Regulation of Maternal Transcript Destabilization During Egg Activation in Drosophila

Wael Tadros,*† Simon A. Houston,*†,1 Arash Bashirullah,*2 Ramona L. Cooperstock,*†,3 Jennifer L. Semotok,*† Bruce H. Reed* and Howard D. Lipshitz*†,4

*Program in Developmental Biology, Research Institute, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada
†Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada
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ABSTRACT

In animals, the transfer of developmental control from maternal RNAs and proteins to zygotically derived products occurs at the midblastula transition. This is accompanied by the destabilization of a subset of maternal transcripts. In Drosophila, maternal transcript destabilization occurs in the absence of fertilization and requires specific cis-acting instability elements. We show here that egg activation is necessary and sufficient to trigger transcript destabilization. We have identified 13 maternal-effect lethal loci that, when mutated, result in failure of maternal transcript degradation. All mutants identified are defective in one or more additional processes associated with egg activation. These include vitelline membrane reorganization, cortical microtubule depolymerization, translation of maternal mRNA, completion of meiosis, and chromosome condensation (the S-to-M transition) after meiosis. The least pleiotropic class of transcript destabilization mutants consists of three genes: pan gu, plutonium, and giant nuclei. These three genes regulate the S-to-M transition at the end of meiosis and are thought to be required for the maintenance of cyclin-dependent kinase (CDK) activity during this cell cycle transition. Consistent with a possible functional connection between this S-to-M transition and transcript destabilization, we show that in vitro-activated eggs, which exhibit aberrant postmeiotic chromosome condensation, fail to initiate transcript degradation. Several genetic tests exclude the possibility that reduction of CDK/cyclin complex activity per se is responsible for the failure to trigger transcript destabilization in these mutants. We propose that the trigger for transcript destabilization occurs coincidently with the S-to-M transition at the end of meiosis and that pan gu, plutonium, and giant nuclei regulate maternal transcript destabilization independent of their role in cell cycle regulation.

In a variety of animal species, maternal transcripts and proteins loaded into the developing oocyte control early embryonic development (Davidson 1986). A subset of these maternal products is eliminated during early embryogenesis, presumably to allow zygotic products to assume control of development at the midblastula transition. We focus here on that subset of maternal transcripts that is stable in the mature oocyte but is destabilized and degraded in the early embryo. The destabilization of maternal transcripts has been observed in several animal models, including mouse, rabbit, zebrafish, and Xenopus (Bachvarova et al. 1998; Duval et al. 1999; Henrion et al. 1997, 2000; Brunet-Simon et al. 2001; Kishida and Callard 2001).

In Drosophila, destabilization of several maternal transcripts—including Hsp70, Hsp83, nanos, Pgc, string, and twine—has been analyzed in early embryos (Edgar and Datar 1996; Bashirullah et al. 1999). Destabilization of string and twine transcripts, which encode Drosophila homologs of the CDC25 cell cycle regulator, may be important for the proper timing of early embryogenesis since reducing the maternal dosage of these genes causes cellularization to occur earlier than normal, whereas increasing the dosage of twine shifts cellularization to a later time point (Edgar and Datar 1996).

Transcript destabilization is required for localization of certain maternal transcripts within the cytoplasm of the early Drosophila embryo via a mechanism that combines generalized transcript degradation with localized protection (Ding et al. 1993; Bashirullah et al. 1999, 2001). We previously showed that specific cis-acting elements are responsible for both the degradation and the protection components of transcript localization (Bashirullah et al. 1999, 2001). Transcript localization was found to occur in activated, unfertilized eggs, indicating that maternal products are sufficient to accomplish both degradation and protection (Bashirullah et al. 1999). The unstable class of maternal transcripts can thus be
subdivided into two categories: those that are uniformly unstable (e.g., string Hsp70) and those that are protected in a subdomain of the cytoplasm and hence become localized (e.g., Hsp83, nano, Pgc; Bashirullah et al. 1999, 2001).

The Drosophila egg and early embryo thus serve as a model in which to study the regulated destabilization of maternal transcripts during early development and the role of transcript degradation in RNA localization. Central to understanding both processes is identifying the regulatory events that trigger the destabilization of maternal transcripts. Of the animals in which this process has been investigated, only Drosophila possesses the advantage that genetic analyses can be combined with molecular strategies to elucidate the control of transcript instability.

Here we show that egg activation is necessary and sufficient to trigger maternal transcript destabilization. We report the results of a genetic screen for maternal-effect lethal mutants that fail to undergo maternal transcript destabilization. Mutations in all 13 genetic loci identified fail in additional aspects of egg activation. By investigating the least pleiotropic mutant class (composed of pan gw, plutonium, and giant nuclei), as well as in vitro-activated wild-type eggs that also fail to initiate transcript degradation, we show that transcript destabilization is likely to be triggered coincidently with the Sto-M transition that occurs upon completion of meiosis. The PNG, PLU, and GNU proteins are thought to promote this Sto-M transition by maintaining high cyclin-B levels and hence activity of the cyclin-dependent kinase (CDK)/cyclin complex (Feng et al. 2000; Lee et al. 2001). Using a variety of genetic manipulations we show that the trigger for transcript degradation is independent of CDK/cyclin complex activity. We conclude that PNG, PLU, and GNU regulate transcript destabilization independent of their role in regulation of chromosome condensation upon completion of meiosis. Since PNG is a novel S/T kinase (Feng et al. 2000), it is possible that distinct cytoplasmic signal transduction pathways are used to trigger chromosome condensation and transcript destabilization.

MATERIALS AND METHODS

Drosophila stocks: The wild-type stocks used were Oregon-R and y w1118. We assayed the following X chromosomal maternal-effect lethal lines generated by A. Hifiker and J. Lucchesi (Swan et al. 2001): 1, 11, 16a, 16g, 22, 23, 32, 33, 34, 37, 39, 40, 41, 42, 43, 48, 49, 50, 58, 61, 68, 69, 75, 78, 81, 85, 87, 92, 99, 118, 122, 123, 126, 131, 135, 172, 181, 185, 187, 189, 193, 195, 203, 205, 209, 210, 214, 215, 224, 231, 232, 238, 246, 248, 249, 254, 255. We also tested class “2” and class “3” maternal-effect lethal mutants on the second chromosome (Schüpbach and Wieschaus 1989): cibele, scopa, luckenhaf, cell/HA10/RP45, volos, cell/PK42, cell/QE1, cell/RH36, presto, syn-H10, syn-PL63, early, early-HM21/R170, heistost, fs(2)TW1, early-PG44, early-RL4, granzin, cortex. EP lines and deficiency stocks used for mapping as well as Vm26AfBQ2 (Savant and Waring 1989), Df(2L)cd7, and Df(2L)y10 were obtained from the Bloomington Drosophila Stock Center; ndl1, ndl2, ndl3, ndl4, ndl10, ndl17, ndl14, ndl6, ndl11, ndl10, ndl65 (Hong and Hashimoto 1995, 1996), and Df(3L)CH12 were provided by E. LeMoy; wisp14300, wisp161317, and wisp161409 (Brent et al. 2000) by T. Hazelrigg; twiR105 (Schüpbach and Wieschaus 1989) by T. Schüpbach; fs(1)Ya4 (Lin and Wolynier 1991) by M. Wolthy; png11120, png113317, png141118, plu2; gnu650 (Freeman et al. 1986; Shamanski and Orr-Weaver 1991), png154100, fs(1)Ya4, as well as stocks of png, plu, and gnu with extra doses of cyclin B or B3 (Lee et al. 2001), were provided by T. Orr-Weaver; fzy and fzy′ (Dawson et al. 1993) were obtained from I. Dawson; and cdkD12-13 (Stern et al. 1993) was obtained from C. Lehner.

RNA extraction and analysis: Northern blot analyses were carried out as described previously (Bashirullah et al. 1999). For RNA dot blots, RNA was extracted from staged embryo collections (10 or 20 embryos per sample) using Trizol (Invitrogen, San Diego) and applied to solid support using a 96-well Minifold I dot-blot apparatus (Schleicher & Schuell, Keene, NH) following the protocol for slot hybridization of RNA described in Sambrook et al. (1989). Probe generation, hybridization, exposure, and quantification were as for the Northern blots.

Maternal-effect lethal (MEL) screen: We screened MEL mutants by either whole-mount RNA tissue in situ hybridization or RNA dot-blot analysis. RNA in situ hybridization was performed as described previously (Bashirullah et al. 1999) on collections of 0- to 5-hr embryos. RNA for dot blots was extracted from collections of 0- to 1-hr and 4- to 5-hr embryos. We identified mutants defective in degradation as those that produced embryos staining darkly in an RNA tissue in situ for Hsp83 transcripts and/or showing significantly above wild-type levels of the transcript at the 4- to 5-hr time point on a dot blot.

Mapping methods: The convention of Schüpbach and Wieschaus (1989) of naming complementation groups with defects in the early syncytial stages, “early” in different languages, was followed: “temprano” is the Spanish word for “early” while “prage” is the Sanskrit word for “early.” The temprano and prage complementation groups were mapped by recombination using flies carrying w+ markers (EP1421, EP55, EP1347, EP1452, EP1547, EP1150) at known cytological positions on the X chromosome. The complementation groups were further mapped using chromosomal deficiencies: Df(1)HA85, Df(1)K6, and Df(1)R147 failed to complement temprano while Df(1)m259-4 and Df(1)N105 complemented temprano. This mapped temprano to 10F1-7. Complementation tests subsequently revealed that all eight temprano mutants are, in fact, alleles of wispy (wisp; Brent et al. 2000). Df(1)BA1, Df(1)R15, and Df(1)x-f4 failed to complement prage while Df(1)m260-1, Df(1)S39, and Df(1)A94 complemented prage. This mapped prage to 1B4-1E2.

Bleach resistance test: Bleach resistance is a useful assay for the vitelline membrane reorganization and crosslinking that occurs upon egg activation (Mahowald et al. 1983; Savant and Waring 1989; Heifetz et al. 2000; LeMoy and Hashimoto 2000). Bleach resistance was assayed by incubating 0- to 1-hr-old embryos in a 50% bleach (2.5% sodium hypochlorite) solution for 2 min. Mutants that produced embryos that lysed or collapsed during observation under a dissecting scope were classified as “fragile.”

Immunohistochemistry, immunoblots, and visualization of DNA: Standard embryo fixation and immunostaining procedures were followed (Rothwell and Sullivan 2000) except where noted. DNA was visualized using either 0.3 μg/ml of 4’6-diamidino-2-phenylindole (Sigma, St. Louis) or a 1:1000 dilution of PicoGreen (Molecular Probes, Eugene, OR) in PBS, 0.1% Triton X-100 for 5 min. Spindles were visualized using the E7 anti-β-tubulin antibody (obtained from the Devel-
opamental Studies Hybridoma Bank, Iowa City, IA; CHU and KLYMOWSKY 1989) at a dilution of 1:200 followed by a goat anti-mouse IgG (H + L) rhodamine TRITC (Jackson, West Grove, PA) secondary at a dilution of 1:300. To visualize bicolored (BCD) protein in wild-type, wisp, pgc, plu, and gnu mutants we used either a rabbit or a rat anti-BCD antibody (DRIEVER and NÜSSLIN-VOLHARD 1988) followed by a donkey anti-rabbit or anti-rat IgG (H + L) HRP (Jackson) secondary. Both antibodies were preadsorbed against a 0- to 18-hr collection of embryos and used at a 1:10 dilution. For cortical microtubule staining, dechorionated embryos were fixed in a 1:1 mixture of 37% formaldehyde:heptane for 5 min and then devitinized in a 3:1 mixture of methanol:heptane. Cortical microtubules were visualized using an anti-α-tubulin antibody (New England Nuclear) at a dilution of 1:5 followed by goat anti-mouse IgG (H + L) rhodamine TRITC (Jackson) secondary at a dilution of 1:300. For Western analysis, protein from 0- to 3-hr-old embryos was extracted, electrophoresed on an 8% SDS-polyacrylamide gel, and immunoblotted according to the methods used by SMIBERT et al. (1996). BCD protein was visualized in wild type and fregr mutants using a polyclonal guinea pig anti-BCD antibody (GAMBERI et al. 2002) at 1:250 dilution after preadsorption overnight at 4° against 4 to 20-hr-old embryos. The secondary antibody was goat anti-guinea pig IgG (H + L; Jackson) used at 1:10,000 dilution.

In vitro activation of oocytes: Stage 14 oocytes were activated as described in PAGE and ORR-WEAVER (1997). Unactivated eggs were aged in isolation buffer (PAGE and ORR-WEAVER 1997).

Cdk1 temperature shift experiment: Adult flies homozygous and heterozygous for cdk1^{+/2} were obtained from a stock maintained at 18° (permissive temperature). RNA was extracted from embryos 4 hr 15 min (±15 min) after egg deposition. Embryo samples were treated as follows: collection and incubation at 18° (permissive temperature), 30-min collection at 18° followed by incubation at 29° (restrictive temperature), or collection and incubation at 29°.

Microscopy: Embryos were cleared in 70% glycerol with 2.5% 1,4-diazabicyclo-[2.2.2]octane and mounted in DAKO fluorescent mounting medium. Images were captured using a cooled-CCD camera (Spot, Diagnostic Instruments) mounted on a Zeiss Axioplan microscope. A Leica TCS 4D or a Zeiss Axiovert 100 was used for confocal microscopy. In the former case, images were obtained using “scanware” software, while in the latter case LSM510 software was used. Adobe Photoshop software was used to process the images.

RESULTS

Egg activation is required for maternal transcript destabilization: We previously showed that the destabilization of a subset of maternal transcripts initiates in activated, unfertilized eggs (BASHIRULLAH et al. 1999). For example, transcripts such as Hsp83, nanos, Pgc, and string are degraded over a 5-hr period in unfertilized eggs while rpA1 transcripts are stable over this same period (Figure 1A). To test whether egg activation is required for transcript destabilization, stage 14 oocytes were bulk isolated (PAGE and ORR-WEAVER 1997) and RNA levels were assayed at various times after isolation. Hsp83, nanos, Pgc, rpA1, and string transcripts are stable in stage 14 oocytes (Figure 1B). Since these transcripts are stable in unactivated mature oocytes but are destabilized in activated, unfertilized eggs, we conclude that egg activation is necessary and sufficient for destabilization of a subset of maternal transcripts.

Identification of maternal-effect lethal loci required for destabilization of maternal transcripts: Since there is no transcription in activated, unfertilized eggs (ANDERSON and LENGYEL 1979), egg activation must trigger either the synthesis or the activation of a maternally encoded transcript degradation machinery. To identify genes required for destabilization of maternal transcripts, we screened collections of EMS-induced MEL mutants. These included 57 uncharacterized mutations on the X chromosome (SWAN et al. 2001) and mutations in 19 previously identified loci on the second chromosome (SCHÜPBACH and WIESCHAUS 1989). The mutants were selected for analysis because their progeny exhibit defects early in embryogenesis, consistent with a possible role for maternal transcript degradation prior to or at cellularization (EDGAR and DATAR 1996; BASHIRULLAH et al. 1999). Using Hsp83 transcripts as a diagnostic “marker,” we identified 21 X-linked mutant lines that fail to degrade maternal transcripts. These lines represent seven complementation groups. The loci identified in the screens are listed in Table 1 along with four additional loci that we identified in candidate mutant tests (see below). Although the mutants screened represent
TABLE 1

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>No. of lines</th>
<th>Cytogenetic map information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vm26Ab</td>
<td>1</td>
<td>26A1 (cloned)</td>
</tr>
<tr>
<td>nudel (ndl)</td>
<td>5</td>
<td>65B5 (cloned)</td>
</tr>
<tr>
<td>cortex (cort)</td>
<td>2</td>
<td>26E4 (cloned)</td>
</tr>
<tr>
<td>grauzone (grau)</td>
<td>2</td>
<td>57B5-14 (cloned)</td>
</tr>
<tr>
<td>wispy (wisp)</td>
<td>8</td>
<td>10F1-7</td>
</tr>
<tr>
<td>prage (prg)</td>
<td>2</td>
<td>1B4-1E2</td>
</tr>
<tr>
<td>pan gu (png)</td>
<td>4</td>
<td>1F4 (cloned)</td>
</tr>
<tr>
<td>plutonium (plu)</td>
<td>2</td>
<td>56F11 (cloned)</td>
</tr>
<tr>
<td>giant nuclei (gnu)</td>
<td>1</td>
<td>70E8-71E5</td>
</tr>
<tr>
<td>fragile locus A (92)</td>
<td>1</td>
<td>X chromosome</td>
</tr>
<tr>
<td>fragile locus B (205)</td>
<td>1</td>
<td>X chromosome</td>
</tr>
<tr>
<td>fragile locus C (215)</td>
<td>1</td>
<td>X chromosome</td>
</tr>
<tr>
<td>fragile locus D (189, 193, 255)</td>
<td>3</td>
<td>X chromosome</td>
</tr>
</tbody>
</table>

Two lines (22 and 214) gave ambiguous results in our assays and thus are not listed. One line (87) exhibited fragility but was not used in complementation tests because the female sterile phenotype reverted or was modified.

a selected subset of all maternal-effect lethals available, the frequency with which degradation-defective mutants was found is remarkably high (X chromosome mutants: 21/57 = 37%; second chromosome loci: 2/19 = 11%).

Transcript destabilization fails in mutants with a defective vitelline membrane: Since maternal transcript destabilization requires egg activation, it seemed likely that at least some of the identified instability mutants would be defective for other aspects of egg activation and that the inability to destabilize transcripts was thus an indirect effect of failure to undergo normal egg activation. One of the first events that occurs upon egg activation is reorganization and crosslinking of the vitelline membrane that makes this structure impermeable to aqueous solutions. In particular, this reorganization renders the egg resistant to lysis after a 2-min incubation in 50% bleach (2.5% sodium hypochlorite; Mahowald et al. 1983; Heifetz et al. 2001). In contrast, stage 14 oocytes lyse by the end of such an incubation. Of the 21 X-linked degradation-defective lines recovered from our screen, seven lines (representing four complementation groups) produce eggs that are not resistant to bleach. These are referred to as “fragile” mutants (see Table 1) since eggs from these seven lines either lyse or collapse by the end of the 2-min bleach treatment.

The fragile phenotype observed is similar to the previously reported phenotype of Vm26Ab mutants. Vm26Ab mutants lack sV23, a major vitelline membrane protein, and are defective in vitelline membrane composition and crosslinking (Savant and Waring 1989). Maternal transcripts fail to be degraded in eggs from Vm26AbQJ42 mutant females (Figure 2A). This is consistent with the trigger for egg activation and transcript instability relying on the organization, and/or on specific components, of the vitelline membrane.

To address whether vitelline membrane crosslinking per se is required to trigger transcript instability, we assayed degradation in several alleles of nudel (ndl). NDL is a serine protease that is proposed to function in the proteolytic cascade responsible for the production of the ligand that binds Toll (Hong and Hashimoto 1995) and thus is required for proper dorsal-ventral patterning of the embryo. In addition to its catalytic function, NDL appears to play a role, possibly structural, in the vitelline membrane (Hong and Hashimoto 1996; LeMosy and Hashimoto 2000; LeMosy et al. 2000). Alleles of nudel thus fall into two major classes: class I alleles, which are apparent nulls, produce fragile eggs similar to the above-mentioned mutants; class II alleles, which appear to interfere with the enzymatic function of NDL, result in dorsalized embryos but not in egg fragility (Hong and Hashimoto 1995, 1996; LeMosy et al. 1998, 2000). Despite the obvious difference in the overt phenotype (i.e., fragility, as assayed by bleach resistance), both classes of mutants have been shown to be defective in vitelline

Figure 2.—Destabilization of maternal Hsp83 transcripts requires proper vitelline membrane organization. RNA was extracted from staged embryo collections (age after egg deposition is indicated), samples were split, and dot blots were probed for Hsp83 or rpA1, the latter as a loading control. (A) RNA degradation occurs in wild-type embryos but not in embryos from Vm26AbQJ42 mutant females. (B) RNA degradation occurs in wild-type embryos and in embryos from ndl141 (a class II allele) and ndl70 (class I) but not in embryos from ndl14 (class I) females.
Egg activation defects in transcript destabilization mutants

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Vitelline membrane reorganized</th>
<th>mRNA translated</th>
<th>Microtubules depolymerized</th>
<th>Meiosis completed</th>
<th>S-to-M transition occurs</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortex</td>
<td>+</td>
<td>−ab</td>
<td>−b</td>
<td>−b</td>
<td>NT</td>
</tr>
<tr>
<td>grauzone</td>
<td>+</td>
<td>−ab</td>
<td>−b</td>
<td>−b</td>
<td>NT</td>
</tr>
<tr>
<td>prage</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>NT</td>
</tr>
<tr>
<td>wispy</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>pan gu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>plutonium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>giant nuclei</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Only nonfragile mutants were assayed. Where no reference is cited, the results of this study are summarized.

*a* Lieberfarb *et al.* (1996).


*c* Brent *et al.* (2000).


*e* Freeman *et al.* (1986).

Membrane crosslinking in biochemical tests (LeMosy and Hashimoto 2000). We assayed maternal transcript degradation in embryos from four class I and six class II alleles (alleles are listed in MATERIALS AND METHODS). All of the class I alleles produced embryos that failed to undergo maternal transcript degradation, whereas five out of the six class II alleles were normal for degradation (Figure 2B).

Together these results show that there is no correlation between failure of vitelline membrane crosslinking and failure to undergo transcript destabilization (all 10 ndl alleles tested fail vitelline membrane crosslinking; however, 5/10 fail transcript degradation while 5/10 undergo transcript degradation). There is, however, a strong correlation between embryo fragility and failure of transcript degradation.

Failure of transcript destabilization does not correlate with failure to initiate maternal mRNA translation: Upon egg activation, a subset of maternal transcripts, including bicoid, Toll, and torso mRNAs, is translated (Driever and Nüsslein-Volhard 1988; Gay and Keith 1992; Lieberfarb *et al.* 1996; Page and Orr-Weaver 1996). The mutants recovered in our screen fall into two classes with respect to translation of these maternal mRNAs (only the nonfragile mutants were tested). The first class is composed of two previously identified RNA destabilization mutants—cortex and grauzone—that fail to initiate translation of a subset of maternal mRNAs, including bicoid, torso, and Toll (Lieberfarb *et al.* 1996; Page and Orr-Weaver 1996; Bashirullah *et al.* 1999), along with a new mutant, prage, identified in this study, which fails to translate bicoid mRNA as assayed by Western analysis (Table 2). The second class, composed of wispy, pan gu, plutonium, and giant nuclei, initiates translation normally as assayed by immunostaining of BCD protein in early embryos (Table 2 and Figure 3, A–C; for identification of plutonium and giant nuclei as destabilization mutants, see below). Thus, failure of transcript destabilization in the second class of mutants is unlikely to be caused by a general failure to translate maternal mRNAs. Instead, these mutants must disrupt components of an egg activation pathway downstream of, or parallel to, the pathway that triggers mRNA translation.

Failure of transcript destabilization does not correlate with failure to reorganize the microtubule-based cytoskeleton: Microtubules are present at the cortex of stage 14 oocytes (Theurkauf *et al.* 1992; Theurkauf 1994). Upon egg activation, these disassemble, presumably for use in the ensuing mitotic divisions (Theurkauf *et al.* 1992; Page and Orr-Weaver 1996). Microtubule depolymerization does not occur in embryos from cortex and grauzone females (Page and Orr-Weaver 1996), two mutants that we identified as being defective in mRNA degradation (Bashirullah *et al.* 1999). However, depolymerization of microtubules is unaffected in five other instability mutants (pan gu, plutonium, giant nuclei, wispy, and prage; Table 2). Thus failure of transcript destabilization in the latter collection of mutants cannot be caused by failure of microtubule depolymerization. Instead, these mutants must disrupt components of an egg activation pathway downstream of, or parallel to, the pathway that triggers microtubule depolymerization.

Transcript destabilization is triggered independent of normal meiotic progression: Mature stage 14 oocytes are arrested in metaphase I of meiosis. Progression through the remainder of meiosis is triggered by egg activation and initiates shortly after the crosslinking of the vitelline membrane (Heifetz *et al.* 2001). The meiotic stages can be visualized using dyes that stain DNA in early embryos. The current screen identified 10 non-fragile degradation-defective lines that fail to complete meiosis, 8 of which form a complementation group mapping to 10F1-7. This complementation group was originally referred to as temprano but was subsequently found to be allelic to wispy (Table 1 and see MATERIALS AND
meiosis:
The final class of transcript destabilization mutants. We conclude that RNA degradation and meiosis were tested by RNA in situ perturbed. Conversely, completion of meiosis can be 

Freeman (Figure 4). Thus transcript destabilization can be either ( 

twine mutants, metaphase I arrest fails to occur in stage 14 oocytes and aberrant nuclear divisions ensue after that the PNG and PLU proteins co-immunoprecipitate White-Cooper et al. (1993). Transcript destabilization correlates with failure of transcript degradation. In twine mutants, metaphase I arrest fails to occur in stage 14 oocytes and aberrant nuclear divisions ensue after egg activation (Alper et al. 1992; Courtot et al. 1992; White-Cooper et al. 1993). Transcript destabilization occurs normally in embryos from twine mutant females (Figure 4). Thus transcript destabilization can be either normal or compromised in mutants in which meiosis is perturbed. Conversely, completion of meiosis can be either normal or compromised in transcript instability mutants. We conclude that RNA degradation and meiosis are regulated independent of each other.

Transcript destabilization fails in mutants that fail to undergo the S-to-M transition upon completion of meiosis: The final class of transcript destabilization mutants identified in our screen of X-linked mutants comprised a single complementation group with four alleles (Table 1, Figure 5). In all four cases the embryos produced by mutant females had a small number of apparently polyploid nuclei rather than the normal, large number of diploid syncytial nuclei. Since this phenotype resembles that of pan gu (png; Shamanski and Orr-Weaver 1991), we tested for allelism and found all of the mutants to be png alleles: our screen reisolated the png172, png246 alleles (Fenger et al. 2000) and one new allele, png48. In wild-type embryos, one of the four female meiotic products fuses with the male pronucleus and undergoes 13 rapid nuclear cycles alternating between S and M phases without intervening gap phases to generate thousands of syncytial nuclei. The pan gu gene encodes a S/T kinase that regulates these S-M cycles (Fenger et al. 2000). Mutations in this gene result in reduced mitosis while DNA synthesis continues unchecked, resulting in a small number of highly polyploid nuclei (Shamanski and Orr-Weaver 1991). DNA overreplication also occurs in activated, unfertilized eggs from png mothers, suggesting that defects in the S-to-M transition lead to failure of chromosome condensation and formation of the “rosette” configuration of the chromosomes after completion of meiosis (Shamanski and Orr-Weaver 1991).

It has been shown that png mutations interact with mutations in two other genes, plutonium (plu) and giant nuclei (gnu), that regulate the S-to-M transition, and that the PNG and PLU proteins co-immunoprecipitate (Shamanski and Orr-Weaver 1991; Fenger et al. 2000). Mutations in the plu and gnu genes give a similar overreplication phenotype to that seen in png mutants (Freeman et al. 1986; Shamanski and Orr-Weaver 1991; Axton et al. 1994). We therefore asked whether plu and gnu mutations affect transcript destabilization. Several alleles of plu and the only published gnu allele were tested by RNA in situ (data not shown) and RNA dot-blot analysis: embryos from mutant females showed complete failure to degrade several maternal transcripts (Figure 5).

Embryos from png, plu, and gnu mutant females are
Maternal Transcript Destabilization in Drosophila

Figure 5.—Maternal transcript destabilization fails in S-to-M transition mutants. Dot blots of RNA extracted from embryos produced by wild-type, png50, png12-3318, png13-1920, plu6, and gnu305 females were sequentially probed for Hsp83, nos, Pgc, and rpA1 (stable control) RNA. The age of the embryos is indicated above (0–1 hr or 4–5 hr).

normal for several other aspects of egg activation (Table 2), including bleach resistance, cortical microtubule depolymerization, completion of meiosis, and maternal mRNA translation (Figure 3B). These results prove that failure of transcript destabilization in these overreplication mutants is not caused by failure to complete meiosis, failure to translate maternal mRNA, or failure to depolymerize microtubules. We conclude that failure to undergo the S-to-M transition at the end of meiosis correlates with failure to initiate transcript degradation.

Maternal transcript destabilization fails in in vitro-activated eggs: Further evidence that defects in the S-to-M transition at the end of meiosis correlate with defects in maternal transcript destabilization came from our analysis of in vitro-activated eggs. Since we had shown (above) that in vivo egg activation is necessary and sufficient to trigger transcript destabilization, we asked whether in vitro egg activation triggers transcript instability. It has previously been shown that incubation of mature stage 14 oocytes in a hypotonic buffer causes them to swell and exhibit several of the characteristics of in vivo-activated eggs: these include bleach resistance, completion of meiosis, and maternal mRNA translation (Mahowald et al. 1983; Page and Orr-Weaver 1997). Normally, after telophase II there is a postmeiotic interphase followed by condensation of the postmeiotic chromosomes to form rosette structures in the dorsal anterior portion of the egg. However, in in vitro-activated eggs, often this recondensation either fails or is delayed, and the meiotic products undergo aberrant replication and division instead of remaining in condensed rosettes (Page and Orr-Weaver 1997). Such defects are reminiscent of those seen in png, plu, and gnu mutants, leading us to assay whether maternal transcript destabilization occurs in in vitro-activated eggs. Strikingly, transcript degradation is not triggered in in vitro-activated eggs, as shown on Northern blots (Figure 6) and RNA dot blots (data not shown). This result provides independent evidence for a correlation between failure of the postmeiotic S-to-M transition and failure to initiate transcript destabilization, suggesting that these two processes may be coregulated.

Overreplication does not cause failure of maternal transcript destabilization: The results of our analyses of RNA destabilization mutants and in vitro-activated eggs focused our attention on the S-to-M transition at the end of meiosis. In addition, the S-M class of RNA instability mutants (png, plu, and gnu) progress further in development than all other classes of mutants identified in our screen, and they are the least pleiotropic in terms of egg activation defects. Our subsequent analyses therefore focused on these mutants with the primary goal of determining whether png, plu, and gnu regulate the S-to-M cell cycle transition independent of transcript destabilization or whether the defect in this transition is the cause of the RNA degradation defect.
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We therefore assayed maternal transcript destabilization in *png fs(1)Ya* double mutants. Strikingly, there was no suppression of the RNA degradation defect (Figure 7). We conclude that overreplication is not the cause of the transcript destabilization defect in *png, plu,* and *gnu* mutants.

**Restoring CDK/cyclin activity in S-to-M transition mutants does not rescue the transcript degradation defect:** The normal progression of cells into mitosis is driven by a heterodimer consisting of a catalytic subunit, CDK, and a regulatory subunit, a mitotic cyclin (reviewed in Doree and Galas 1994). Embryos from *png, plu,* or *gnu* females have been shown to have decreased CDK activity, probably because of reduced mitotic cyclin levels (Fenger et al. 2000). To assay whether this decreased cyclin protein abundance is caused by a reduction in cyclin transcript levels, we carried out Northern analysis. Like all other transcripts assayed, cyclin B transcripts failed to undergo degradation in *png* mutants (data not shown). Thus, the reduced levels of cyclin protein in *png* must be a consequence of defects in translation or protein stability.

Lee et al. (2001) have recently shown that increasing the gene dosage of cyclin B or cyclin B3 in *png, plu,* and *Pgc* transcripts fails in *in vitro*-activated eggs. A Northern blot is shown comparing these four unstable RNAs to *rpA1*, or *gnu* mutant females can partially suppress the early which serves as a stable loading control. Extracts were from *in vitro*-activated stage 14 oocytes aged for 27 min, 2.5 hr, and 5 hr. Quantification of *Hsp83, string, nanos,* and *Pgc* transcripts, normalized to *rpA1,* is shown below the RNA blot.

One striking aspect of the *png, plu,* and *gnu* phenotypes is the overreplication of chromosomal DNA that occurs in place of chromosome condensation (Freeman et al. 1986; Shamanski and Orr-Weaver 1991; Axton et al. 1994). We, therefore, asked whether overreplication *per se* is the cause of the RNA degradation defect. To do this we took advantage of another mutant, *fs(1)Ya* (Lopez et al. 1994; Liu et al. 1995). The *fs(1)Ya* gene encodes a maternally provided component of the nuclear lamina that binds chromatin and is required for DNA replication (Lopez et al. 1994; Lopez and Wolfner 1997; Yu and Wolfner 2002). Embryos from *fs(1)Ya* mutant mothers arrest at the stage of pronuclear fusion but maternal transcript degradation occurs normally (Figure 7). Mutations in *fs(1)Ya* had previously been shown to suppress the overreplication defect of S-M mutants (Shamanski and Orr-Weaver 1991; Liu et al. 1997). We therefore assayed maternal transcript destabilization in *png fs(1)Ya* double mutants. Strikingly, there was no suppression of the RNA degradation defect (Figure 7). We conclude that overreplication is not the cause of the transcript destabilization defect in *png, plu,* and *gnu* mutants.

**Figure 6.**—Destabilization of maternal *Hsp83, string, nanos,* and *Pgc* transcripts fails in *in vitro*-activated eggs. A Northern blot is shown comparing these four unstable RNAs to *rpA1,* which serves as a stable loading control. Extracts were from *in vitro*-activated stage 14 oocytes aged for 27 min, 2.5 hr, and 5 hr. Quantification of *Hsp83, string, nanos,* and *Pgc* transcripts, normalized to *rpA1,* is shown below the RNA blot.

**Figure 7.**—Suppression of overreplication does not suppress failure to undergo transcript destabilization. Samples of RNA extracted from embryos produced by wild-type, *png* single-mutant, *Ya* single-mutant, or *png* double-mutant females were split, loaded onto a dot blot, and probed for *Hsp83* or *rpA1* transcripts.
Figure 8.—Restoration of CDK/cyclin activity does not suppress the defect in transcript destabilization. (A) Extra doses of cyclin B suppress the mitotic phenotype of png. Embryos were stained with PicoGreen to visualize DNA. Shown are representative embryos from mothers mutant for png50 alone (top), png50 with six extra copies of cyclin B (middle), and png50 with eight extra copies of cyclin B (bottom). (B) Extra doses of cyclin B or cyclin B3 do not suppress the transcript degradation defect of png, plu, or gnu mutants. Dot blots are shown for RNA extracted from embryos produced by females that were wild type, png50 with six extra copies of cyclin B, png50 with eight extra copies of cyclin B, png2370 with eight extra copies of cyclin B3, plu6 with four extra copies of cyclin B, or gnu305 with four extra copies of cyclin B. Blots were probed for Hsp83 transcripts and then stripped and reprobed for rpA1.

in triggering transcript destabilization is to lower CDK activity in otherwise wild-type embryos and ask whether transcripts are then stable rather than unstable. This was done using two strategies.

First we assayed transcript instability in embryos with reduced CDK activity. This was done by mutating CDC25, a phosphatase that removes inhibitory phosphates from, and thus activates, CDK (reviewed in Berry and Gould 1996). There are two Drosophila homologs of CDC25, encoded by string (stg) and twine (twe; Edgar et al. 1989; Alphey et al. 1992; Courtot et al. 1992). Both stg and twe are present in oocytes and early embryos; however, only twe has a nonredundant function prior to the midblastula transition (Edgar and Datar 1996); twe mutants do not maintain meiotic arrest at stage 14 of oogenesis (Courtot et al. 1992; White-Cooper et al. 1993). Transcript destabilization occurs normally in embryos from twe mutant mothers (Figure 4). These data indicate that transcript destabilization can still occur under conditions of lowered CDK activity.

For a second, definitive test of the possible requirement for CDK activity, we took advantage of a temperature-sensitive allele of cdk1, cdk1E1-24 (Stern et al. 1993). In control experiments, cdk1 homozygous mutant females were allowed to lay eggs at the permissive temperature of 18°C, the embryos were aged for 4 hr, and transcript degradation was shown to occur normally (Figure 9). Upon transfer to the restrictive temperature of 29°C, DNA staining showed that the embryos had severe cell cycle defects (Figure 9A), consistent with the reported cdk1 phenotype (Stern et al. 1993). Despite these defects, the embryos displayed normal transcript destabilization, even after the females had been kept at the restrictive temperature for 6 hr (Figure 9B). We conclude that transcript destabilization occurs even when CDK activity is low or absent. This result argues against the transcript instability defect in embryos from png, plu, and gnu females being a consequence of reduced CDK/cyclin activity in these mutants and supports the hypothesis that these loci regulate transcript destabilization independent of the S-to-M transition.

DISCUSSION

Almost all analyses of animal egg activation have been carried out in systems that are refractory to detailed genetic analysis (e.g., amphibians, echinoderms) and there have been few systematic studies of egg activation in Drosophila. Most recently, careful analyses of the time course of egg activation events have shown that vitelline membrane reorganization initiates prior to the resumption of meiosis (Heifetz et al. 2001). However, it has not yet been reported exactly when other processes, such as the depolymerization of cortical microtubules and maternal mRNA translation, initiate. Our previous analyses (Bashirullah et al. 1999), together with the results reported here, clearly indicate that destabilization of a subset of maternal transcripts should also be regarded as one of the events that is triggered upon egg activation. The exact timing of transcript destabilization relative to the other events of egg activation remains to be determined.

At this time it is not known which aspects of egg activation are interdependent. Specifically, the fact that egg activation is required to trigger maternal transcript destabilization does not address whether degradation is dependent on the normal progression of one or more of the other processes. For example, it is possible that
translation of certain maternal transcripts at egg activation may be required for the production or activation of the degradation machinery. Another possibility is that translation-mediated events may be important for targeting the transcripts themselves for degradation. In vertebrates such as mouse, Xenopus, and zebrafish, transcripts are translationally masked during early oogenesis and then unmasked upon either egg maturation or fertilization (reviewed in Davidson 1986). Translation of these mRNAs has been shown to be dependent on the cytoplasmic polyadenylation of these transcripts (see, for example, Groisman et al. 2000). Upon egg activation in Drosophila, several transcripts, such as Hsp83 (R. L. Cooperstock and H. D. Lipshitz, unpublished data), bicoid, Toll, and torso, are cytoplasmically polyadenylated and translated (Salles et al. 1994; Lieberfarb et al. 1996; Page and Orr-Weaver 1996). The exact relationship between cytoplasmic polyadenylation, translation, and transcript degradation in Drosophila thus remains to be determined.

Genetic analyses of egg activation and its constituent processes have been initiated recently. For example, cortex and grauzone affect cytoplasmic polyadenylation and translation of several maternal mRNAs (Lieberfarb et al. 1996; Page and Orr-Weaver 1996). These mutants have additional egg activation defects, including failure to complete meiosis (Lieberfarb et al. 1996; Page and Orr-Weaver 1996), and were also identified as failing to trigger transcript destabilization (this study; Bashirullah et al. 1999). Additional examples of egg activation mutants include nudel, which fails in vitellin membrane reorganization (Hong and Hashimoto 1995, 1996; LeMosy et al. 1998, 2000); Vm26Ab, which is missing a key component of the vitelline membrane (Savant and Waring 1989); wispy, which fails to progress normally through meiosis (Brent et al. 2000); as well as png, plu, and gnu, all of which fail to condense their chromosomes at the end of meiosis (Freeman et al. 1986; Shamanski and Orr-Weaver 1991; Axton et al. 1994).

Our genetic screens for maternal-effect lethal mutants that are defective in maternal transcript destabi-
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zation, together with our tests of candidate genes, have resulted in the reisolation of several of the above-mentioned loci. This has enabled us to begin to position the trigger for transcript destabilization relative to the other processes of egg activation. For example, we have shown that failure of transcript degradation does not correlate with failure of vitelline membrane crosslinking, cortical microtubule depolymerization, mRNA translation, and progression through meiosis. This lack of correlation cannot be taken as evidence that transcript destabilization is regulated independently of the above processes. For example, the fact that some of the degradation mutants undergo normal mRNA translation does not mean that normal mRNA translation is not a prerequisite for transcript destabilization. However, that transcript destabilization can fail even when maternal mRNA translation occurs normally supports the idea that transcript degradation requires components additional to, and possibly functioning independent of, the translation machinery.

We found a strong correlation between the S-to-M transition at the end of meiosis and the trigger for transcript instability: in vitro-activated eggs proceed normally through vitelline membrane reorganization, mRNA translation, and meiosis but then begin to show abnormalities at the S-to-M transition that follows meiosis (Page and Orr-Weaver 1997). Unexpectedly, transcript destabilization fails to be triggered in these eggs. The simplest interpretation of this result is that transcript destabilization is triggered coincidently with this S-to-M transition and that it fails in in vitro-activated eggs because the temporal progression of egg activation is disrupted at this point. The fact that the least-pleiotropic RNA destabilization mutants (png, plu, and gnu) also proceed normally to this point and then fail to undergo both the S-to-M transition and transcript destabilization is fully consistent with this interpretation.

Previous analyses indicated that the S-to-M transition defect in png, plu, and gnu was likely to be a result of reduced cyclin-B levels and thus of reduced CDK activity (Fenger et al. 2000). We have presented several lines of evidence that argue against a simple interpretation of the png, plu, and gnu mutant effects on transcript instability, namely, that the S-to-M transition and high CDK activity per se is required for transcript destabilization. Briefly, we have shown that genetic suppression of the S-to-M transition defect in these mutants, under conditions in which CDK/cyclin activity is restored (Fenger et al. 2000; Lee et al. 2001), does not result in suppression of the transcript degradation defect. Reciprocally, we have shown that a severe reduction of CDK activity does not abrogate transcript destabilization. In addition, we have shown here that mutations that suppress the overreplication phenotype of the S-to-M transition mutants by means other than restoration of CDK/cyclin activity (e.g., by preventing replication as in fs(1)Ya mutants; see Lopez et al. 1994; Lopez and Wolfner 1997; Yu and Wolfner 2002) do not suppress the transcript destabilization defect. We recently extended these analyses by analyzing three additional dosage-sensitive suppressors of png (Lee et al. 2001): eIF-5A, which is involved in translation initiation and replication and mutation of which, like that of fs(1)Ya, does not result in restoration of cyclin-B levels, and two protein phosphatases, Ppl-87B and Pp2A-28D. In all three cases, while the mitotic defect of png was clearly suppressed, the RNA degradation phenotype was indistinguishable from that of png single mutants (W. Tadros and H. D. Lipshtiz, unpublished data).

PNG is a S/T kinase that is likely to be in a complex with PLU and GNU (Fenger et al. 2000). A model consistent with all of the above data is that transcript destabilization is triggered by the PNG-PLU-GNU signaling complex coincident with, but independent of, the complex’s role in regulating the S-to-M transition at the end of meiosis. To test this hypothesis it will be necessary to carry out genetic screens for suppressors of the png, plu, or gnu transcript degradation phenotype per se, rather than relying upon suppressors of the mitotic phenotype as we have done here. Identification of molecular targets of the PNG-PLU-GNU signaling complex may lead to the definition of independent pathways through which transcript destabilization and the S-to-M transition are regulated.

To fully understand the trigger for transcript destabilization it will be necessary to carry out a more systematic analysis of egg activation in Drosophila. The failure of both postmeiotic chromosome condensation (Page and Orr-Weaver 1997) and transcript degradation in eggs activated in vitro in a hypotonic buffer suggests that this method does not fully replicate the in vivoegg activation trigger. A difference previously pointed out (Heifetz et al. 2001) is that in vitro-activated eggs do not undergo any of the mechanical contractions that normally occur during transit through the oviducts of the female reproductive tract. Such mechanical pressures have been shown to be sufficient to activate late-stage Hymenopteran oocytes in the absence of hydration (Went and Krause 1973). Drosophilaeocytes can be activated to complete meiosis simply by the removal of the chorion (Endow and Komma 1997). If mechanically activated oocytes (or oocytes that are subjected to both mechanical activation and a hypotonic environment) undergo chromosome condensation and maternal transcript destabilization, then mechanosensory receptors would be implicated in the regulatory pathway for these aspects of egg activation.

There are several possible reasons why the fragile class of vitelline membrane mutants fails egg activation and transcript destabilization. First, particular chemical components of the vitelline membrane may be missing or incorrectly organized. For example, Vm26Ab or nudel class I mutants lack components of the vitelline membrane (LeMosy and Hashimoto 2000; LeMosy et al.
2000; Waring 2000). These proteins, and/or proteins that rely on interaction with Vn23 (the product of Vn26Ab) or NDL, may thus be unable to signal to the egg to activate it. A related possibility is that the defective vitelline membrane may fail to act as a scaffold for signals that are secreted during oogenesis but are required for egg activation later, as the egg leaves the ovary (examples of mutants with vitelline membrane defects that affect extra-embryonic signals are ndl, fs(1)Nasrat, and fs(1)polehole; Degelmann et al. 1990; LeMosy et al. 1998, 2000; LeMosy and Hashimoto 2000; Jimenez et al. 2002). Alternatively, if mechanical pressure is required to trigger certain aspects of egg activation, the fragile mutants may fail to activate because an intact eggshell is required for proper hydration and swelling, which, in turn, may be a prerequisite for mechanical pressure on the oocyte as it enters the oviduct.

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