The *Drosophila* prage Gene, Required for Maternal Transcript Destabilization in Embryos, Encodes a Predicted RNA Exonuclease

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**ABSTRACT** Egg activation, the transition of mature oocytes into developing embryos, is critical for the initiation of embryogenesis. This process is characterized by resumption of meiosis, changes in the egg's coverings and by alterations in the transcriptome and proteome of the egg; all of these occur in the absence of new transcription. Activation of the egg is prompted by ionic changes in the cytoplasm (usually a rise in cytosolic calcium levels) that are triggered by fertilization in some animals and by mechanosensitive cues in others. The egg's transcriptome is dramatically altered during the process, including by the removal of many maternal mRNAs that are not needed for embryogenesis. However, the mechanisms and regulators of this selective RNA degradation are not yet fully known. Forward genetic approaches in *Drosophila* have identified maternal-effect genes whose mutations prevent the transcriptome changes. One of these genes, *prage* (*prg*), was identified by Tadros et al. in a screen for mutants that fail to destabilize maternal transcripts. We identified the molecular nature of the *prg* gene through a combination of deficiency mapping, complementation analysis, and DNA sequencing of both extant *prg* mutant alleles. We find that *prg* encodes a ubiquitously expressed predicted exonuclease, consistent with its role in maternal mRNA destabilization during egg activation.

The transition from egg to embryo involves major changes in cell fate and potential, including progression of the cell cycle from meiotic arrest through completion of meiosis to the initiation of mitosis (reviewed in Clift and Schuh 2013; Horner and Wolfner 2008). This developmental transition involves major molecular changes in the egg including the polyadenylation of some maternal mRNAs (e.g., for *Drosophila*: Benoit et al. 2008; Cui et al. 2008, 2013) and the degradation of others (e.g., Chen et al. 2014; Giraldez et al. 2006; Kugler et al. 2013), synthesis of new proteins, and phospho-modulation of others (Guo et al. 2015; Krauchunas et al. 2012; Roux et al. 2006, 2008). The molecular changes of “egg activation” are critical for pronuclear formation and cell cycle modulation, for embryonic patterning and morphogenesis, and for structural and chemical changes to the egg’s outer coverings to block polyspermy and support the developing embryo.

Remarkably, most of this transition is driven or conducted entirely by parental (mostly maternal) molecules. In particular, maternally encoded mRNAs drive production of proteins needed for oocyte maturation and maintenance, for reversing this differentiated state after fertilization to permit totipotency, and for initiating early embryonic cell divisions and cell fate decisions. The maternal mRNAs needed for embryonic development must be kept stable until fertilization, and then must be translated at the appropriate time and place (e.g., reviewed in Kugler and Lasko 2009; Tadros and Lipshitz 2009, 2005; Tadros et al. 2007b; Yartseva and Giraldez 2015). At the same time, for embryogenesis to proceed normally, maternal RNAs must be eliminated at the appropriate time and place (e.g., Giraldez et al. 2006; Kugler et al. 2013) so...
that the zygotic genome can take over. For example, some cell cycle regulators must be eliminated in order for meiosis to resume (e.g., Swan and Schüpbach 2007; Pesin and Orr-Weaver 2007) and complete, in preparation for pronuclear fusion and embryonic mitoses; indeed many of the maternally stored mRNAs that are degraded at egg activation have roles in cell cycle regulation, (e.g., meiotic cyclins; Tadros et al. 2007a). Additionally, some mRNAs encoding localized proteins are initially present throughout the oocyte and undergo massive destabilization during egg activation except in protected local areas. Transcripts that fall into this latter category in Drosophila include those from the Hsp83, nanos, and Pgr genes (Bashirullah et al. 1999, 2001). Destruction of maternal mRNAs occurs in two general phases (reviewed in Laver et al. 2015). The first phase is maternally driven: products that had been loaded into the oocyte during oogenesis are activated, and they degrade certain mRNAs; in Drosophila, 20% of stored maternal transcripts are subject to this degradation (Tadros and Lipshitz 2009). The second phase of degradation of maternal transcripts is dependent on zygotic gene expression. In Drosophila, an additional 15% of maternal transcripts are degraded under this control.

Identifying the regulators of stability and degradation of maternal RNAs has been challenging, both because egg activation is rapid and because many of its regulators are maternally encoded and therefore cannot be detected by looking for changes in the egg’s transcriptome. However, studies in model systems have identified some regulators of the fate of maternal mRNAs. For example, in Drosophila, the maternal phase of degradation requires activity of the PAN GU (PNG) kinase complex, causing translation and activation of another key component the SMAUG (SMG) protein (Tadros et al. 2007a). SMG binds to specific elements in certain maternal mRNAs, and targets these mRNAs for degradation by recruiting a deadenylase complex (Benoit et al. 2009; Chen et al. 2014; Semotok et al. 2008). In another example, a zygotically encoded miR small-RNA has been shown to mediate the degradation of maternal RNAs in zebrafish (Giraldez et al. 2006; Bazzini et al. 2012). A similar mechanism, with a different miR, likely operates in Drosophila (Bushati et al. 2008), and the piRNA pathway is also involved in regulating maternal mRNA stability in Drosophila embryos (Rouget et al. 2010). But knowledge of the machinery that selectively degrades maternal mRNAs is incomplete.

A genetic approach, such as that taken by Tadros et al. (2003) in Drosophila melanogaster, provides a way to identify important regulators of maternal mRNA stability. These authors identified several X-linked genes whose female sterile mutations affected the destabilization of maternally encoded Hsp83 mRNA in early embryos (Tadros et al. 2003). Many of these loci were linked to key pathways during egg activation. Among the molecules identified in this screen was a conserved GLD2 poly(A) polymerase, wisp, which extends poly(A) tails of a large number of maternal mRNAs (Cui et al. 2008, 2013; Benoit et al. 2008), permitting their stability and, where tested, their translation (Benoit et al. 2008; Cui et al. 2008). Two loci were identified as graguzone and cortex, which were known to be required for completion of female meiosis (Page and Orr-Weaver 1996; Lieberfarb et al. 1996; Chen et al. 2000; Swan and Schupbach 2007). Genes encoding subunits of the early embryonic cell cycle regulator PNG kinase complex, including pgr, plutonium (plu), and giant nuclei (gun) (Lee et al. 2003), were also detected in the screen. Another mutation discovered in that screen was pgr, whose molecular identity was unknown. Offspring from pgr mutant mothers fail to destabilize maternal Hsp83 mRNA, suggesting that PGR plays some role in maternal mRNA degradation. Here, we report molecular mapping and sequence analysis of pgr mutant alleles that demonstrate that pgr encodes a predicted RNA exonuclease, suggesting a role as part of the enzymatic machinery that degrades maternal mRNAs.

**MATERIALS AND METHODS**

**Drosophila stocks and complementation tests**

pgr^{64A}/FM6 and pgr^{22}/FM6 (Tadros et al. 2003) were kindly provided by W. Tadros and H. Lipshitz (Hospital for Sick Children, University of Toronto, Canada). Drosophila strains carrying deficiencies [Df(1)Bsc719/Binsincys, Df(1)Ed6565/FM7h, Df(1)A94/FM6, Df(1)Bsc530/Binsincys, Df(1)D260-1/FM4 and Df(1)AD11/FM7c] or P-element insertion in the pgr region (P[MaeMts.6.11]CG42666[CG0337], P[Eyg2]CG42666[ET1466] and P[XP]CG42666[ET1828]) were obtained from the Bloomington Stock Center. For complementation tests, we crossed approximately five 3- to 5-d-old virgin females of each strain to pgr^{64A} and to pgr^{22}, and scored the fertility of their pgr/DF (or P-insertion) female progeny.

**Nucleic acid extraction and PCRs**

To identify the location of the mutant lesions in pgr, whole fly genomic DNA was extracted from pgr^{64A} and pgr^{22} males as in Sirot et al. (2014) and used as template to amplify target regions using GoTaq PCR amplification kit (Promega, Madison, WI). DNA sequencing was performed by Cornell Life Sciences Core Laboratories Center (Cornell University, Ithaca, NY). To examine pgr expression, total RNA was extracted from 3- to 5-d-old adult males, adult females, and embryos collected 0-2, 2-4, or 4-6 hr after egg laying, cDNA was synthesized, and RT-PCR carried out as described previously (Cui et al. 2008; Findlay et al. 2014). Primers for genomic and RT-PCRs are listed in Supplemental Material, Table S1.

**Data availability**

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.
RESULTS

prage alleles carry nonsense mutations in CG42666, a gene that encodes a predicted exonuclease

The prage gene was previously reported as in polytene chromosome region 1B4-1E2 (Tadros et al. 2003). To localize the prage gene more precisely, we carried out complementation analysis between both prage mutant alleles (prage16A and prage32) and six deficiencies in or near the 1B4-1E2 region (Figure 1 and Table S2). Df(1)BSC719 failed to complement both prage alleles, while another line, Df(1)A94, carrying a partially overlapping deficiency complemented both alleles. These results suggested that the prage mutation was in chromosome region 2B12-13. Genomic DNA corresponding to the predicted exons of four genes in this region [CG14812, deltaCOP (CG14813), CG14814, Med18 (CG14802)] was PCR-amplified and then sequenced in both prage mutant lines. We found no difference from wild type in these four genes for either prage allele. Considering the possibility that the cytological breakpoint in the deficiency might not have been perfectly annotated relative to the genome sequence, we expanded our search to include three additional genes [CG42666 (originally called CG14801), CG14810, CG14811] from the adjacent region, 2B10. No differences from wild-type sequence were seen in either prage mutant chromosome for CG14810 and CG14811. However, as shown in Figure 2 and Figure 3 and detailed below, we found that both prage alleles have molecular lesions in the predicted ORF of CG42666. Each mutant contains a C-to-T single nucleotide change. In each allele this change generates a premature stop codon in the reading frame that results in a truncated protein. These data suggest that CG42666 is the prage gene. Based on the prage mutant phenotype, one would expect prage RNA to be present in oocytes and early embryos. Our RT-PCR for CG42666 RNA confirmed this expected expression pattern (Figure S1; see also Gelbart and Emmert 2013).

To confirm that the prage gene corresponds to CG42666, we carried out complementation tests of prage mutations with P-element insertions in CG42666. We tested for complementation between both prage mutant alleles and three P-element insertion lines available from Bloomington Stock Center. Two insertions, P[Mae UAS.6.11]/CG42666fl1689 (Mae) and P[EPgy2]/CG42666EY21466 (EPgy2), failed to complement both prage mutant alleles. However, P[XP]/CG42666EY21466 (XP) unexpectedly complemented both prage alleles. We confirmed, by RT-PCR with primers specific to the XP line, that this line had an insertion in CG42666 (Figure S2). Insertions Mae and EPgy2 are expected to disrupt all six RNA isoforms of CG42666, whereas insertion XP only interrupts the PE isoform (Figure 3). Our data suggest that disruption of this single isoform by the XP insertion does not eliminate function of CG42666 gene; the other isoforms are likely expressed and produce functional PRG protein. Whether and how CG42666 isoforms can compensate for each other requires further study, but the results from the Mae and EPgy2 insertion lines confirm that CG42666 is the prage gene.

CG42666 encodes 6 RNA isoforms (PE, PB, PD, PF, PC, PA) with differing 5’ ends, according to the latest annotation of the Drosophila genome (http://www.flybase.org) (Figure 3). The PA isoform, for example, encodes a predicted protein of 761 amino acids. Sequence similarity analysis reveals that the CG42666 protein (hereafter called the PRG protein) is a putative RNA exonuclease: Interpro sequence analysis and classification identified a single conserved domain near the C-terminal end of the protein with terms “Exonuclease” (IPR006055), “Ribonuclease H-like domain” (IPR012337), and “Exonuclease, RNase T/DNA polymerase III” (IPR013520). Each of the mutant prage alleles has a single base pair change toward the 3’ end of CG42666, in the region that is shared by all six PRG isoforms. prage16A and prage32 are both nonsense mutations, truncating their PRG proteins to 72 amino acids and 373 amino acids (relative to the PA isoform), respectively, and deleting the conserved exonuclease domain from each. Database searches revealed that the REX1_like exonuclease domain in the PRG protein is conserved among eukaryotes (Figure 4). In fruit flies, a domain of this type is also found in three additional genes. Of these three genes, the sequence of the predicted exonuclease domain of CG12877 is the most similar to that of prage.

DISCUSSION

Egg activation is a coordinated process that is critical to initiate embryonic development (reviewed in Clift and Schuh 2013; Horner and Wolfiner 2008; Krauchunas and Wolfiner 2013; Tadros and Lipshitz 2009; Tadros et al. 2007b; Yartseva and Giraldez 2015). Changes in the transcriptome during egg activation (Giraldez et al. 2006; Kugler et al. 2013) are fundamental, as they will allow changes in the spectrum of proteins in the cell that transition its state from that of differentiated mature oocyte to totipotent dividing embryo. One enzymatic player in transcriptome dynamics during Drosophila egg activation is known: the GLD2-family poly(A) polymerase encoded by wispy is essential for egg activation and early embryogenesis (Benoit et al. 2008; Cui et al. 2008). WISPY polyadenylates a large fraction of the maternally loaded mRNAs in the egg (Cui et al. 2013), presumably facilitating their efficient translation. But the machinery that catalyzes the degradation of maternal mRNAs during this transition is less fully understood. A genetic screen in Drosophila was successful in pinpointing candidates for roles in this degradation; genes whose mutants disrupted the destabilization of maternal mRNAs (Tadros et al. 2003). In this study we discovered that one of those genes, prage, encodes a predicted RNA exonuclease.

Approximately 55% of the Drosophila genome is represented as mRNA in the mature oocyte (Tadros et al. 2007b). Approximately 1600 (20%) of these maternally stored mRNAs are degraded upon egg activation. Tadros et al. (2007a) showed that two-thirds of these destabilized transcripts are regulated through the SMG protein, and are
enriched for elements critical for cell cycle regulation. The remaining one-third are enriched for genes required for oogenesis (Tadros et al. 2007b). Evidence from yeast and *Xenopus* suggests that the first and often rate-limiting step in eukaryotic mRNA decay is the shortening of the poly(A) tail and the major deadenylase activity in *Drosophila* embryos is from the CCR4/POP2/NOT complex (reviewed in Temme et al. 2014). For two different mRNAs, SMG has been shown to recruit the CCR4/POP2/NOT deadenylase complex to the target mRNA, which shortens the poly(A) tail (Semotok et al. 2005; Zaessinger et al. 2006). Specific sequences in the 3′ UTR can target cytoplasmic mRNA for deadenylation, followed by either exosome (3′ to 5′) degradation or exonuclease (5′ to 3′) decapping/degradation (Houseley and Tollervey 2009). Both mechanisms require an exonucleolytic activity to complete the degradation (Houseley and Tollervey 2009). It is not known which exonuclease(s) degrade maternal mRNAs in *Drosophila* embryos.

The function of REX1-like proteins like PRG is unknown in most organisms. The only role that has been reported is in yeast; its *REXO1* gene’s function is required for RNA editing and maturation (Nariai et al. 2005; van Hoof et al. 2000). The sequence data presented here, in conjunction with the phenotypic data reported by Tadros et al. (2003) make it tempting to speculate that prg encodes an exonuclease that is actively involved in degrading maternal mRNA during the egg-to-embryo transition. Although it still remains to be demonstrated that the PRG protein has exonuclease activity, both *prg* alleles that fail to destabilize maternal mRNAs (Tadros et al. 2003) remove PRG’s predicted exonuclease domain.

PRG’s identity as a predicted RNA exonuclease raises several intriguing questions, beyond the obvious ones of its mechanism, potential partners, and targets. First, RNAseq (Gelbart and Emmert 2013) and microarray (Chintapalli et al. 2007) data indicate that the *prg* gene is expressed in stages and tissues that are unrelated to the egg-to-embryo transition (for example, it is expressed in adult males); we have verified some of these data (Figure S2). Although the existing *prg* mutant alleles remove its exonuclease domain and thus are likely null for this function in the germline (supported by the fact that homozygotes and hemizygotes are equally sterile), both are viable. This suggests that either *prg’s* activity is not needed in later somatic tissues, or that there are compensatory activities (perhaps from *CG12877* and/or the other two genes that encode proteins with exonuclease domains with some similarity to PRG’s). Alternatively, PRG’s translation might be regulated to restrict the protein’s presence to the female’s germline and early embryos. All of these will be fertile areas for future study.

Second, since some of the machinery required for the maternal/zygotic transition of the transcriptome is known, it will be intriguing to

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**Figure 3** *prg* gene structure. The map shows the positions of the *prg* RNA and protein isoforms, and the locations of the *prg* mutant lesions, as well as the P-element insertions that were used in complementation tests to confirm gene assignment. Data are from Flybase (http://www.flybase.org). Orange denotes regions of the transcript that are translated into protein (purple shows those regions within the protein). Gray indicates exon regions that are not translated. Black bars mark the positions of the mutations in the two extant *prg* alleles. Blue triangles show the locations of P-element insertions.
determine how prg relates to it. For example, the machinery includes the PNG kinase complex that upon egg activation triggers the translation of several maternal mRNAs including the one encoding SMG, the major factor that destabilizes maternal mRNAs in the early embryos (Tadros et al. 2007a). How does prg activity interface with the SMG-dependent pathway? Are prg and smg parts of independent pathways that act at different times? Or might prg control the stability of smg mRNA (assuming that smg mRNA must be translated upon egg activation), thus potentially regulating the amount of SMG or of components or assemblers of PNG kinase? Moreover, how is PRG itself regulated to act, including potentially to interface with the SMG pathway, so that it only degrades its targets after fertilization? Perhaps its targets are only modified appropriately at this time. Alternatively, perhaps PRG’s translation requires progression past a critical stage of early development, such as the meiotic progression mediated by the products of the cortex or graunzone genes (Lieberfarb et al. 1996; Pesin and Orr-Weaver 2007; Swan and Schüpbach 2007), or requires elongation of its poly(A) tail by the WISPY cytoplasmic poly(A) polymerase, as is the case for BCD (Cui et al. 2008; Benoit et al. 2008). It is also possible that PRG protein may be present in a nonfunctional state in oocytes, requiring post-translational modification during egg activation (e.g., Krauchunas et al. 2012), or activation of a cofactor, or both, for its activity.

Finally, PolII binding assays (Chen et al. 2013) have identified prg as one of ~100 genes that start significant transcription during cycles 8–12. That prg mRNA is both maternally loaded and also zygotically transcribed prior to the maternal-to-zygotic transition (MZT) raises the intriguing possibility that PRG could play roles in regulating RNA stability before the MZT (potentially even contributing to the initiation of the MZT), and also in the second wave of maternal mRNA degradation that occurs subsequently. Our identification of prg as CG42666, a predicted RNA exonuclease, permits the future investigation of these intriguing questions and further dissection of the molecular mechanisms that modulate maternal mRNA stability during the egg-to-embryo transition.

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Figure 4 Alignment of REX_1-family genes. Amino acid sequence of the putative exonuclease domain of PRG was aligned against those of homologs from fly (CG12877, CG6833, and CG8368), human, mouse, rat, worm, and yeast. Identical or similar residues are highlighted in colored boxes.
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