# Functional characterization of the *Drosophila MRP* (mitochondrial RNA processing) RNA gene

MARY D. SCHNEIDER,[1,5] ANUPINDER K. BAINS,[1] T.K. RAJENDRA,[2,3] ZBIGNIEW DOMINSKI,[4] A. GREGORY MATERA,[2,3] and ANDREW J. SIMMONDS[1]

[1]Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G2H7, Canada
[2]Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599, USA
[3]Department of Genetics, University of North Carolina, Chapel Hill, North Carolina 27599, USA
[4]Department of Biochemistry & Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599, USA

## ABSTRACT

***MRP* RNA is a noncoding RNA component of RNase mitochondrial RNA processing (MRP), a multi-protein eukaryotic endoribonuclease reported to function in multiple cellular processes, including ribosomal RNA processing, mitochondrial DNA replication, and cell cycle regulation. A recent study predicted a potential *Drosophila* ortholog of *MRP* RNA (CR33682) by computer-based genome analysis. We have confirmed the expression of this gene and characterized the phenotype associated with this locus. Flies with mutations that specifically affect *MRP* RNA show defects in growth and development that begin in the early larval period and end in larval death during the second instar stage. We present several lines of evidence demonstrating a role for *Drosophila MRP* RNA in rRNA processing. The nuclear fraction of *Drosophila MRP* RNA localizes to the nucleolus. Further, a mutant strain shows defects in rRNA processing that include a defect in 5.8S rRNA processing, typical of *MRP* RNA mutants in other species, as well as defects in early stages of rRNA processing.**

**Keywords: *Drosophila*; RNAse MRP; ribosomal RNA processing; noncoding RNA**

## INTRODUCTION

*MRP* RNA was initially identified as a component of RNase mitochondrial RNA processing (*MRP*), an essential, highly conserved eukaryotic ribonucleoprotein complex that also contains approximately 10 proteins (Yuan et al. 1991; Schmitt and Clayton 1992; Chamberlain et al. 1998; Welting et al. 2004; Salinas et al. 2005). Studies in *Saccharomyces cerevisiae* and mammals have identified several RNase MRP substrates, associated with diverse cellular processes that are cleaved in a site-specific manner. In mammalian cells, RNase MRP was implicated in processing mitochondrial transcripts complementary to the origin of replication of the mitochondrial chromosome, to produce primers for mitochondrial DNA replication (Chang and Clayton 1987). In *S. cerevisiae*, it was known for some time to cleave the rRNA precursor at a single site, leading to the formation of the 5′ end of 5.8S rRNA (Schmitt and Clayton 1993; Lygerou et al. 1996). However, the results of a recent study show that inactivation of *MRP* RNA results in additional rRNA processing defects that occur in early stages of the pathway (Lindahl et al. 2009).

Cleavage of B-type cyclin (*CLB2*) mRNA mediated by RNase MRP to regulate cell cycle progression was also observed in *S. cerevisiae* (Gill et al. 2004). In human cells, a defect in rRNA processing and regulation of cyclin B mRNA were also observed; however, direct cleavage of these substrates by RNase MRP has not yet been demonstrated (Hermanns et al. 2005; Thiel et al. 2005, 2007). Recently, human *MRP* RNA (*RMRP*) was identified as a component of a novel complex with telomerase reverse transcriptase (TERT) that functions as an RNA-dependent RNA polymerase. In this complex, *RMRP* acts as a template for the synthesis of ds*RMRP* RNA, primed by a 3′ fold-back structure. ds*RMRP* RNA associates with Ago2 and is processed into 22-nucleotide (nt) dsRNAs in a Dicer-dependent fashion. It has been proposed that these dsRNAs represent a novel class of endogenous siRNAs that regulate *RMRP* levels by a negative feedback mechanism (Maida et al. 2009).

*MRP* RNA was the first noncoding RNA to be associated with a human disease. Ridanpää et al. (2001) described a set of mutations in the *RMRP* gene that cosegregate with the phenotype of the autosomal recessive disease cartilage-hair

[5]**Present address:** Department of Medical Genetics, University of Alberta, Edmonton, Alberta T6G2H7, Canada.

**Reprint requests to:** Andrew J. Simmonds, Department of Cell Biology, Faculty of Medicine and Dentistry, 5-14 Medical Sciences Building, University of Alberta, Edmonton, Alberta T6G 0A5, Canada; e-mail: andrew.simmonds@ualberta.ca; fax: (780) 492-8450.

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hypoplasia (CHH). Various *RMRP* mutations are now known to be associated with other disorders such as Omenn syndrome and anauxetic dysplasia (Thiel et al. 2005; Martin and Li 2007). Short stature is common to all of these diseases; however, each disease presents a characteristic spectrum of other defects affecting a broad range of organs (Makitie et al. 2001a,b; Martin and Li 2007; Toiviainen-Salo et al. 2008). Recent studies have identified correlations between specific molecular defects and disease symptoms in patients with various *RMRP* mutations. Mutations that affect rRNA processing are correlated with the characteristic anauxetic dysplasia symptom of severe skeletal defects. Mutations that result in increased cyclin B mRNA levels are correlated with symptoms characteristic of CHH. These patients show less severe skeletal defects but have higher incidences of immunodeficiency, hematological abnormalities, and cancer (Thiel et al. 2005, 2007).

We report the expression of the *Drosophila* ortholog of *MRP* RNA (CR33682), previously predicted by a bioinformatics screen for *MRP* RNA sequences (Fig 1A; Piccinelli et al. 2005). Characterization of a mutant strain shows that *Drosophila* MRP (*dMRP*) is an essential gene. *dMRP* mutants display a severe impairment in growth, a characteristic shared with human diseases carrying mutations in this gene (Martin and Li 2007). These phenotypic defects could be the result of impairments at different stages of rRNA processing that we have observed. These include the classic defect in processing 5.8S rRNA that has been associated with human and *S. cerevisiae* RNase MRP mutants (Schmitt and Clayton 1993; Lygerou et al. 1996; Hermanns et al. 2005; Thiel et al. 2005), as well as a defect in early rRNA processing similar to a defect recently reported in *S. cerevisiae* (Lindahl et al. 2009).

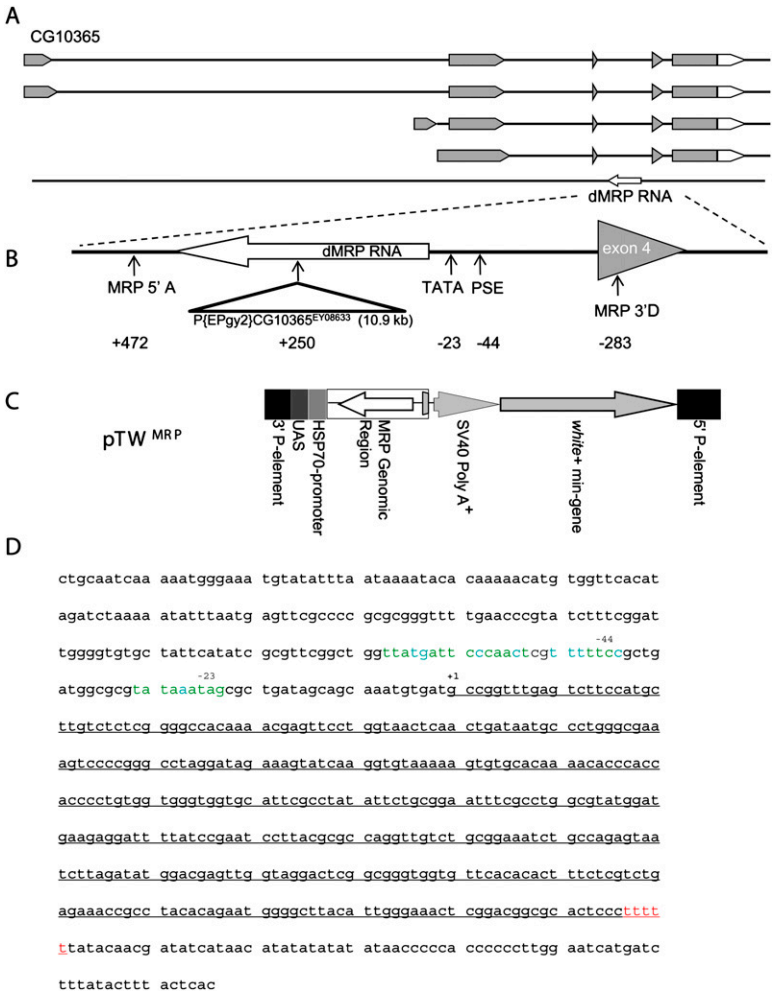


**FIGURE 1.** The *Drosophila* (*dMRP*) RNA gene. (*A*) Piccinelli et al. (2005) predicted a potential *dMRP* RNA encoded by a gene located in the third intron of CG10365, transcribed from the opposite strand. (Gray) Translated sequences. (*B*) Expanded view of the *dMRP* gene. P{EPgy2} CG10365$^{EY08633}$ disrupts *dMRP* in the *dMRP*$^{EY08633}$ mutant strain. Positions of primers used to amplify the genomic region used to make the *pTW -MRP* strain (MRP 5′A and MRP 5′D) are indicated. (*C*) Structure of *pTW* $^{MRP}$ used to make the *pTW -MRP* strain. Regions inserted into chromosome 2 by untargeted integration via 5′ and 3′ P-element sequences are shown. *dMRP* genomic sequences were inserted in the opposite orientation relative to the UAS/Hsp70 transcriptional regulatory sequences so that *dMRP* RNA was expressed from its own regulatory sequences. The *white* (+) mini-gene encodes a red eye-color gene for selecting transgenic individuals. (*D*) *dMRP* sequence with potential Pol III regulatory elements. The sequence of the entire intron containing *dMRP* is shown. Transcribed sequences are underlined. Positions of PSE (−44 nt) and TATA-like element (−23 nt) upstream of the transcription start site (+1) are indicated *above* the sequence. (Green nucleotides in promoter elements) Identical nucleotides in all reference sequences (Hernandez et al. 2007), (blue nucleotides) the same as at least one of the reference sequences, (red nucleotides) consensus Pol III terminator (Hernandez et al. 2007).

## RESULTS

### *dMRP* RNA is expressed throughout the *Drosophila* life cycle and localizes to the nucleolus

Expression of *dMRP* RNA was detected throughout the *Drosophila* life cycle (Fig. 2A,B). This is consistent with its role as a component of RNase MRP in the essential cellular processes of ribosome biogenesis, mitochondrial DNA replication, and cell cycle regulation. The major fraction of RNase MRP in *S. cerevisiae* (Schmitt and Clayton 1993) and *MRP* RNA in mammalian cells is in the nucleolus, specifically in the dense fibrillar component marked by fibrillarin (Li et al. 1994; Jacobson et al. 1995). In syncytial *Drosophila* embryos, *dMRP* RNA colocalizes with *Drosophila* fibrillarin in the nucleolus (Fig. 2C). Similar results were

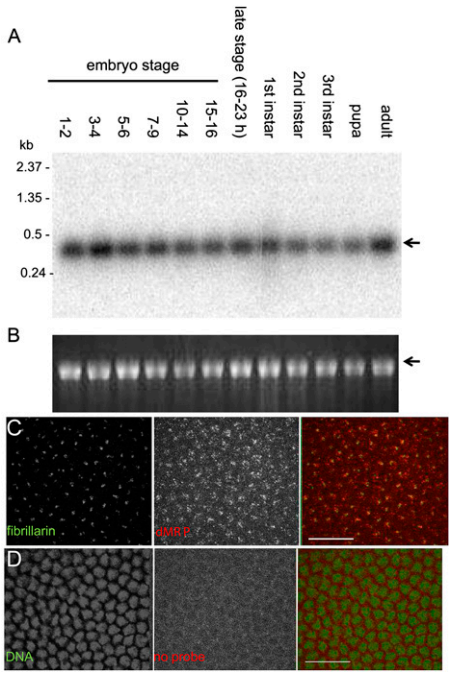


**FIGURE 2.** Expression and nucleolar localization of *dMRP* RNA. (*A*) *dMRP* RNA is expressed throughout *Drosophila* development. Northern blot of total *Drosophila* RNA probed with *dMRP* antisense RNA. *dMRP* RNA (arrow) is predicted to be 383 nt long, including the termination signal. (*B*) rRNA (arrow) as a loading control is visualized by ethidium bromide staining. (*C*) *dMRP* RNA colocalizes with fibrillarin in syncytial *Drosophila* embryos. *dMRP* RNA was detected by FISH, fibrillarin with rabbit anti-*Drosophila* fibrillarin antibody. (*D*) Control FISH without RNA probe. DNA stained with PicoGreen was used as a counterstain instead of fibrillarin to show a lack of nuclear localization of low signal levels in the red channel. Fibrillarin distribution varies considerably with cell cycle and does not consistently mark each nucleus. (*C*,*D*) Images of a single confocal plane. Scale bar, 200 μm.

observed in S2 cells (data not shown). Nucleolar localization of *dMRP* RNA suggests that this is a conserved feature of *MRP* RNA.

## Characterization of a *dMRP* mutant strain

### *Rescue of a* dMRP *mutant strain with genomic DNA containing an intact* dMRP *gene*

*dMRP* is encoded within an intron of the uncharacterized gene CG10365, but is transcribed in the opposite direction. We determined that a P-element, $P\{EPgy2\}CG10365^{EY08633}$, inserted in transcribed *dMRP* sequences results in a lethal phenotype (Fig. 1A). Due to the large size of the P-element (10.9 kb), it is unlikely that a product expressed from the *dMRP* RNA polymerase III (Pol III) promoter could form a product with *dMRP* RNA function. This product would likely be degraded, resulting in a functional null mutation. We note that this P-element is inserted in an intron of the surrounding *CG10365* gene, and as such does not disrupt its translated sequences. Thus, it is possible that the lethal phenotype was caused by disruption of the *dMRP* gene, leaving *CG10365* function largely intact. To test this idea, a P-element vector ($pTW^{MRP}$) containing a genomic fragment that encompassed the entire intronic region of the *dMRP* gene was inserted into chromosome II and used to rescue the lethal P-element insertion. The $pTW^{MRP}$ transgene was then tested in P{Epgy2} $CG10365^{EY08633}$ mutants (Table 1). Individuals homozygous for the *dMRP* mutation P{Epgy2} $CG10365^{EY08633}$ and the $pTW^{MRP}$ transgene were present in numbers consistent with random segregation of both insertions, suggesting a full rescue of the lethal phenotype in these individuals. The viability of *dMRP* mutant individuals carrying $pTW^{MRP}$ indicates that the mutant phenotype resulting from the insertion of P{Epgy2} $CG10365^{EY08633}$ is caused by an impairment of the *dMRP* gene and not *CG10365*. *CG10365* may therefore be a nonessential gene, or the P{Epgy2} $CG10365^{EY08633}$ insertion may not impair its function to a physiologically significant degree. As the phenotypes associated with P{Epgy2} $CG10365^{EY08633}$ appear to be specific to impairment of *dMRP* function, we will hereafter refer to this mutation as $dMRP^{EY08633}$.

### dMRP$^{EY08633}$ *mutants are impaired in growth and development and die as second instar larvae*

A significant defect in growth of homozygous $dMRP^{EY08633}$ mutants compared with the reference $w^{1118}$ strain was observed beginning at 3 d after egg deposition (AED) (Fig. 3A). At 5 d AED the mean cross-sectional area of homozygous $dMRP^{EY08633}$ mutants was approximately one-third that of the $w^{1118}$ strain. Homozygous $dMRP^{EY08633}$ mutants were also delayed in development, undergoing the first molt ~1 d later than normal. Despite the defects in growth and delayed development, homozygous $dMRP^{EY08633}$ mutants showed no gross physical deformities. Growth and development of heterozygous $dMRP^{EY08633}$ larvae were indistinguishable from the normal $w^{1118}$ strain (Fig. 3A,B).

Homozygous $dMRP^{EY08633}$ mutants showed a bimodal pattern of mortality, with ~70% of individuals dying between 5 and 6 days of age while the remaining individuals

**TABLE 1.** Rescue of the $dMRP^{EY08633}$mutant strain

| Genotype | No. observed | No. expected |
|---|---|---|
| $\frac{pTW-MRP}{\text{CyO}}$; $\frac{P\{EPgy2\}CG10365^{EY08633}}{P\{EPgy2\}CG10365^{EY08633}}$ | 25 | 92.6 |
| $\frac{pTW-MRP}{\text{CyO}}$; $\frac{P\{EPgy2\}CG10365^{EY08633}}{\text{TM3,Sb1}}$ | 110 | 185.3 |
| $\frac{pTW-MRP}{pTW-MRP}$; $\frac{P\{EPgy2\}CG10365^{EY08633}}{P\{EPgy2\}CG10365^{EY08633}}$ | 39 | 46.3 |
| $\frac{pTW-MRP}{pTW-MRP}$; $\frac{P\{EPgy2\}CG10365^{EY08633}}{\text{TM3,Sb1}}$ | 243 | 92.6 |
| Total | 417 | — |

Individuals homozygous for P{EPgy2} $CG10365^{EY08633}$ were observed, indicating rescue of the lethal phenotype. Individuals homozygous for both P{EPgy2} $CG10365^{EY08633}$ and *pTW -MRP* appear in numbers consistent with random segregation of both insertions. Genotypes were analyzed approximately seven generations after establishing the strain.

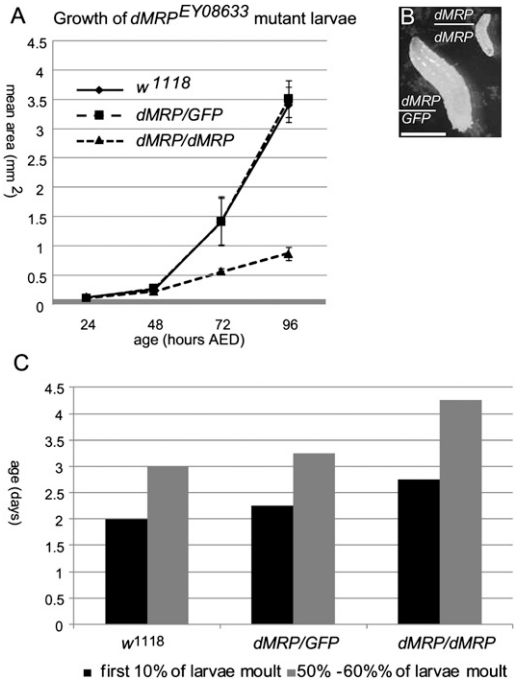


**FIGURE 3.** Homozygous $dMRP^{EY08633}$ mutant larvae are delayed in growth and development. (*A*) $dMRP^{EY08633}/dMRP^{EY08633}$ mutants showed a significant growth delay (mean cross sectional area for 20 individuals) beginning before 72 h AED compared with control ($w^{1118}$) or heterozygous $dMRP^{EY08633}$/GFP siblings. (*B*) Comparison of 5-d-old $dMRP^{EY08633}/dMRP^{EY08633}$ and $dMR^{EY08633}$/GFP mutant individuals. Scale bar, 1 mm. (*C*) The first molt in $dMRP^{EY08633}/dMR^{EY08633}$ mutants occurred ~1 d later than control ($w^{1118}$) or heterozygous $dMRP^{EY08633}$/GFP siblings raised in the same vial(s). (*dMRP*) $dMRP^{EY08633}$.

lingered as second instar larvae for up to 17 d AED (Fig. 4A), which is several days after normal individuals have reached adulthood. Mouth hooks examined after death had two to four teeth, characteristic of the second instar stage (Fig. 4B; Apatov 1929). Northern blot analysis of *dMRP* RNA expression levels showed a progressive decline in homozygous $dMRP^{EY08633}$ mutants from 1 to 5 d of age while levels remained constant in $dMRP^{EY08633}/GFP$ heterozygotes (Fig. 5). This progressive decline in *dMRP* RNA levels is likely due to gradual depletion of maternal *dMRP* RNA and correlates with the timing of impairments in growth and development prior to mortality.

### $dMRP^{EY08633}$ *mutants show impaired rRNA processing*

To identify a potential physiological defect affecting growth and/or development of $dMRP^{EY08633}$ mutants, we examined the well-documented role for *MRP* RNA as a component of RNase MRP in the endonucleolytic cleavage of the rRNA precursor leading to formation of the 5′ end of 5.8S rRNA. Mutations in *S. cerevisiae* and human *MRP* RNAs result in accumulation of unprocessed or long forms of 5.8S RNA ($5.8S_L$) relative to the processed short form ($5.8S_S$) (Schmitt and Clayton 1993; Lygerou et al. 1996; Hermanns et al. 2005; Thiel et al. 2005). Our analysis of the relative abundance of these two forms of 5.8S rRNA in $dMRP^{EY08633}$ mutant larvae showed an increase in the abundance of the $5.8S_L$ form (Fig. 6). This result is consistent with a defect in a subset of previously described RNase MRP mutants that has been described in other species (Schmitt and Clayton 1993; Lygerou et al. 1996; Hermanns et al. 2005; Thiel et al. 2005, 2007).

A recent study identified an additional role for *S. cerevisiae* RNase MRP in early stages of rRNA processing. In this study, a reduction in levels of normal early intermediates was observed in *MRP* RNA mutants, which was accompanied by the appearance of abnormal intermediates (Lindahl et al. 2009). To investigate a potential role for dMRP in regulating early processing steps, we examined rRNA intermediates in homozygous $dMRP^{EY08633}$ mutants. Figure 7 is a schematic representation of two pathways for processing the primary pre-rRNA transcript in *Drosophila melanogaster*. The canonical pathway is analogous to that described in vertebrates (Levis and Penman 1978). An alternative pathway was subsequently proposed by Long and Dawid (1980) that differs from the canonical pathway in the order of early cleavages steps.

Northern blots of total RNA from homozygous $dMRP^{EY08633}$ mutants, $dMRP^{EY08633}/GFP$ heterozygotes of the same age, and $w^{1118}$ second instar larvae were hybridized with oligonucleotide probes (oligos) complementary to several regions of the primary pre-rRNA transcript (Fig. 8B; Table 2). RNA levels for each genotype were normalized for similar levels of mature rRNA to compare processed with unprocessed rRNA levels. Probing with an oligo complementary to the external transcribed spacer (ETS) revealed a 4.2-kb RNA species in homozygous $dMRP^{EY08633}$

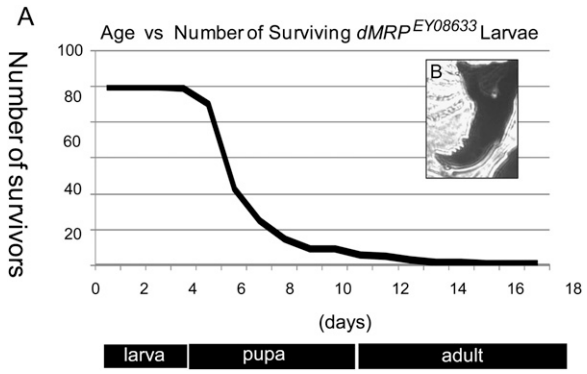


**FIGURE 4.** Homozygous $dMRP^{EY08633}$ mutants die during the second larval instar. (*A*) A bimodal pattern of mortality was observed, with ~70% of individuals dying 5–6 d AED. The remaining individuals lingered as second instar larvae for up to 17 d compared with control populations, which reached adulthood at ~10 d AED. (*B*) Representative mouth hook from a homozygous $dMRP^{EY08633}$ larva examined post mortem. All mouth hooks had two to four teeth, indicative of the second instar stage. (*Below*) Approximate development timeline for wild-type individuals. (dMRP) $dMRP^{EY08633}$.

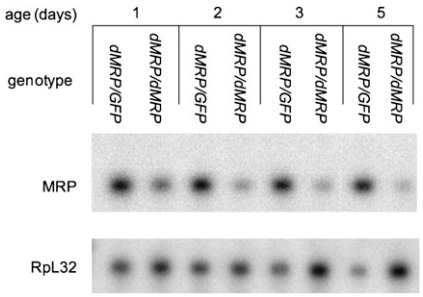


**FIGURE 5.** *dMRP* RNA levels decline at the time of growth arrest and death in $dMRP^{EY08633}$ mutants. A Northern blot of total RNA from homozygous $dMRP^{EY08633}/dMRP^{EY08633}$ mutants and their heterozygous $dMRP^{EY08633}$/GPF siblings during larval development shows levels of *dMRP* RNA (MRP). The same blot was probed for RpL32 mRNA (RpL32) as a loading control. (*dMRP*) $dMRP^{EY08633}$.

mutants that was not detected in phenotypically normal larvae. A 4.2-kb molecule (designated intermediate α) was also seen when blots were probed with oligos complementary to the internal transcribed spacer (ITS)1, ITS2, and 5.8S regions (Fig. 8A). This molecule spans from the 5′ end of the primary transcript to the ITS2 region. Assuming that it is a product of a cleavage at one of the normal sites, it would be the product of an initial cleavage at site 5 (Fig. 7). A 3.0-kb molecule (designated intermediate β) was detected only in RNA from homozygous $dMRP^{EY08633}$ mutants on blots probed with ITS1, ITS2, and 5.8S oligos but not the ETS oligo (Fig. 8A). This could represent the product of intermediate α, following cleavage at site 1, which removes the ETS from the primary transcript. The experimentally determined sizes of both of these molecules are consistent with their expected sizes, which were determined by comparison with sizes of previously characterized precursors (Long and Dawid 1980) or calculated from the sequence of the primary RNA polymerase I pre-rRNA transcript based on the annotation by Tautz et al. (1988) (see Table 3). Neither of these molecules corresponds to products of either of the previously described *Drosophila* rRNA processing pathways (Fig. 7).

Accumulation of a 0.58-kb molecule (designated intermediate e) was detected in RNA from homozygous $dMRP^{EY08633}$ mutants with ITS2 and 5.8S oligos but not with ETS or ITS1 oligos (Fig. 8). The size (Table 3) and structure of this molecule suggest that it may correspond to a previously observed normal precursor of 5.8S and 2S rRNAs, designated intermediate e by Long and Dawid (1980). However, the boundaries of both of these intermediates need to be defined more accurately to determine their relationship. Intermediate e, identified in Figure 8, could be the product of further processing of intermediate β or result from abnormal accumulation of an intermediate produced by a normal processing pathway. We were unable to detect this intermediate in RNA from phenotypically normal larvae. If intermediate is a processed product of intermediate β, then the three intermediates α, β, and that accumulates in homozygous $dMRP^{EY08633}$ mutants may represent a series of intermediates in an atypical processing pathway generated by cleavages at normal sites used in an atypical order (Fig. 8B).

Our probes detected several RNAs that were present in RNA from homozygous $dMRP^{EY08633}$ mutants as well as the two phenotypically normal genotypes, whose identities we could not determine. 5.8S and ITS1 oligos both hybridized to a 2.0-kb fragment that is close in size to the 28Sb mature rRNA and could result from nonspecific hybridization to this abundant RNA species. The 0.35-kb fragment seen with the ITS2 and 5.8S probes and the 0.10-kb fragment seen with the ITS1 probe do not correspond to known products of *Drosophila* rRNA processing pathways. They may represent novel processing intermediates or may be detected as a result of nonspecific hybridization with unrelated small RNAs.

## DISCUSSION

### *Drosophila MRP* RNA is orthologous to *MRP* RNA genes in other species

Our results support the idea that *dMRP* RNA shares structural and functional homology with conserved *MRP* RNA genes previously characterized in other eukaryotes. In addition to the structural and sequence similarities between *dMRP* RNA and *MRP* RNAs in other species described by Piccinelli et al. (2005), we identified genomic sequences flanking *dMRP* RNA resembling RNA Pol III regulatory elements (Fig. 1; Hernandez et al. 2007). The presence of these elements suggests that in *Drosophila*, as in most species,

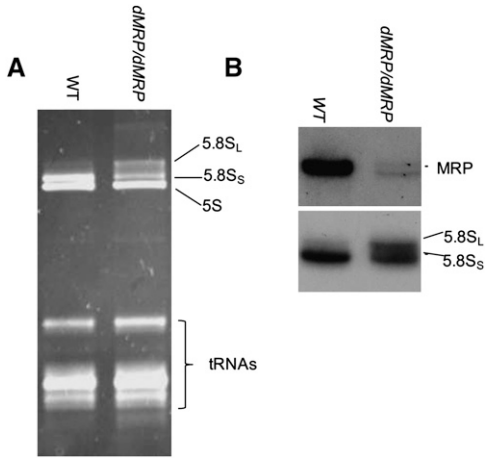


**FIGURE 6.** 5.8S rRNA processing is impaired in *dMRP* mutants. Total RNA isolated from wild-type (WT) and $dMRP^{EY08633}$ mutant larvae was separated in a denaturing polyacrylamide gel and either directly analyzed by staining with ethidium bromide (*A*) or used for Northern blotting with probes specific to the *dMRP* RNA or 5.8 rRNA (*B*). The two forms of 5.8S rRNA are indicated. (*dMRP*) $dMRP^{EY08633}$, (WT) Oregon R strain.

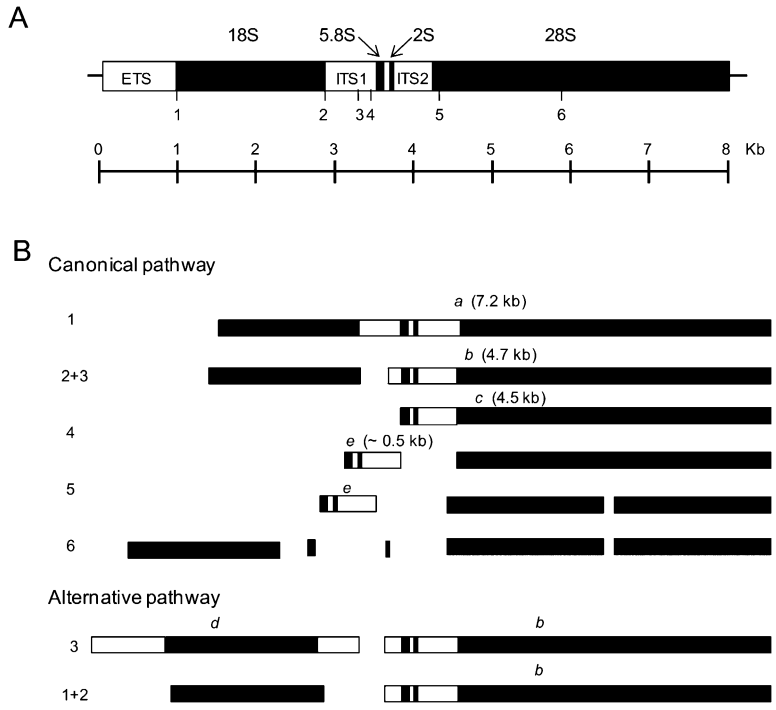


**FIGURE 7.** A *D. melanogaster* ribosome gene and previously described rRNA processing pathways. (*A*) The primary rRNA transcript is drawn approximately to scale. (Solid boxes) Regions that are processed into mature rRNA. ETS (external transcribed spacer), ITS1 (internal transcribed spacer 1), and ITS2 (internal transcribed spacer 2) (open boxes) are removed during processing. Endonucleolytic cleavage sites are indicated by numbered marks (1–6). (*B*) The canonical and alternative rRNA processing pathways that have been previously described in *D. melanogaster*. Processing intermediates designated according to Long and Dawid (1980) are indicated by letters *above* each fragment.

*MRP* RNA is transcribed by RNA Pol III (Dieci et al. 2007). Our success in rescuing the $dMRP^{EY08633}$ mutant strain with a genomic fragment containing these sequences is consistent with the idea that these are functional *dMRP* transcriptional regulatory sequences.

A number of additional observations support the idea that this gene is orthologous to the gene encoding *MRP* RNA in other eukaryotes. Essentially uniform expression of *dMRP* RNA throughout the *Drosophila* life cycle (Fig. 2A) is consistent with its role in fundamental cellular processes such as ribosome biogenesis, mitochondrial DNA replication, and cell cycle regulation (Chang and Clayton 1987; Schmitt and Clayton 1993; Lygerou et al. 1996; Gill et al. 2004; Thiel et al. 2007). Further, *dMRP* RNA localizes to the nucleolus, the same compartment to which the bulk of *S. cerevisiae* RNase MRP (Gill et al. 2006) and mammalian *MRP* RNA localize (Fig. 2C; Li et al. 1994; Jacobson et al. 1995).

The rRNA processing pathway has not been characterized as extensively in *Drosophila* as in other species. There have been few additional studies of this pathway since mapping of cleavage sites by Long and Dawid (1980). In their study, a single form of 5.8S was identified. We have identified both long and short forms of 5.8S rRNA in normal larvae (Fig. 6). Our data also reveal similarities in rRNA processing between *dMRP* RNA and *MRP* RNA orthologs in other species. *S. cerevisiae* and human *MRP* RNA genes have been known for some time to cleave the rRNA precursor leading to the formation of the 5′ end of the $5.8S_S$ rRNA. A reduction in the level of $5.8S_S$, accompanied by an increase in the level of the unprocessed $5.8S_L$, in *MRP* RNA mutants is a strong indication of this function (Schmitt and Clayton 1993; Lygerou et al. 1996; Hermanns et al. 2005; Thiel et al. 2005, 2007). Homozygous $dMRP^{EY08633}$ mutants display a similar change in relative abundances of the two forms of 5.8S rRNAs, indicating a similar function for this gene in *Drosophila* (Fig. 6).

## *Drosophila* and *S. cerevisiae MRP* RNA genes show similar effects on early rRNA processing

We have observed an additional defect in early rRNA processing events that resembles one that was recently reported in *S. cerevisiae MRP* RNA mutants. In these mutants, an atypical 24S intermediate, spanning from the 5′ end of the ETS to the 3′ end of 5.8S, was detected. It was proposed that this intermediate was generated by a premature cleavage in ITS2 followed by 3′ to 5′ exonuclease trimming to the 3′ end of 5.8S (Lindahl et al. 2009). The 24S intermediate resembles intermediate α in homozygous $dMRP^{EY08633}$ mutants (Fig. 8). Intermediate α, like the 24S intermediate, begins with the ETS at the 5′ end. ITS2 sequences at the 3′ end suggest that it was generated by premature cleavage at the normal cleavage site 5 (Fig. 7). However, unlike the 24S intermediate, intermediate α retains ITS2-derived sequences (Fig. 8). Accumulation of intermediate β and intermediate e in addition to intermediate α suggests further processing of intermediate α. Together, these fragments could represent a series of intermediates in an atypical processing pathway (Fig. 8).

Normally, the pre-rRNA transcript is cleaved at defined sites in a consistent order to produce a defined set of rRNA intermediates that are ultimately processed into mature rRNAs. To account for the atypical pathway in homozygous $dMRP^{EY08633}$ mutants, we propose a role for RNAse MRP in maintaining the order of early cleavage steps. Specifically, RNase MRP could contribute to suppressing premature cleavage at site 5 in ITS2 (Fig. 7). This function could be performed as part of a large complex, associating with the pre-rRNA and masking cleavage site 5. Subsequent conformational changes in this complex could expose site 5 for

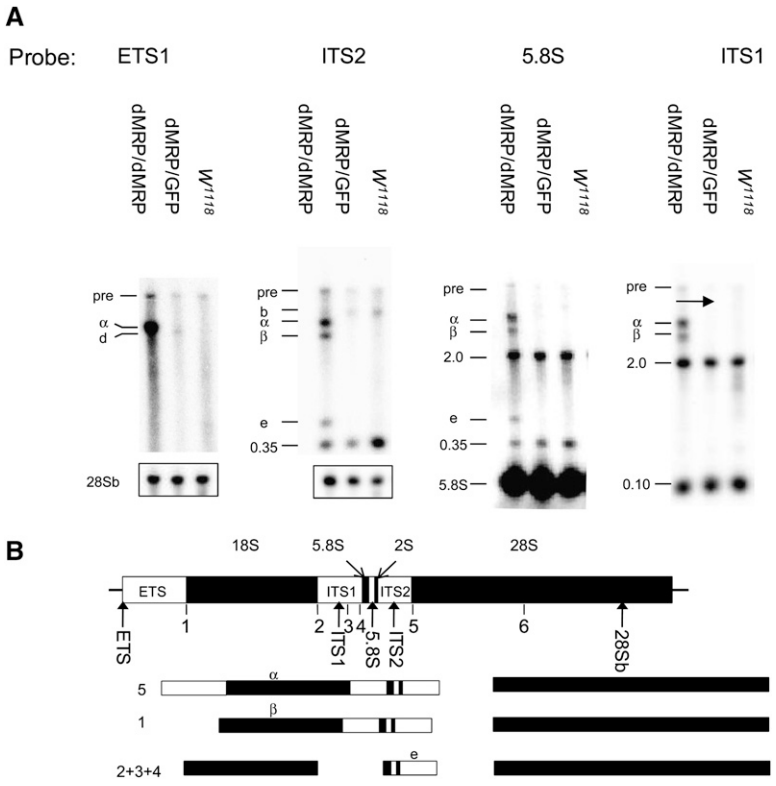


**FIGURE 8.** rRNA precursors in *dMRP*$^{EY08633}$ mutants and a novel processing pathway that they may represent. (*A*) Northern blot analysis of rRNA precursors in *dMRP*$^{EY08633}$/*dMRP*$^{EY08633}$ mutants (5 d AED), their heterozygous *dMRP*$^{EY08633}$/GPF siblings (5 d AED), and second instar wild-type (WT) (*w*$^{1118}$) larvae. Loading was normalized to similar levels of mature rRNA. Blots probed with ETS1 and ITS2 oligos were subsequently probed with a 28Sb oligo to show similar loading. Similar loading for the blot probed with the 5.8S oligo is indicated by the mature 5.8S product. The blot probed with ITS2 was stripped and reprobed with ITS1. The primary transcript (pre) and mature products are indicated. Novel processing intermediates are identified by letters that correspond to intermediates shown in the pathway below. Previously characterized intermediates b (seen in RNA from *dMRP*$^{EY08633}$/GPF larvae probed with ETS probe) and d (seen in RNA from *dMRP*$^{EY08633}$/GPF and *w*$^{1118}$ larvae) probed with ITS2 probes are identified as in Figure 7. Fragments of unknown identities are indicated by their apparent molecular weight (kb). 5.8S and ITS1 oligos both hybridized to a 2.0-kb fragment that is approximately the size of the 28Sb mature rRNA and could result from nonspecific hybridization to this abundant RNA species. The 0.35-kb and 0.10-kb fragments seen with the 5.88 and ITS1 probes, respectively, do not correspond to known products of the *Drosophila* rRNA processing pathway. They may represent novel intermediates of may result from nonspecific hybridization with unrelated small RNAs. (*B*) Map of the primary rRNA transcript is represented as in Figure 7. (Arrows) Positions of oligonucleotide hybridization probes. Probes are complementary to the following regions: ETS, ITS2, 5.8S, ITS1, and 28S. Initial cleavages of the rRNA processing pathway represented by these fragments are shown *below* the primary transcript. Further processing is likely to follow the canonical pathway.

cleavage at the appropriate processing step. At a later stage in the pathway RNase MRP would perform its previously documented function of an endoribonuclease, cleaving the pre-rRNA precursor at a single site in ITS1 to process the 5′ end of the 5.8S rRNA.

Similar aberrant intermediates in *Drosophila* and *S. cerevisiae* mutant *MRP* RNA genes could be formed by a common mechanism, such as the one described above. Alternatively, they could form as a result of the mechanism proposed by Lindahl et al. (2009). In this mechanism RNase MRP could function as an endoribonuclease to directly cleave the pre-rRNA transcript at multiple sites. In addition to its previously characterized role of processing the 5′ end of the 5.8S rRNA, it would also cleave early intermediates. The absence of this activity in *MRP* RNA mutants this would lead to reduction of normal early rRNA precursors. The 24S intermediate would be produced in the absence of this activity while cleavage in ITS2 continues.

Elevated levels of atypical intermediates in the pathway outlined in Figure 8 could accumulate as a result of inefficient processing. Normally, early cleavages of the primary pre-rRNA transcript separate the 18S region from the 28S region, followed by further processing along separate pathways to form distinct 40S and 60S ribosomal subunits (Fatica and Tollervey 2002). In intermediate α of the atypical pathway, the 5.8S and 2S regions are inappropriately linked to the 18S region, which is destined to become part of the 40S subunit. An inability by the normal pathway to process intermediate α could result in an increase in its levels. Inefficient processing of intermediate α by an atypical pathway could lead to accumulation of additional intermediates such as intermediates β and e. Eventually, accumulation of atypical intermediates surpassing critical levels could interfere with normal rRNA processing pathways and lead to defects in ribosome biogenesis. This model is consistent with a reduction in levels of normal early processing intermediates in *S. cerevisiae MRP* RNA mutants reported by Lindahl et al. (2009) and predicts that analysis of physical and morphological features of ribosomal subunits or nucleoli where they are assembled could reveal additional defects.

Intermediate e resembles a precursor of 5.8S and 2S rRNAs previously identified by Long and Dawid (1980). Both molecules span from 5.8S to ITS2; however, their precise boundaries have not yet been determined. Based on analogy with the *S. cerevisiae* rRNA processing pathway (Fatica and Tollervey 2002), two forms of intermediate e would be expected in normal individuals, one the product of processing initiated by RNase MRP cleavage and another unprocessed form. In *Drosophila*, intermediate b is the likely

**TABLE 2.** Sequences and positions of oligonucleotide probes used in Northern blots

| Oligo | Sequence | Position[a] |
|---|---|---|
| ETS | CGAACAATGCGAGGTCGGCAACCACTGCCTACC | 1–33 |
| ITS1 | GGTTGTTGCATTAGCCAACGTATGCCCATAACTAAGATG | 3281–3319 |
| ITS2 | AGAAAATATTTCTCTTCGTTTTTCACATTCAAATGTGAGATAATG | 3866–3910 |
| 5.8S | GTCGATGTTCATGTGTCCTGCAGTTCACACGATGACGCACAG | 3634–3675 |
| 28Sb | GTAACTAGCGCGGCATCAGGTGATCGAAGATCCTCCC | 7917–7953 |

[a]Indicates the complementary position on primary transcript.

substrate for these alternative cleavages (Fig. 7). At this time we cannot determine which of the two predicted forms of this intermediate (or perhaps both) we detect in homozygous *dMRP*$^{EY08633}$ mutants since their size difference is likely to be very small. In *S. cerevisiae* there is only a 7-nt difference between the two forms (Shuai and Warner 1991). However, accumulation of intermediate e in homozygous *dMRP*$^{EY08633}$ mutants with reduced levels of *dMRP* RNA suggests that it could be the precursor of the long form of the 5.8S rRNA that has not been cleaved by RNase MRP. Determining the precise 5′ boundary of this intermediate would determine if it is indeed the 5.8S$_L$ precursor. If intermediate e in homozygous *dMRP*$^{EY08633}$ mutants is a precursor of 5.8 S$_L$, it is tempting to speculate that the pathway in Fig. 8B could function in normal individuals as a result of incomplete suppression of premature cleavage in ITS2 to produce low levels of 5.8 S$_L$.

## Mutation of *dMRP* phenotypically resembles human *RMRP* mutants

The similarity in phenotype of the *dMRP*$^{EY08633}$ mutant strain with human patients with *RMRP* mutations further supports the idea of functional homology between these genes. The impairment in growth of *dMRP*$^{EY08633}$ mutant larvae resembles the short stature thought to be caused by an intrinsic defect in proliferation of cells in human patients with *RMRP* mutations (Pierce and Polmar 1982). It is not clear which of the three RNase MRP functions may be causing the defects in growth and development seen in the *dMRP*$^{EY08633}$ mutants. It is unlikely that it is an impairment of cell cycle regulation by a defect in cyclin B mRNA degradation because cyclins A, B, and B3 are not expressed in endoreplicating cells (Lehner and O'Farrell 1989, 1990), which constitute most of the larval tissues. The *dMRP*$^{EY08633}$ mutant phenotype may be most similar to a class of human *RMRP* mutations that is associated with impaired rRNA processing that does not alter cyclin B mRNA degradation. This class of mutations is associated with a severe growth defect seen in the disease anauxetic dysplasia.

The potential impact of a defect in mitochondrial function on *dMRP*$^{EY08633}$ mutants cannot be inferred from studies of human *RMRP* mutations because defects in mitochondrial function have not yet been detected in these patients (Hermanns et al. 2005). Indeed a potential role for RNase MRP in mitochondrial DNA replication has been controversial. The enzyme was initially isolated from mouse cells and shown to cleave an RNA representing the primer for mitochondrial DNA replication (Chang and Clayton 1987). This activity was dependent on complementarity of a segment of *MRP* RNA to sequences of the RNA substrate (Bennett and Clayton 1990). However, the cleavage site on the substrate is 6–10 nt from the in vivo cleavage site, suggesting that the in vitro activity may be an artifact (Kiss and Filipowicz 1992). In favor of a role for RNase MRP in mitochondria, there is good evidence for localization of *MRP* RNA to mitochondria of mouse cardiomyocytes by in situ hybridization (Li et al. 1994). The recent purification of a mitochondrial enzyme consisting of *MRP* RNA with a protein composition distinct from nucleolar RNase MRP provides convincing evidence for a mitochondrial function in *S. cerevisiae* (Lu et al. 2010).

In *Drosophila*, mutations in a variety of mitochondrial functions have been described. These mutations can affect a number of diverse processes such as apoptosis (Abdelwahid et al. 2007), spermatogenesis (Hales and Fuller 1997), and growth in both mitotic and endoreplicating tissues (Morris et al. 2008). The latter effect is consistent with the impairment in growth displayed by *dMRP*$^{EY08633}$ mutants. We have been unable to detect localization of *dMRP* RNA to mitochondria in several tissues that we examined, with the exception of occasional localization to mitochondria in third instar larval muscle (data not shown). Additional analysis employing a variety of experimental approaches could establish whether *dMRP* RNA functions in mitochondria. These could include additional attempts at localization of *dMRP* RNA to mitochondria by in situ hybridization as well as purification of RNase MRP activity from mitochondria. In *Drosophila* and other metazoans, identifying a mitochondrial RNase MRP would be more

**TABLE 3.** Sizes of rRNA precursors in Figure 8

| Precursor | Expected size (kb) | Measured size (kb) |
|---|---|---|
| pre | 8.1[a] | 9.0 |
| b | 4.7[b] | 5.0 |
| α | 4.15[a] | 4.2 |
| d | 3.4[b] | 3.5 |
| β | 3.3[a] | 3.0 |
| e | ~ 0.5[b] | 0.58 |

[a]Expected sizes were calculated from the sequence of the primary RNA polymerase I pre-rRNA transcript annotated by Tautz et al. (1988) based on positions of cleavage sites identified by Long and Dawid (1980).
[b]Expected sizes are based on sizes of precursors previously characterized Long and Dawid (1980).

likely in tissues that are known to be actively replicating mitochondrial DNA.

The defects that we report in growth of *dMRP*$^{EY08633}$ mutants are consistent with impaired rRNA processing and/or impaired mitochondrial functions, based on phenotypic comparisons of other genetic defects in these processes. Defects in development may be an indirect consequence of growth defects. In *Drosophila* larvae there may be a requirement to reach a certain size before proceeding to the next stage of development.

We have shown that the single *Drosophila* homolog of *MRP* functions analogously to mammalian *MRP*. The well-characterized RNAi pathway in *Drosophila* (Kim et al. 2009) together with the recent discovery of *RMRP*-derived endogenous siRNAs in human cells (Maida et al. 2009) facilitates future studies of the relatively unexplored area of RNAi-mediated regulation of noncoding RNAs. Our identification of a *Drosophila* strain with a mutation in *MRP* RNA will be a useful tool in these future endeavors.

## MATERIALS AND METHODS

### *Drosophila* culture and strains

*Drosophila melanogaster* strains were maintained at 18°C or 25°C according to standard maintenance procedures on semisolid medium consisting of 1.2% brewer's yeast, 1.2% agar, 8% cornmeal, 7.5% v/v blackstrap molasses supplemented with 0.25 g of methyl 4-hydroxybenzoate (Sigma, dissolved in 1.5 mL of 95% ethanol) as a preservative. The following stocks, obtained from the Bloomington *Drosophila* Stock Center, were used: the normal $w^{1118}$ strain; second chromosome balancer CyO/*Sco*, third chromosome balancers $w^-$; TM3 $Sb^1$/*Ly* and $w^-$; $Sb^1$/TM3 P($w^+$mC = ActGFP) $JMR^2$,$Ser^1$ and strains bearing the lethal P-element insertion in *CG10365*; P[EPgy2] *CG10365*$^{EY08633}$.

### Generation of transgenic animals

A genomic region that included the d*MRP* gene was PCR-amplified from pBAC11E7 (GenBank accession no. AC008201.8) with *MRP*5′A: CACCCGTTGAGGACAAAGAGGTGAGTA and *MRP*3′D: GCTGCTTGAGATAATCCAGTGCCG primers that were derived from flanking CG10365 coding sequences and cloned into pENTR/D (Invitrogen) to make pENTR$^{MRP}$. This entry vector was then recombined into *pTW* (provided by T. Murphy, Carnegie Institute, Troy, MI), inserting the *dMRP* gene into pTW to make pTW$^{MRP}$. Note that the *dMRP* genomic region was inserted in the opposite orientation to the UAS promoter in this vector so that *dMRP* RNA would be expressed from its own regulatory sequences. Wild-type $w^{1118}$ flies were transformed with pTW$^{MRP}$ to make the w$^-$; *pTW -MRP* strain.

### Observations of *Drosophila* growth and development

Embryos were collected on apple juice-agar plates. After hatching, larvae were maintained at 25°C on the same plates supplemented with yeast paste spread for the period of observation. Homozygous *dMRP*$^{EY08633}$ mutants were identified as non-GFP individuals of the strain P{Epgy2} *CG10365*$^{EY08633}$/$w^+$; $Sb^1$/TM3 P ($w^+$mC = ActGFP) JMR2,$Ser^1$ (abbreviated as *dMRP*/GFP). The lethal stage of mutant larvae was determined by observation of mouth hooks mounted under #1.5 coverslips in standard tissue mounting medium (5 g of Mowiol 40-88, 20 mL of PBS, 10 mL of glycerol, 2.5% DABCO [1,4-diazabicyclo(2.2.2)octane]).

### rRNA processing analysis

Total RNA isolated from Oregon-R and *dMRP*$^{EY08633}$mutant larvae (~2.5 μg per lane) was separated in an 8%/7 M urea polyacrylamide denaturing gel and either stained with ethidium bromide for direct RNA visualization or transferred to Hybond-N+ membrane (Amersham Biosciences) for Northern analysis with $^{32}$P-labeled probes specific to the *MRP* RNA or 5.8S rRNA (see below). Templates for probe labeling were generated by PCR using the primers MRP fwd: 5′-AAGTCCCCGGGCCTAGGATAGAAAG-3′; MRP rev: 5′-CGGTTTCTCAGACGAGAAAGTGTGTG-3′; 5.8S rRNA fwd: 5′-AACTCTAGGCGGTGGATCACTCGGC-3′; 5.8S rRNA rev: 5′-CAGCATGGACTGCGATATGCGTTCA-3′. Note: 5.8S primers were chosen from the sequence annotated as CR40454 (Flybase). For analysis of early rRNA intermediates, oligonucleotide probes were $^{32}$P end-labeled with [γ-$^{32}$P]ATP (3000 Ci/mmol, 10 mCi/mL, Perkin Elmer) using T4 polynucleotide kinase (Invitrogen) by the forward reaction according to the manufacturer's protocol.

### RNA extraction and Northern blot analysis

Total RNA was extracted using TRIzol (Invitrogen) from adults and staged collections throughout development. Equal amounts of denatured RNA (1–2 μg) were loaded in RNA gel loading buffer (Eppendorf); 2.5–5 μL of buffer for each 1.0 μL of RNA. Redistilled formamide (Invitrogen) was added to samples to a minimum of 60% final concentration to allow fractionation on a native 2% agarose gel (Masek et al. 2005). RNA was capillary-transferred to a Brightstar-Plus Membrane (Ambion) and UV cross-linked with a 120-mJ burst for 30 sec. The membrane was pre-hybridized in 5–10 mL of hybridization buffer (3 M urea, 5× SSC, 0.1% [w/v] N-laurylsarcosine, 0.02% SDS, 0.5% BSA, 0.1 mg/mL sonicated salmon sperm DNA) in a glass hybridization tube for 1 h at 60°C.

Hybridization buffer was replaced with 5 mL of fresh buffer containing the appropriate antisense RNA probe labeled with [α-$^{32}$P]UTP, and the membrane was hybridized overnight at 60°C. A probe encompassing the entire transcribed region of *dMRP* (Piccinelli et al. 2005) was used to detect *dMRP* RNA. This region was PCR-amplified with *MRP* forward (GCCGGTTTGAGTCTTCC) and *MRP* reverse (TAATACGACTCACTATAGGGAAAAAAAGT GCGCCG) primers from pBAC11E7. The antisense probe was transcribed with T7 polymerase from T7 promoter sequences added to the template in the *MRP* reverse primer. The membrane was stripped and hybridized to a probe detect the RpL32 gene as a loading control. The template for RpL32 (CG7939) was amplified from the cDNA RH03940 using T3 and T7 primers from the corresponding promoter sequences in the polylinker of pFLC-1. The antisense probe was transcribed using T3 polymerase. Probes were labeled with $^{32}$P in a 20-μL transcription reaction containing T7/T3 Buffer (Invitrogen); 0.5 mM each of ATP, CTP, and GTP; 12 μM UTP; 20 U of SUPERase-In (Ambion); 50 U of T7 polymerase

(Invitrogen); 100 ng of DNA template; and 50 μCi of [α-$^{32}$P]UTP (800 Ci/mmol, 20 mCi/mL, Perkin Elmer).

### In situ hybridization

Transcribed *dMRP* sequences were PCR-amplified from pBAC11E7 with the primers *MRP* fwd GCCGGTTTGAGTCTTCC and *MRP* rev GGAGTGCGCCGTCCGAGTT. Using this product as a template, a subregion of the *dMRP* gene, consisting of nucleotides 126–366 with T7 promoter sequences appended, was amplified in a subsequent step using the primers *MRP*2 5′ CACAAAACACCCAC CCCTGTG and *MRP*-2 3′ + T7 TAATACGACTCACTATAGGG TCCGAGTTTCCCAATGTAA. A digoxygenin-labeled antisense RNA probe transcribed from this template was hybridized to fixed embryos as described in Hughes and Krause (1999). RNA probes were visualized by fluorescent antibody staining using a primary sheep anti-DIG antibody (1:200, Roche) and secondary anti-sheep Alexi-conjugated fluorescent antibody (1:2000, Molecular Probes). Fibrillarin was detected with rabbit anti-fibrillarin antibody (1:4000, Abcam).

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