Regulation of Maternal Transcript Destabilization During Egg Activation in Drosophila

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Manuscript received January 8, 2003
Accepted for publication March 11, 2003

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 and De Leon 1980; Duval *et al.* 1990; 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

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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*, *nanos*, *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 gu*, *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 S-to-M transition that occurs upon completion of meiosis. The PNG, PLU, and GNU proteins are thought to promote this S-to-M transition by maintaining high cyclin-B levels and hence activity of the cyclin-dependent kinase (CDK)/cyclin complex (Fenger *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 (Fenger *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 $y^1\ w^{1118}$. We assayed the following X chromosomal maternal-effect lethal lines generated by A. Hilfiker 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, 155, 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): *cribble*, *scraps*, *luckenhaft*, *cell-HA10/RF45*, *valois*, *cell-PK42*, *cell-QE1*, *cell-RH36*, *presto*, *syn-Hi10*, *syn-PL63*, *early*, *early-HM21/RU70*, *beintot*, *fs(2)TW1*, *early-PG44*, *early-RL4*, *grauzone*, *cortex*. EP lines and deficiency stocks used for mapping as well as $Vm26AB^{Q942}$ (Savant and Waring 1989), *Df(2L)cl7*, and *Df(2L)r10* were obtained from the Bloomington Drosophila Stock Center; ndl^2, ndl^6, ndl^9, ndl^{10}, ndl^{13}, ndl^{14}, ndl^{46}, ndl^{111}, ndl^{133}, ndl^{rm5} (Hong and Hashimoto 1995, 1996), and *Df(3L)CH12* were provided by E. LeMosy; $wisp^{11\text{-}600}$, $wisp^{12\text{-}3147}$, and $wisp^{14\text{-}1299}$ (Brent *et al.* 2000) by T. Hazelrigg; $twine^{HB5}$ (Schüpbach and Wieschaus 1989) by T. Schüpbach; $fs(1)Ya^2$ (Lin and Wolfner 1991) by M. Wolfner; $png^{13\text{-}1920}$, $png^{12\text{-}3318}$, $png^{13\text{-}1058}$, plu^6; gnu^{305} (Freeman *et al.* 1986; Shamanski and Orr-Weaver 1991), $png^{13\text{-}1920}\ fs(1)Ya^2$, 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^6 and fzy^7 (Dawson *et al.* 1993) were obtained from I. Dawson; and $cdk1^{E1\text{-}24}$ (Stern *et al.* 1993) was obtained from C. Lehner.

RNA extraction and analysis: Northern blots 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)KA6*, and *Df(1)RA47* 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)tR15*, and *Df(1)sc-J4* failed to complement *prage* while *Df(1)260-1*, *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; LeMosy 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 0.5 mg/ml of 4′,6-diamidino-2-phenylindole (Sigma, St. Louis) or a 1:4000 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-

opmental Studies Hybridoma Bank, Iowa City, IA; Chu and Klymkowsky 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 bicoid (BCD) protein in wild-type, *wisp*, *png*, *plu*, and *gnu* mutants we used either a rabbit or a rat anti-BCD antibody (Driever and Nüsslein-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 devitellinized 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 *prage* 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 $cdk1^{E1\text{-}24}$ 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.

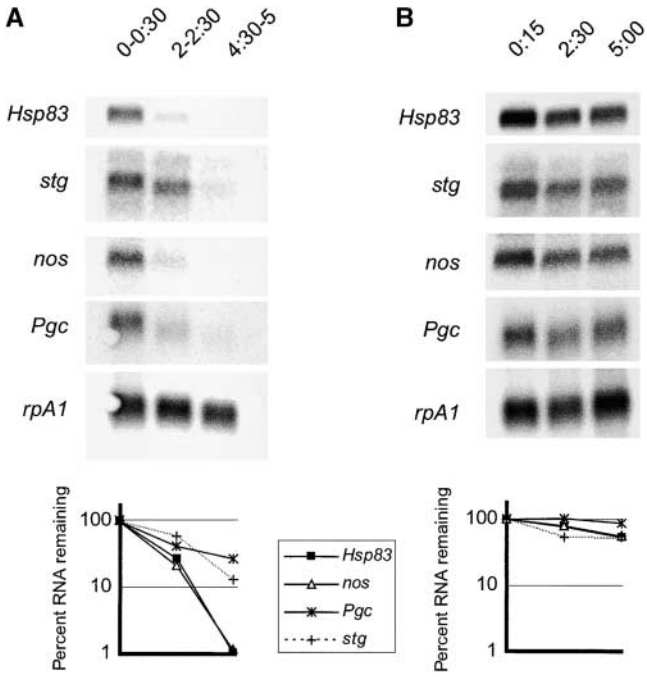

Figure 1.—Destabilization of maternal *Hsp83*, *string*, *nanos*, and *Pgc* transcripts requires egg activation. Northern blots are shown comparing these four unstable RNAs to *rpA1*, which serves as a stable loading control. Quantification of *Hsp83*, *string*, *nanos*, and *Pgc* transcripts, normalized to *rpA1*, is shown below each of the RNA blots. (A) *Hsp83*, *string*, *nanos*, and *Pgc* transcripts are unstable in unfertilized eggs activated *in vivo*. RNA was extracted from 30-min collections aged for 0, 2, and 4.5 hr. (B) *Hsp83*, *string*, *nanos*, and *Pgc* transcripts are stable in stage 14 oocytes aged for 15 min, 2.5 hr, and 5 hr.

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

Transcript destabilization loci

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

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 $Vm26Ab^{QJ42}$ 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.

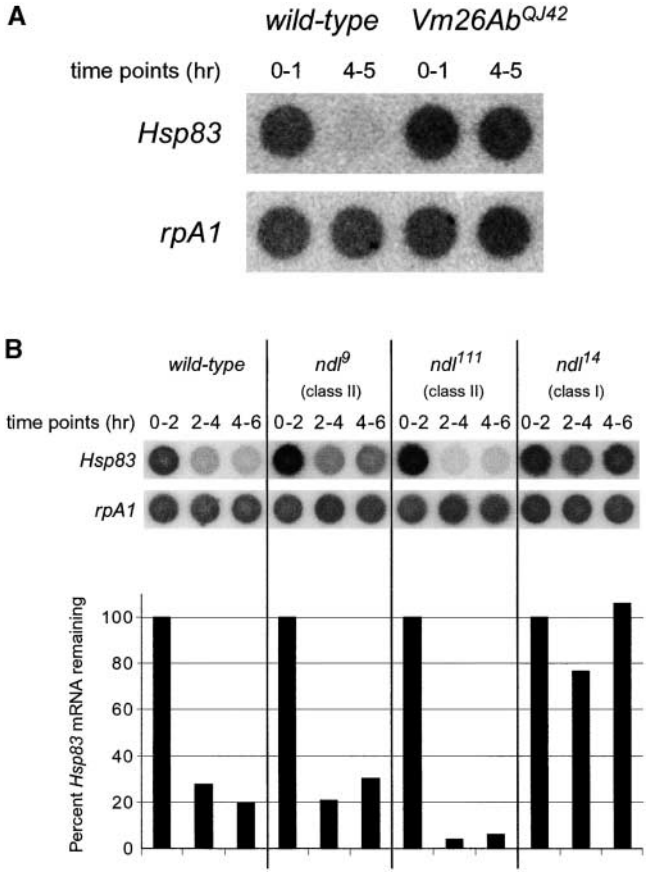

Figure 2.—Destabilization of maternal *Hsp83* transcripts requires proper vitelline membrane organization. RNA was extracted from staged embryo collections (age after egg deposition is indicated), samples were split, and dot blots were probed for *Hsp83* or *rpA1*, the latter as a loading control. (A) RNA degradation occurs in wild-type embryos but not in embryos from $Vm26Ab^{QJ42}$ mutant females. (B) RNA degradation occurs in wild-type embryos and in embryos from ndl^{111} (a class II allele) and ndl^{9} (class II) but not in embryos from ndl^{14} (class I) females.

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

TABLE 2

Egg activation defects in transcript destabilization mutants

Complementation group	Vitelline membrane reorganized	mRNA translated	Microtubules depolymerized	Meiosis completed	S-to-M transition occurs
cortex	+	−[a,b]	−[b]	−[b]	NT
grauzone	+	−[a,b]	−[b]	−[b]	NT
prage	+	−	+	−	NT
wispy	+	+	+	−[c]	NT
pan gu	+	+	+	+[d]	−
plutonium	+	+	+	+[d]	−
giant nuclei	+	+	+	+[e]	−

Only nonfragile mutants were assayed. Where no reference is cited, the results of this study are summarized. +, normal; −, defective; NT, not testable since meiotic progression fails.

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

[b] Page and Orr-Weaver (1996).

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

[d] Shamanski and Orr-Weaver (1991).

[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 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 nonfragile 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

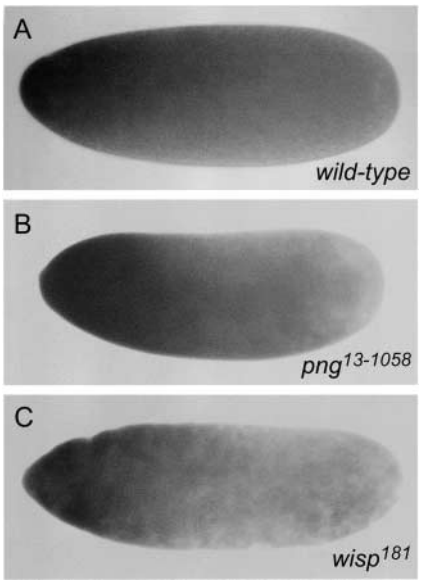

FIGURE 3.—mRNA translation is normal in a subset of the transcript destabilization mutants. (A) In wild-type embryos, BCD protein forms a gradient with its peak at the anterior pole (to the left). BCD is also translated in embryos from $png^{13\text{-}1058}$ (B) and $wispy^{181}$ (C) females.

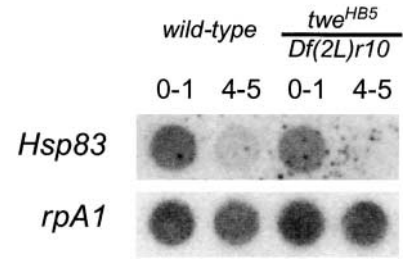

FIGURE 4.—Transcript degradation does not require normal meiotic progression. Dot blots of RNA from wild-type and twe^{HB5} hemizygous mutant females were probed for *Hsp83* transcripts (top) and then stripped and reprobed for *rpA1* transcripts (a loading control; bottom).

METHODS). Eggs from *wispy* mutants have been shown to be defective in both meiosis I and II, and embryos from mutant females fail to produce any syncytial nuclei (BRENT *et al.* 2000). The two remaining lines map to 1B4-E2 and comprise a complementation group that we call *prage* (*prg*; Table 1 and see MATERIALS AND METHODS). *prage* mutants, along with two previously identified transcript instability mutants, *cortex* and *grauzone* (LIEBERFARB *et al.* 1996; PAGE and ORR-WEAVER 1996; BASHIRULLAH *et al.* 1999), fail in meiotic progression.

Despite our recovery of mutations in these four meiotic loci, two lines of evidence prove that mRNA degradation is triggered independent of normal meiotic progression. First, several degradation mutants complete meiosis (*pan gu*, *plutonium*, and *giant nuclei*, described below). Second, we assayed a known meiotic mutant, *twine* (*twe*), to ask whether abnormal meiotic progression 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 (ALPHEY *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 png^{50}, png^{172}, and png^{246} alleles (FENGER *et al.* 2000) and one new allele, png^{48}. 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

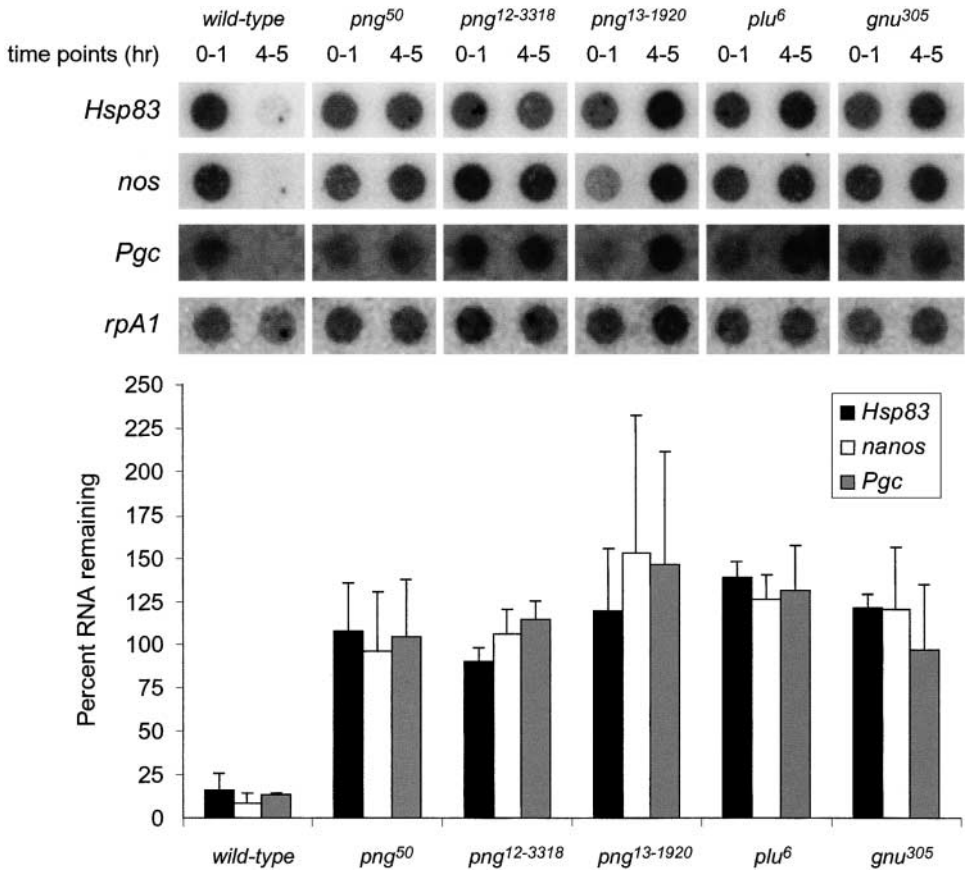

FIGURE 5.—Maternal transcript destabilization fails in S-to-M transition mutants. Dot blots of RNA extracted from embryos produced by wild-type, *png*^{50}, *png*$^{12\text{-}3318}$, *png*$^{13\text{-}1920}$, *plu*^{6}, and *gnu*^{305} females were sequentially probed for *Hsp83*, *nos*, *Pgc*, and *rpA1* (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.

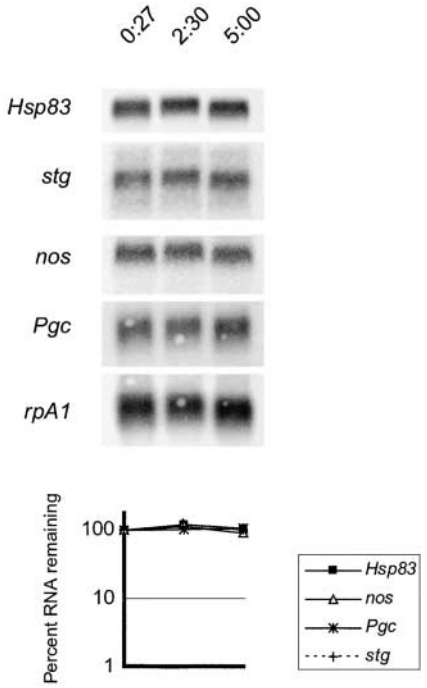

FIGURE 6.—Destabilization of maternal *Hsp83*, *string*, *nanos*, and *Pgc* transcripts fails in *in vitro*-activated eggs. A Northern blot is shown comparing these four unstable RNAs to *rpA1*, which serves as a stable loading control. Extracts were from *in vitro*-activated stage 14 oocytes aged for 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.

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.

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*, or *gnu* mutant females can partially suppress the early embryonic mitotic phenotype. To address whether the failure to destabilize maternal transcripts in *png*, *plu*, and *gnu* embryos might be caused by reduced CDK/cyclin activity, we used this same strategy to increase cyclin B or B3 levels. In genetic backgrounds where the mitotic phenotype was clearly suppressed (Figure 8A), we found no restoration of transcript instability (Figure 8B). These results show that restoration of CDK/cyclin activity to a level capable of substantially rescuing the S-M cell cycle defects is not sufficient to suppress the defects in maternal transcript destabilization. This result is consistent with the hypothesis that *png*, *plu*, and *gnu* regulate both the S-to-M transition and transcript destabilization but do so via distinct pathways.

Transcript destabilization occurs even when CDK activity is reduced or absent: A caveat to the preceding experiments is that the mitotic defect may be more easily suppressed than the transcript instability defect. In other words, if we had been able to restore CDK/cyclin activity to completely wild-type levels, then transcript instability would have been restored. An alternative strategy for testing the role of CDK/cyclin activity

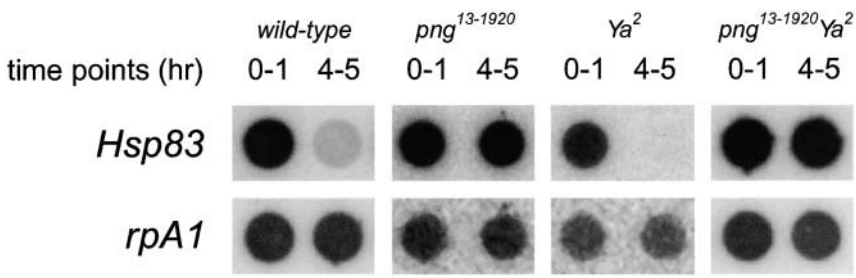

FIGURE 7.—Suppression of overreplication does not suppress failure to undergo transcript destabilization. Samples of RNA extracted from embryos produced by wild-type, $png^{13\text{-}1920}$ single-mutant, Ya^2 single-mutant, or $png^{13\text{-}1920}$ Ya^2 double-mutant females were split, loaded onto a dot blot, and probed for *Hsp83* or *rpA1* transcripts.

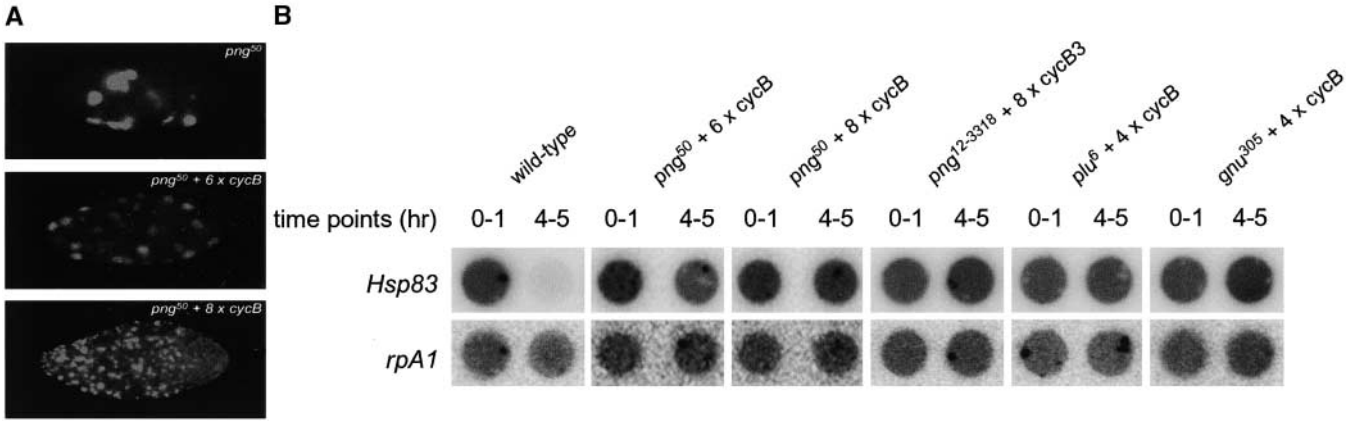

FIGURE 8.—Restoration of CDK/cyclin activity does not suppress the defect in transcript destabilization. (A) Extra doses of cyclin B suppress the mitotic phenotype of *png*. Embryos were stained with PicoGreen to visualize DNA. Shown are representative embryos from mothers mutant for png^{50} alone (top), png^{50} with six extra copies of cyclin B (middle), and png^{50} 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, png^{50} with six extra copies of cyclin B, png^{50} with eight extra copies of cyclin B, $png^{12\text{-}3318}$ with eight extra copies of cyclin B3, plu^{6} with four extra copies of cyclin B, or gnu^{305} 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*, $cdk1^{E1\text{-}24}$ (STERN *et al.* 1993). In control experiments, *cdk1* homozygous mutant females were allowed to lay eggs at the permissive temperature of 18°, 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°, 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

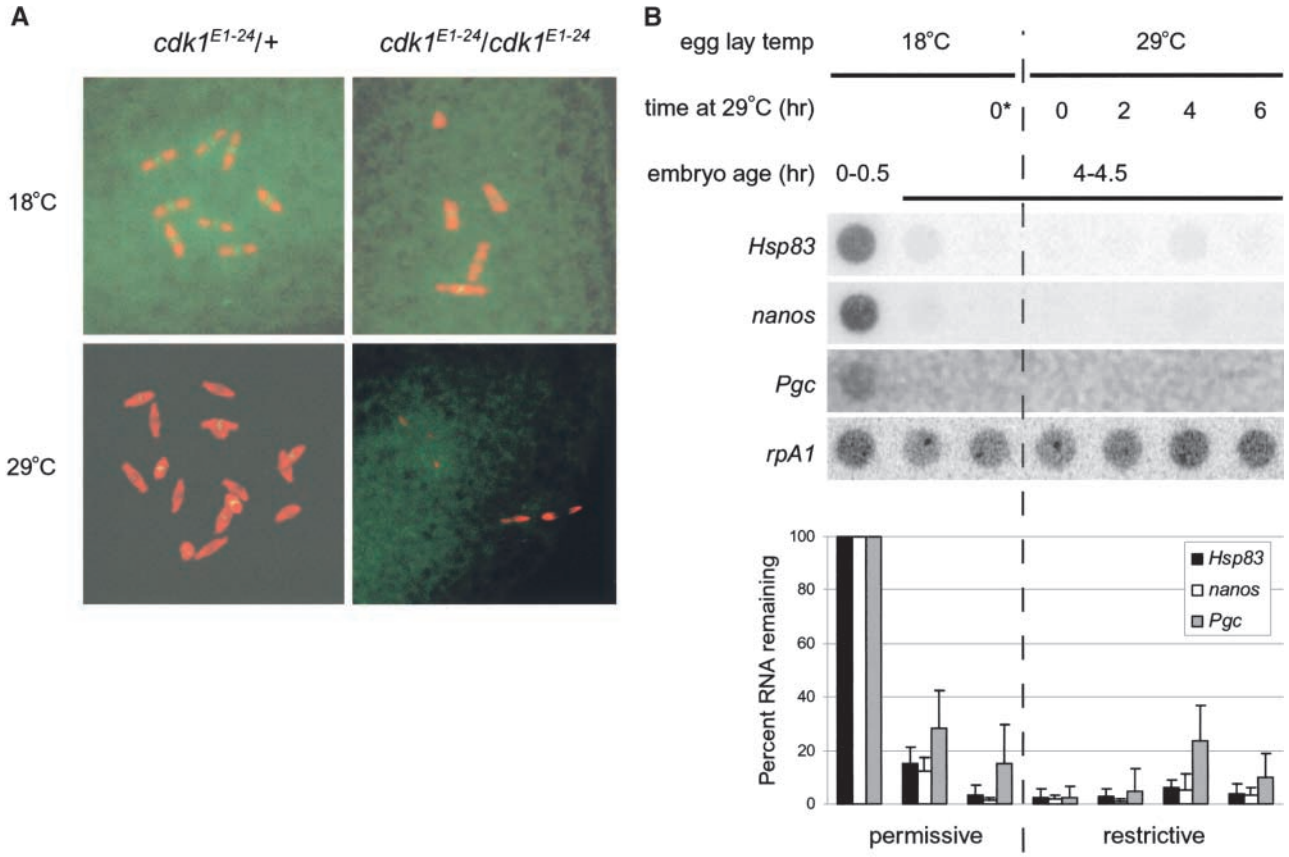

FIGURE 9.—Lowering CDK activity does not affect maternal transcript destabilization. (A) Embryos from *cdk1* temperature-sensitive mutant females show severe cell cycle defects. Embryos 0–1 hr old were stained with PicoGreen to visualize DNA (green) and anti-β-tubulin to visualize spindles (red). Representative embryos are shown from heterozygous $cdk1^{E1\text{-}24}/In(2LR)Gla, wg^{Gla\text{-}1}$ ($cdk1^{E1\text{-}24}/+$), or homozygous $cdk1^{E1\text{-}24}/cdk1^{E1\text{-}24}$ females at the permissive temperature of 18° (top) *vs.* after the females had been at the restrictive temperature of 29° for 4.5 hr (bottom). (B) Dot blot of RNA extracted from embryos produced by $cdk1^{E1\text{-}24}/cdk1^{E1\text{-}24}$ females. The period for which the flies were kept at 29° and the temperature at which the embryos were laid and incubated are indicated above the dot blots. Quantification of *Hsp83*, *nanos*, and *Pgc* transcripts, normalized to *rpA1*, is shown below the dot blots. *, indicates embryos were laid at 18° and then incubated at 29°.

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 (SALLÉS *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 vitelline 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 destabili-

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 independent 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 degradation 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, PpI-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. Lipshitz, 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 vivo* egg activation trigger. A difference previously pointed out (Heifetz *et al.* 2001) is that *in vitro*-activated eggs do not undergo any of the mechanical constrictions 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). Drosophila oocytes 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 sV23 (the product of *Vm26Ab*) 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.

We thank T. Hazelrigg, C. Hashimoto, C. Lehner, E. LeMosy, T. Orr-Weaver, T. Schüpbach, M. Wolfner, and the Bloomington Drosophila Stock Center for mutant lines; E. Gottlieb, C. Goodman, and the Developmental Studies Hybridoma Bank, Iowa City, Iowa for providing antibodies. W.T. was supported in part by an Ontario Graduate Scholarship; S.A.H. and R.L.C. in part by scholarships from the Medical Research Council of Canada; and J.L.S. in part by a scholarship from the National Science and Engineering Research Council of Canada. In all cases supplementary funds were provided by the Research Training Center of the Hospital for Sick Children's Research Institute. H.D.L. is Canada Research Chair (CRC) in Developmental Biology at the University of Toronto. This research was supported by funds from the CRC Program and an operating grant (MOP-14409) to H.D.L. from the Canadian Institutes of Health Research.

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Communicating editor: T. Schüpbach