.

png May Regulate Several Aspects Of Egg Activation

Depolymerization of the fibrous cortical microtubules occurs at egg activation, so that in both mitotically dividing embryos and in activated unfertilized eggs, the cortical microtubules have cleared from the cytoplasm (Page and Orr-Weaver, 1996; Theurkauf et al., 1992). In the egg activation mutants, *cortex* and *grauzone*, the cortical microtubules are defective, remaining in a pre-activated state in mutant eggs and embryos (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). Unlike *cortex* and *grauzone*, *plu*, *gnu*, and *temp* all behave like wild-type in that they all clear the cortical microtubules once the egg is laid. However, *png* is clearly defective in this aspect of egg activation. The phenotype is identical to *cortex* and *grauzone* in that the cortical region of the embryo is full of microtubules in a 0-3 hour collection. This is unexpected because PNG and PLU interact physically and are found as a complex required at the onset of embryogenesis. PLU may be a substrate of the PNG kinase. Alternatively, PLU may act as a regulatory subunit as PNG has been shown (by sequence comparisons) to lack a regulatory domain (Elfring et al., 1997; Fenger et al., 2000; Shamanski and Orr-Weaver, 1996). Also, PNG, PLU, and GNU regulate the same processes, as mutations in both *plu* and *gnu* dominantly enhance the phenotype of weaker *png* alleles by eliminating the transient S-M cycling (Shamanski and Orr-Weaver, 1991). This suggests that PNG may regulate specific aspects of egg activation in conjunction or as a complex with PLU / GNU, in agreement with Elfring et al. (1997) and Fenger et al. (2000), such as regulating the S-M transition following

meiosis (by stabilizing the levels of mitotic cyclins through PNG-dependent kinase activity (see later)) and controlling maternal mRNA degradation. This also suggests that PNG is involved in controlling other aspects of egg activation independently of *plu* and *gnu*, such as depolymerization of the cortical microtubules. Alternatively, it could be argued that this result is consistent with the findings that functional *png* is required for the presence of Plu protein and association of Plu protein with Png protein (Elfring et al., 1997; Fenger et al., 2000). Three strong mutants, png^{1058}, png^{158}, and png^{172} do not physically associate with PLU-myc (Fenger et al., 2000). Therefore *png* may be a key switch that acts just upstream of *plu*, and *gnu* in a linear egg activation pathway.

Relationship Of S-M Cell Cycle Regulation To Maternal mRNA Degradation

Mutations in the three maternal-effect genes, *png*, *plu*, and *gnu* result in unfertilized eggs that complete meiosis but do not arrest. Instead, the four unused meiotic products undergo extensive DNA replication, resulting in the production of a giant polyploid nucleus due to the fusion of polar bodies (Fenger et al., 2000; Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). If fertilization is achieved, *png*, *plu*, and *gnu* embryos undergo defective S-M cycles. In this case DNA replication occurs without mitosis, again resulting in giant polyploid nuclei (Fenger et al., 2000; Freeman et al., 1986; Shamanski and Orr-Weaver, 1991) similar to the case of unfertilized eggs. It has also been reported that the strength of the alleles affects the number of giant nuclei observed in the embryos (Shamanski and Orr-Weaver, 1991). Weaker alleles often possess as many as 16 giant nuclei whereas the stronger alleles usually contain 1-5 giant nuclei. Originally, this rare giant nuclei phenotype was believed to be due to either an

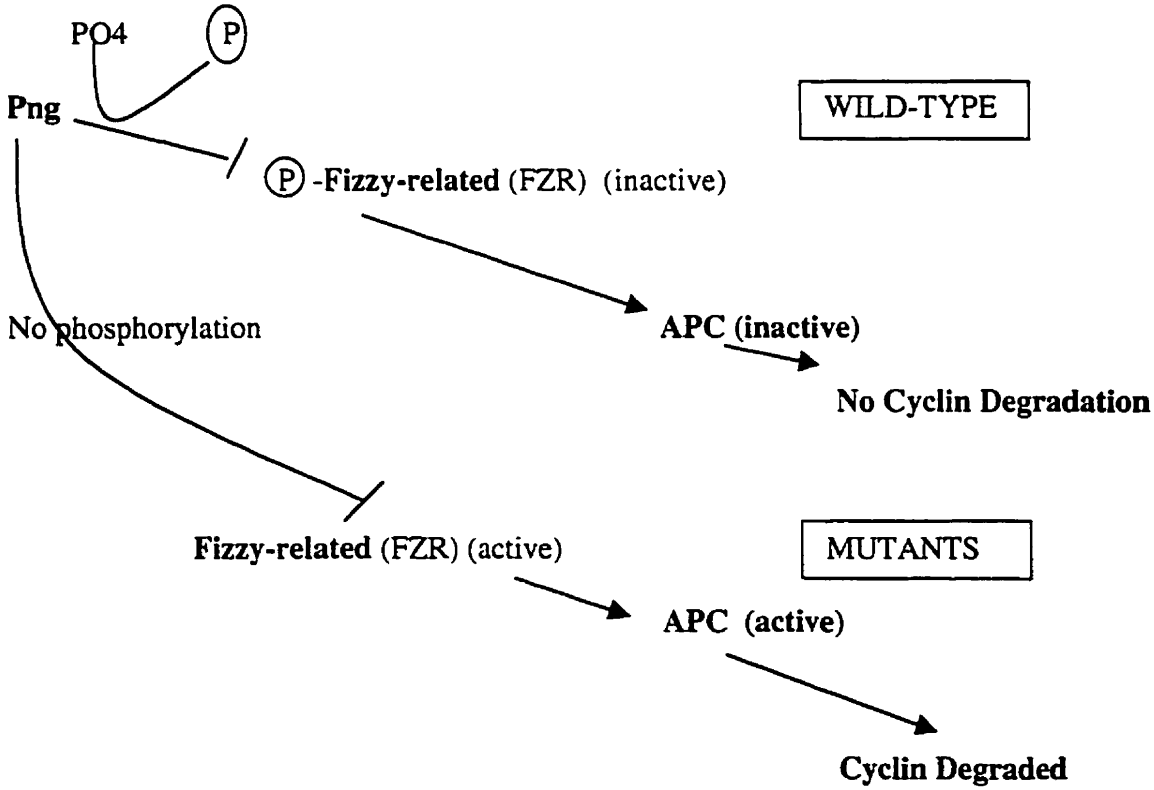
uncoupling of mitosis from DNA replication during the early cleavage divisions in the case of *gnu* (Freeman and Glover, 1987; Freeman et al., 1986) or due to overreplication of DNA due to the fact that all three genes are required to prevent entry into S phase until fertilization has been achieved (Axton *et al.*, 1994; Elfring et al., 1997; Shamanski and Orr-Weaver, 1991).

Fenger et al. (2000) have proceeded to clone PNG (this represents a novel protein kinase in *Drosophila* that has no known homologues in other species) and report that the *png*, *plu*, and *gnu* gene products are required to block DNA replication until fertilization is achieved, and to ensure that S phase is properly coordinated with mitosis during the S-M cell cycles. PNG is a serine threonine kinase that interacts with the ankyrin repeat protein, Plutonium, and plays an important role in the stabilization of mitotic cyclins (Fenger et al., 2000). In wild-type, the mitotic cyclins A and B, and Cdc2 are stable and present at high levels during the first 7 cell cycles (Edgar et al., 1994). Fenger et al. (2000) showed that in *png*, *plu*, and *gnu* mutants there was an allele-specific decrease in the levels of mitotic cyclins A and B and CDK activity. These cyclins may be required to block endoreplication in unfertilized eggs and embryos. All three genes (*png*, *plu*, and *gnu*) may be necessary to promote entry into M phase at the end of meiosis thereby blocking DNA replication until fertilization is achieved, allowing proper controlled oscillation of S-Phase with mitosis during the early cleavage divisions (Fenger et al., 2000). It is unlikely that PNG interacts directly with the mitotic cyclins due to the fact that PNG and PLU do not coimmunoprecipitate with cyclin A and B (Fenger et al., 2000) and because the unphosphorylated form of Cyclin A is lost in *png* mutants (Fenger et al., 2000). It is more likely that in wild-type embryos, PNG may control mitosis by regulating

cyclin stability by interacting with the protein degradation machinery of the APC/Cyclosome. It has been reported that Fizzy-related (FZR), which is the *Drosophila* homologue of yeast Cdh1 and an activator of APC, is inactivated by phosphorylation (Jaspersen et al., 1999; Sigrist and Lehner, 1997; Visintin et al., 1998; Zachariae et al., 1998). Hence, this may prove to be a downstream target of *png* (see below). Therefore, in *png* mutants, Fizzy-related is non-phosphorylated resulting in the abnormal destabilization of the Cyclin proteins as a result of activation of the degradation machinery by the APC/Cyclosome.

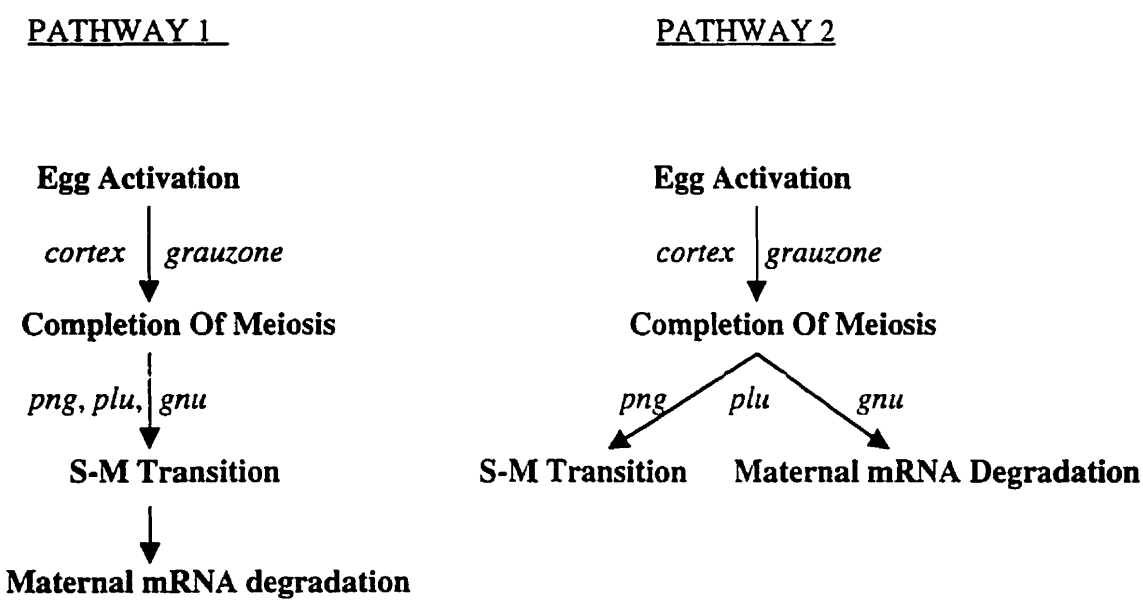
Wild-Type - FZR is inactivated by phosphorylation by Png

Mutants - FZR remains unphosphorylated



This is consistent with the recovery of genes encoding components of the ubiquitin protein degradation machinery in a genetic screen for *png* interactors (Elfring et al., unpublished result from Fenger et al., 2000). The difference between strong and weak alleles in terms of the number of giant nuclei is attributed to a transient S-M cycling before mitosis ends in the weak alleles. In the stronger alleles, mutations are found in the kinase active site which prevents mitosis completely (Fenger et al., 2000). In addition, it is not known whether the cortical microtubule clearing phenotype observed in *png* alleles is related to FZR and APC.

Here it has been shown that all *Drosophila* mutants that are known to be defective in S-phase control at the onset of development (Elfring et al., 1997; Fenger et al., 2000; Freeman and Glover, 1987; Freeman et al., 1986; Shamanski and Orr-Weaver, 1991) also fail to degrade maternal mRNA in the early embryo. This suggests that there may be a correlation between the regulation of S-M cycles and the control of maternal mRNA degradation in the early embryo. The fact that the novel mutation, *temprano*^{181}, which results in maternal mRNA stabilization also fails to properly regulate the S-M transition supports this correlation. Two possible models exist that could explain these findings. In the first model the S-M transition is required for maternal mRNA degradation. If the S-M cell cycle is correctly regulated, then maternal mRNA degradation proceeds. In this model maternal mRNA degradation is downstream of S-M cell cycle control (pathway 1).



An alternative model would suggest that *png*, *plu*, and *gnu* have pleiotropic functions and independently regulate both maternal mRNA degradation and S-M cell cycle control (pathway 2). It is thought that maternal mRNA degradation cannot be upstream of the S-M transition in a linear egg activation pathway because not all maternal mRNA degradation mutants over-replicate their DNA. In addition, *in vitro* activated eggs show normal S-M transition regulation, but fail to degrade maternal mRNA (Unpublished result, Ramona Cooperstock). To determine which model is correct, the female sterile mutation *fs(1)Ya* could be used to ask whether or not S-M transition and maternal mRNA degradation are related. This mutation encodes a nuclear lamina-associated protein that is under both developmental and cell cycle control. A strong mutation in this gene causes development to arrest during the pro-nuclear stage (Lin and Wolfner, 1989). These homozygous mutant embryos have also been shown to degrade maternal mRNA normally (W. Tadros, unpublished result). YA protein function is required during and after maturation of the egg to allow proper chromatin condensation as Ya mutant eggs

and embryos contain nuclei with abnormally condensed chromosomes (Liu et al., 1995). Crucially, it is also known that double mutants of the putative null allele, YA^{76}, with either *png*, *plu*, or *gnu* do not over-replicate their DNA and are indistinguishable from *Ya* embryos suggesting that YA plays a role in the nuclear envelope that allows DNA replication to occur (Liu et al., 1995; Liu et al., 1997) . This is in agreement with a previous study that reports that in *Xenopus* cell-free egg extracts components of the nuclear envelope are required for DNA replication (Blow and Sleeman, 1990). Also, in double mutant unfertilized eggs , abnormally condensed chromatin was observed similar to Ya unfertilized eggs (Liu et al., 1995). Therefore, *fs(1)YA* ; *png* double mutants would be useful to ask whether or not this rescue of the over-replication phenotype also results in degradation of maternal mRNA. If this is the case then it would suggest that the S-M transition is required for maternal mRNA degradation in the early *Drosophila* embryo. If on the other hand maternal RNA still remains undegraded in the double mutant, it would suggest that the two processes are unlinked, which is consistent with the *png*, *plu*, and *gnu* pathway having pleiotropic functions.

png is required for the stability of mitotic cyclins A and B (Fenger et al., 2000). This instability of the mitotic cyclins in the giant nuclei mutants may upset the proper coordinated oscillations between the S and M phases resulting in a shift towards the S-phase which in turn leads to the over-replication phenotype (Shamanski and Orr-Weaver, 1991; Fenger et al., 2000). Therefore, if the two processes appear to be unlinked (maternal mRNA remains undegraded in the double mutant) the epistasis of *Ya* to *png* must be confirmed by looking at the DNA phenotype of the double mutant (to see if the

overreplication phenotype has been rescued), and by examining cyclin stability to determine if it is also rescued in the *Ya Png* double mutant.

Conclusions

The four maternal-effect genes identified in this RNA degradation screen, *png*, *plu*, *gnu*, and *temp*, and the two previously identified non-degraders, *cort* and *grau* (Bashirullah et al., 1999) are all defective in egg activation and early development. These genetic results present the opportunity for determining the regulatory role of each of these genes in maternal mRNA degradation in the early *Drosophila* embryo and how these various genes and the processes they control interact with each other to bring about this destabilization of RNA. For each of the six mutants, phenotypic analyses for the specific processes involved in egg activation were employed to try to dissect a genetic pathway linking the two biological events and to provide insight into how maternal mRNA degradation is regulated.

The high numbers of genes being identified that are required for degradation of maternal mRNA suggests mRNA degradation is a downstream process during egg activation that can be inhibited by mutations in many genes that affect many pathways involved in egg activation. Therefore, to get to the more direct levels of regulation, it will be necessary to carefully select only those mutations that are believed to be involved in downstream regulatory processes, such as *png*. Mutants that fail to degrade maternal mRNA but which are also defective in processes such as rehydration, crosslinking of the vitelline membrane and completion of meiosis might not be as informative as the more downstream regulatory components due to the fact that the former mutations may have

extremely pleiotropic effects. However from both previous analyses and these analyses, a series of conclusions can be made about how maternal mRNA degradation is regulated by six genes involved in very early development. 1) *cort* and *grau*, which are required very early during egg activation, affect many aspects of egg activation including meiosis (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). It is known that *grauzone* activates *cortex* transcription which is necessary for the completion of oocyte meiosis and the regulation of female meiosis (Chen et al., 2000). These genes are therefore considered as upstream components in the regulation of the mRNA degradation pathway(s). 2/ *temp* is unlike *png*, *plu*, and *gnu*, in that some alleles are defective in meiosis. This gene could also be considered to be an upstream component in the regulation of maternal mRNA degradation. 3/ *png*, *plu*, and *gnu* all complete meiosis and are known to initiate defective S-M cell cycles. *png*, *plu*, and *gnu* act to control the S-M transitions. These are considered to be downstream components of *cort*, *grau* and *temp* in the regulation of the maternal mRNA degradation pathway(s). *png* also appears to be defective in cortical microtubule clearing, unlike *plu* and *gnu*. However it has been reported that *png* is required for the presence of the *Plu* protein and that the *png* phenotype inversely reflects the level of Plu protein (Elfring et al., 1997). Therefore *png* may be involved in regulating several aspects of egg activation, both as a complex with *plu* and *gnu* and independently. The *gnu* gene remains to be cloned. GNU may act downstream of *png* and *plu* because the *gnu* mutation has no effect on Plu and Png protein levels (Elfring et al., 1997; Fenger et al., 2000). Another explanation would be that *gnu* controls the same processes as *png* and *plu* but via a parallel independent pathway. From these analyses, a

preliminary picture of how maternal mRNA degradation is regulated in the early *Drosophila* embryo is beginning to be achieved.

Further analyses of *png*, *plu*, *gnu*, *temp* and the identification of additional genes will provide a genetic means for determining how these genes and the processes they control are related, and what their involvement is in maternal mRNA degradation in the early *Drosophila* embryo.

CHAPTER 3

Future Directions

1/ The maternal-effect screen - maternal-effect lethals on the third chromosome have yet to be screened for defects in maternal mRNA degradation. It can be predicted that from the large numbers of third chromosome mutants available for screening, many more RNA degradation mutants will be isolated. Also, to determine if the degradation phenotype is general, and not limited to *Hsp83* RNA, it will be necessary to test the stability of other maternal transcripts that have been shown to be degraded by the same maternal machinery such as *nanos*, *Pgc*, and *string* (Bashirullah et al., 1999) in each of the identified mutants.

2/ Phenotypic analyses of candidate genes - One such example is *morula*. Nurse cells from female-sterile alleles of *morula* begin to become polyploid but revert to a mitotic-like state, condensing the chromosomes and forming spindles (Reed and Orr-Weaver, 1997). In strong, larval lethal alleles of *morula*, the polytene ring gland cells also inappropriately enter mitosis and form spindles (Reed and Orr-Weaver, 1997). *morula* is also necessary for dividing cells to exit mitosis. Embryonic S-M cycles and the archetypal (G1-S-G2-M) cell cycle are both arrested in metaphase in different morula mutants (Reed and Orr-Weaver, 1997). Therefore, *morula* acts to block mitosis-promoting activity in both the endo cycle and at the metaphase/anaphase transition of the mitotic cycle. Hence, this cell cycle regulator, which is similar to *png*, *plu*, and *gnu* in that it is clearly defective in the regulation of the S-M transition, is an interesting RNA degradation mutant candidate.

Another interesting mutant is *wispy* (Brent et al., 2000). The *wispy* phenotype is very similar to that of *temprano* , and is also found on the X chromosome. It has been proposed that this gene functions in microtubule-based events in meiosis and early embryogenesis. Embryos from wispy mothers arrest development after abnormal meiosis and failure of pronuclear fusion. The *wispy*

gene product is also required for proper RNA localization (Brent et al., 2000). The*wispy* alleles that have been analyzed may correspond to strong alleles of *temprano*, such as $temp^{248}$ in which meiosis is clearly defective, in that chromosomes appear fragmented and polar bodies do not properly form. If complementation tests show that the two genes are not the same, this *wispy* mutant will remain an attractive RNA degradation mutant candidate as it is clearly defective in specific aspects of egg activation.

3/ Detailed phenotypic analyses of mutants identified as non-degraders to determine the regulatory role of each gene in maternal mRNA degradation - To determine whether the S-M transition, which follows meiosis, acts upstream of maternal mRNA degradation in the same linear pathway, or whether the three genes (*png*, *plu*, and *gnu*) regulate each process in separate pathways, it will be necessary to look at the *png* ;*Ya* double mutant which does not overreplicate it's DNA (Liu et al., 1997). If rescue of the S-M phenotype rescues maternal mRNA degradation, then it can be assumed that the two processes are related. If on the other hand rescuing the S-M regulation still results in maternal mRNA stabilization, then the two processes are probably not obligatorily linked. Epistasis analyses would have to be performed if this is the case to check that *Ya* is epistatic to *png*. This could be carried out by DNA analyses, in order to determine if giant nuclei are produced in the double mutant. Also, it is known that the levels of mitotic cyclins are reduced in *png* (Fenger et al., 2000). If cyclin stability is normal in the double mutant, it would further suggest that the two processes are separate. If cyclin stability fails to be rescued in the double mutant, then it may be necessary to examine null cyclin mutants to ask whether maternal mRNA degradation is prevented in the absence of cyclins. It will also be informative to determine whether *Fizzy-related* mutants fail to degrade maternal mRNA. This is a homologue of the yeast activator of APC/Cyclosome degradation machinery, Cdh1, which is inactivated by

phosphorylation (Jaspersen et al., 1999; Sigrist and Lehner, 1997; Visintin et al., 1998; Zachariae et al., 1998). Fizzy-related may be the downstream target of the serine threonine kinase, Png, which when mutated results in the destabilization of Mitotic Cyclins. In addition, it may be useful to look at the levels of various mitotic promoting factors such as String and Rsk homologues in each of the egg activation mutants as the actual defect which results in both the overrepliction phenotype and maternal mRNA stabilization is still not determined.

Given the fact that *temprano* may be a novel uncharacterized gene, mapping, cloning and further detailed phenotypic characterization will be required in order to determine its role in the regulation of maternal mRNA levels. One important issue to be addressed is whether *temprano* mutants fail to complete meiosis, whether they complete meiosis but do not enter the S-M cell cycle, or whether *temp* mutants complete meiosis and begin to overreplicate their DNA in a manner similar to *png*, *plu*, and *gnu* mutants. If *temprano* mutants have the giant nuclei phenotype, similar to *png*, *plu*, and *gnu*, (as was reported here for temp^{181}), then identification of the *temprano* gene product and its molecular characterization (if novel) will be informative in understanding how the regulation of the S-M transitions are related to maternal mRNA degradation during early development. Because several alleles of *temp* have been isolated from the maternal-effect screen, it will be necessary to phenotypically characterize each of the alleles using Pico Green in order to place these in an allelic series based on the strength of their mutations. To determine whether an allele is null, it will be necessary to place the strongest *temp* allele over a deficiency. If it is a null, the resulting phenotype should not be stronger. It will also be informative to look in greater detail at other aspects of the *temp* phenotype.

Given the fact that in *png* mutants, PLU protein levels are reduced or absent, and that the giant nuclei phenotype is partially the result of defective mitotic control, it will be interesting to look at whether PLU protein is absent and whether mitotic cyclins are destabilized in the *temp* mutants (Elfring et al., 1997; Fenger et al., 2000).

Finally, to identify the actual machinery of the maternal mRNA degradation pathway as opposed to regulatory components, it may be necessary to perform a germ line clone screen. Preliminary evidence suggests that the same degradation machinery utilized to degrade maternal transcripts by the MBT, may also be used later in development (A. Bashirullah, unpublished result). Hence, a loss of zygotic gene function during development would lead to lethality. Therefore the FLP-FRT technique could be employed to uncover loci involved in the maternal degradation pathway that might have been missed by the maternal-effect screen.

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Appendix I: Unfertilized Maternal-Effect Screen

Maternal *Hsp83* RNA is degraded by 3-4 hours in laid unfertilized eggs by the sole action of the maternal mRNA degradation machinery (Bashirullah et al., 1999). However, maternal *Hsp83* RNA fails to degrade in the pole cells of developing embryos or in the posterior polar plasm of unfertilized eggs (Ding et al., 1993a). This RNA has been shown to be protected from the degradation machinery in this region of the egg/embryo (Bashirullah et al., 1999). The components that are involved in this protection still remain unidentified, however it is possible that components of the polar plasm are involved in masking the transcript.

Initially an unfertilized maternal-effect screen was performed to try to identify mutants defective in maternal mRNA degradation. Any mutations that affect this process would be easily identified because of the absence of the zygotic degradation machinery. Homozygous, unmated females were used for collection of unfertilized egg lays to isolate mutants defective in maternal mRNA degradation. Eggs were collected 3-6 hours post-fertilization and probed for *Hsp83* RNA. Out of a total of 68 lines, 12 were analyzed using unfertilized eggs. From these 12 lines, 3 interesting mutant *in situ* results were identified.

1/ Line 172 failed to show any degradation of maternal *Hsp83* RNA in any of the embryos in 3-6 hour collections, which suggested either a defect in the degradation machinery or a defect in the regulation of maternal mRNA degradation (Fig. 18A). This mutation was later identified as *png*.

2/ Line 37 failed in RNA protection. No RNA was observed at the posterior which may be due to defective / missing polar plasm components (Fig. 18B). 3/ Line 224 showed an abnormal localization pattern at the posterior of the egg suggesting a defect in the RNA localization apparatus (Fig. 18C).

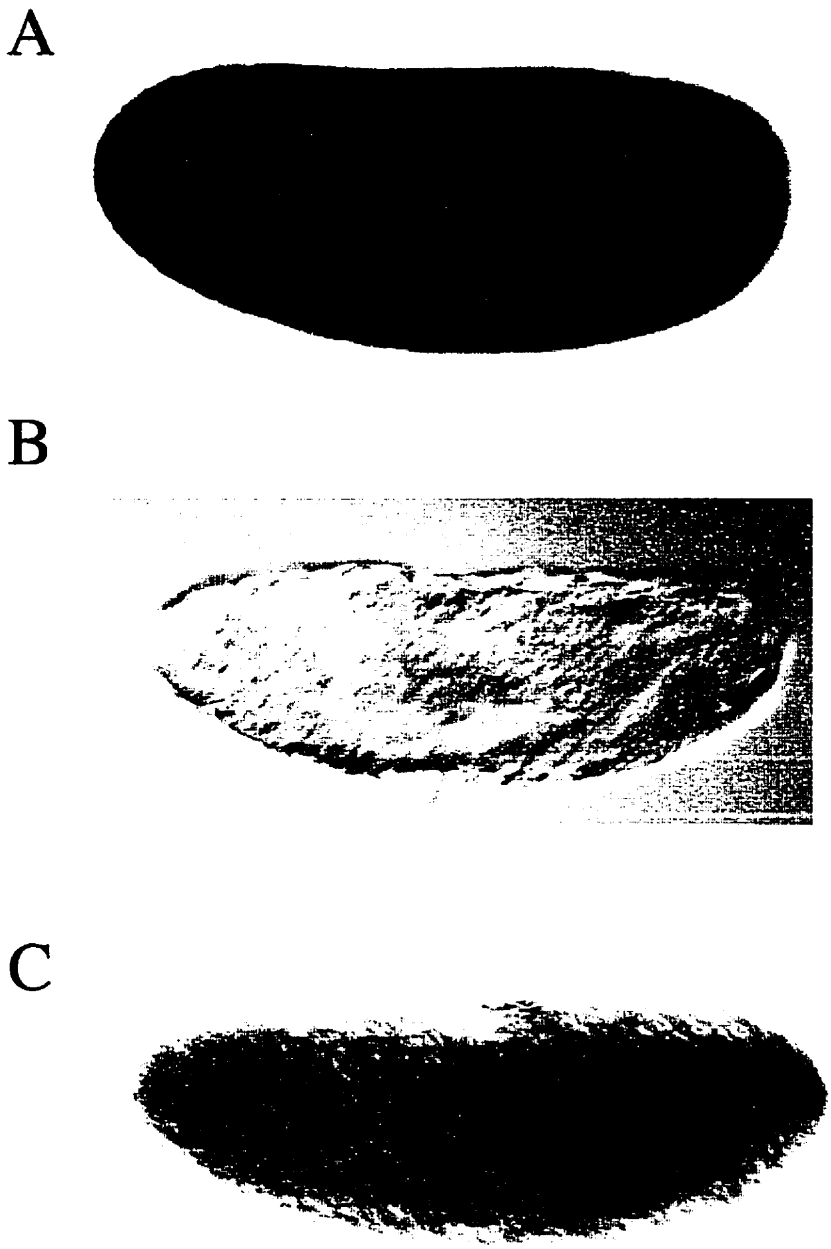


Figure 18. Three lines of unfertilized eggs resulted in defects in maternal mRNA degradation.
These embryos were fixed, and probed for maternal mRNA using a *Hsp83* RNA probe. Anterior is to the left , and dorsal is to the top.
Line 172 (A), which was later identified as *png*, failed to degrade maternal mRNA throughout the embryo. Line 37 (B) failed to protect maternal *Hsp83* RNA at the posterior polar plasm. Line 224 (C) showed an abnormal localization pattern of *Hsp83* RNA which was not tightly localized to the posterior.

The known mutations *cappuccino*, *oskar*, *spire*, *staufen*, *tudor*, *valois*, and *vasa* disrupt the posterior polar plasm and the polar granules (Boswell and Mahowald, 1985; Hay et al., 1988; Lehmann and Nusslein-Volhard, 1986; Manseau and Schupbach, 1989; Schupbach and Wieschaus, 1989; St. Johnston et al., 1991). Similar to line 37, *Hsp83* RNA is not protected at the posterior plasm of eggs produced by females carrying these maternal-effect mutations (Ding et al., 1993a). However, none of these mutations are on the X chromosome, so it can be concluded that this defect could be due to a novel mutation. Line 224 did not localize *Hsp83* RNA tightly to the posterior plasm as in wild-type eggs. This may be due to a defect in RNA localization apparatus genes such as *vasa* and *staufen*. Throughout the unfertilized maternal-effect screen, egg collections were very difficult possibly because these were maternal-effect lines and also due to the fact that unfertilized eggs are laid less frequently than fertilized. The collections were very few in number for the required 3 hour time interval of 3-6 hours, hence embryos 0-5 hours old were subsequently used for the maternal-effect screen.

Appendix II: RNA Degradation Dynamics

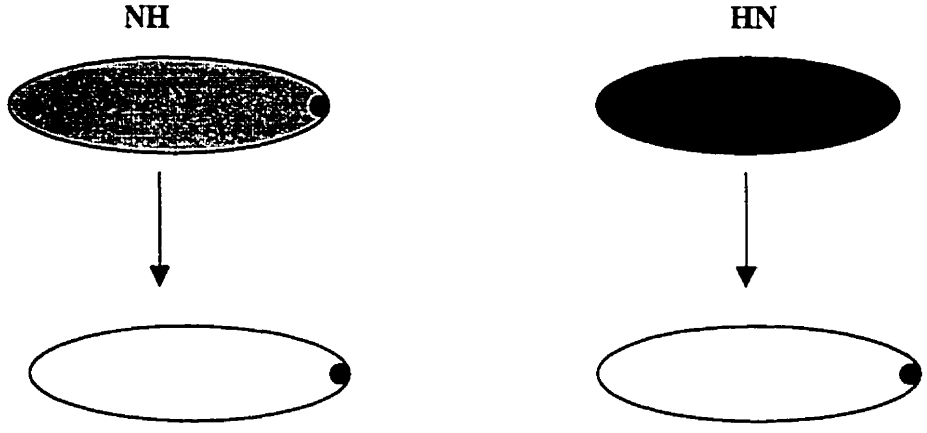
Both *nanos* and *Hsp83* RNAs are localized to the posterior polar plasm of the unfertilized egg by the action of the maternal mRNA degradation machinery. It has been shown by Quantitative Northern blot analyses that 99% of *Hsp83* transcripts are degraded by 4-5 hours after egg activation, whereas 99% of *nanos* transcripts are degraded by 3 hours after egg activation, 1-2 hours earlier than *Hsp83* transcripts (Bashirullah et al., 1999). The posterior localized *nanos* RNA is visible much earlier than localized *Hsp83* transcripts (Bashirullah et al., 1999) which may or may not be due to the fact that the *Hsp83* promoter is very strong and may hide localization in pre-two hour old eggs. In order to test the hypothesis that the differences in the degradation/localization of *Hsp83* and *nanos* are quantitative rather than qualitative, two transgenes were constructed and injected into wild-type flies -

1/ ***nanos*** promoter - *nanos* 5'UTR - lacZ - ***Hsp83*** 3'UTR (NH)

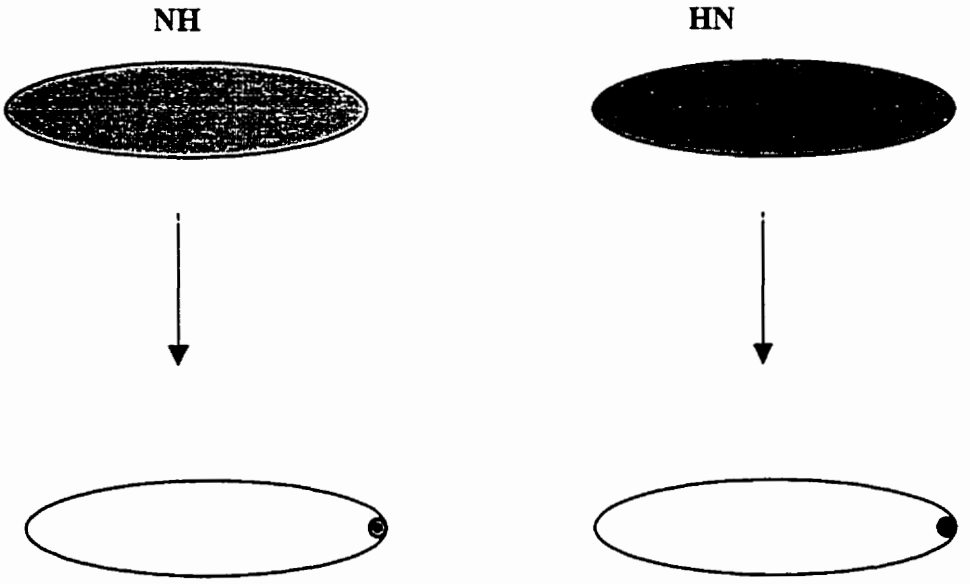
2/ ***Hsp83*** promoter - *Hsp83* 5'UTR - lacZ - ***nanos*** 3'UTR (HN)

Two outcomes were predicted (see below).

1/ Same pathway



2/ Different pathway



To test the hypothesis, *in situ* hybridization using a lac Z probe was carried out on three NH lines and three HN lines. The *in situ* of homozygous unfertilized eggs from the HN lines at 0-2 hours yielded wild-type *nanos* like localization. A slower degradation rate due to the increased expression of HN was not observed (Fig. 19A). Unfertilized eggs from NH lines showed lower than wild-type *Hsp83* RNA levels (Fig. 19B). NH transcripts did not localize to the posterior pole like wild-type *Hsp83* RNA after 4 hours (Fig. 19C). Also, this NH transgene was not fully degraded throughout the somatic region of the egg by 4 hours after egg activation (unlike wild-type *Hsp83* RNA) even though the levels of LacZ mRNA expression were lower than the levels of wild-type *Hsp83* RNA (Fig. 19D). The lack of degradation and localization of the NH transcripts may be due to a cis-regulatory element in the 5'UTR of *Hsp83*. No further analyses were carried out.

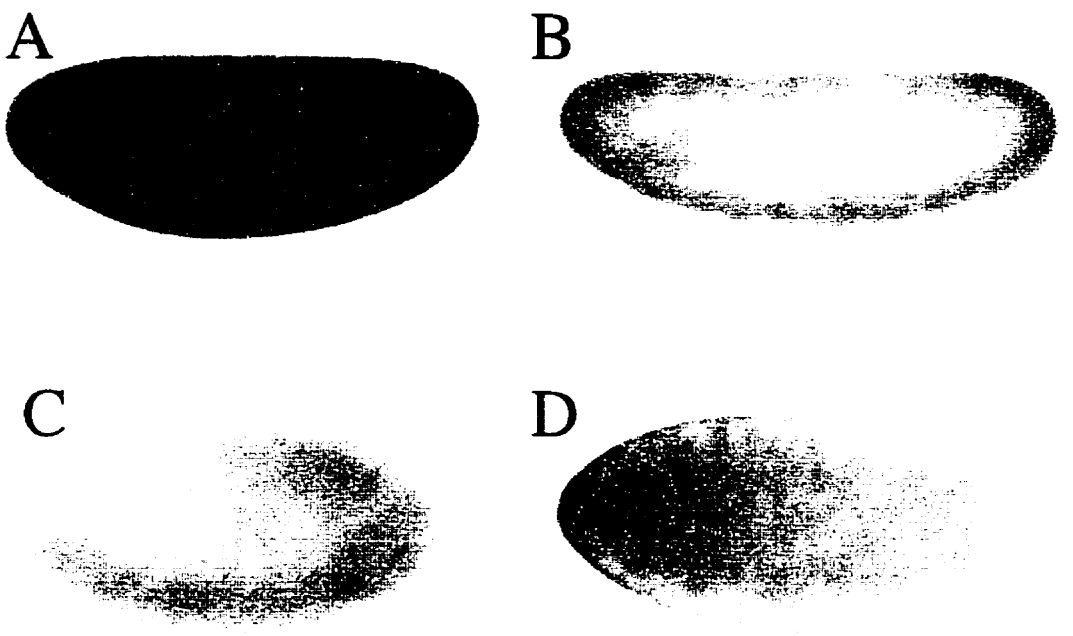


Fig 19. RNA degradation dynamics.
A. 0-2 hour homozygous unfertilized eggs from the HN lines produced wild-type *nanos*-like localization.
B. Unfertilized eggs from NH lines showed lower than wild-type *Hsp83* RNA levels.
C, D. NH transcripts did not localize to the posterior pole like wild-type *Hsp83* RNA 4 hours after egg activation. In addition, transcripts were not totally degraded throughout the somatic region of the egg.

Anterior is to the left, and dorsal on top.

Standard protocols were used for all subcloning steps (Sambrook et al., 1989).

Germline transformation was carried out by Angelo Karaiskakis using standard procedures (Rubin and Spradling, 1982). w^{1118} embryos were coinjected with 500ug/ml of the constructs and 100 ug/ml helper plasmid (Steller and Pirrotta, 1985). All transgenics were homozygosed or balanced over *CyO* or *TM3*.

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