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Control of poly(A) polymerase level is essential to cytoplasmic polyadenylation and early development in *Drosophila*

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Poly(A) polymerase (PAP) has a role in two processes, polyadenylation of mRNA precursors in the nucleus and translational control of certain mRNAs by cytoplasmic elongation of their poly(A) tails, particularly during early development. It was found recently that at least three different PAP genes exist in mammals, encoding several PAP isoforms. The *in vivo* specificity of function of each PAP isoform currently is unknown. Here, we analyse PAP function in *Drosophila*. We show that a single PAP isoform exists in *Drosophila* that is encoded by the *hiiragi* gene. This single *Drosophila* PAP is active in specific polyadenylation *in vitro* and is involved in both nuclear and cytoplasmic polyadenylation *in vivo*. Therefore, the same PAP can be responsible for both processes. In addition, *in vivo* overexpression of PAP does not affect poly(A) tail length during nuclear polyadenylation, but leads to a dramatic elongation of poly(A) tails and a loss of specificity during cytoplasmic polyadenylation, resulting in embryonic lethality. This demonstrates that regulation of the PAP level is essential for controlled cytoplasmic polyadenylation and early development.
Keywords: cytoplasmic polyadenylation/*Drosophila*/polyadenylation/poly(A) tail/translational regulation

Introduction

Early steps of development in many species rely on maternally inherited mRNAs because transcription is quiescent at these stages. Therefore, changes in protein synthesis that control early developmental events depend on translational control. One way to regulate translation is by changing the poly(A) tail length of mRNAs in the cytoplasm. Shortening of poly(A) tails correlates with translational repression, whereas lengthening of poly(A) tails induces translation (Richter, 2000; Wickens *et al*., 2000). In *Drosophila* embryos, cytoplasmic polyadenylation is crucial for initiation of development; it activates translation of several molecules essential for axis formation, such as the anterior morphogen Bicoid (Salles *et al*., 1994), Hunchback (Wreden *et al*., 1997) and Toll (Schisa and Strickland, 1998). Cytoplasmic polyadenylation has also been proposed to regulate translation of the posterior determinant Oskar during *Drosophila* oogenesis (Chang *et al*., 1999).

The molecular mechanism of cytoplasmic polyadenylation has been analysed extensively in *Xenopus* oocytes, and some aspects of the reaction are similar to that of nuclear polyadenylation. Nuclear polyadenylation consists of endonucleolytic cleavage of pre-mRNAs followed by the synthesis of a poly(A) tail onto the upstream cleavage product (Zhao *et al*., 1999). Poly(A) addition can be reconstituted *in vitro* from three purified mammalian factors: poly(A) polymerase (PAP), cleavage and polyadenylation specificity factor (CPSF) and poly(A)-binding protein II [PABP2, the nuclear poly(A)-binding protein]. CPSF is a complex of four proteins that binds the polyadenylation signal AAUAAA located upstream of the cleavage site. Recognition of the poly(A) site also requires cleavage stimulation factor (CstF) that binds to a GU/U-rich element downstream of the cleavage site and interacts with CPSF. PAP catalyses the polyadenylation reaction, but is also required for efficient cleavage of pre-mRNAs *in vitro* (Christofori and Keller, 1989; Takagaki *et al*., 1989). PAP by itself does not recognize pre-mRNAs specifically. Specificity requires the AAUAAA element and CPSF that binds PAP through its 160 kDa subunit (Murthy and Manley, 1995). Even in the presence of CPSF, PAP activity remains weak; it is again stimulated by binding of PABP2 to the poly(A) tail (Wahle, 1991). Together, CPSF and PABP2 stimulate PAP activity by holding PAP on the RNA such that a full-length poly(A) tail is synthesized in a single processive event (Bienroth *et al*., 1993). When the poly(A) tail has reached its complete length, elongation is no longer processive and becomes slow and distributive. PABP2 is required for this poly(A) tail length control (Wahle, 1995).

Cytoplasmic polyadenylation in *Xenopus* relies on two sequences: the nuclear polyadenylation signal, AAUAAA, and an upstream U-rich element called the cytoplasmic polyadenylation element (CPE). CPE-dependent polyadenylation can be recapitulated *in vitro* in the presence of purified bovine CPSF and PAP (Bilger *et al*., 1994), indicating a role for CPSF. Indeed, a cytoplasmic form of CPSF has been identified in *Xenopus* oocytes (Dickson *et al*., 1999). CPEs are bound by CPEB, a major component of the reaction (Hake and Richter, 1994; Mendez and Richter, 2001). Cytoplasmic polyadenylation during *Xenopus* oocyte maturation is triggered by phosphorylation of CPEB, which stimulates a direct interaction between CPEB and the 160 kDa subunit of CPSF (Mendez *et al*., 2000). Thus, the role of CPEB during cytoplasmic polyadenylation would be to recruit CPSF into an active polyadenylation complex containing a PAP.

In *Drosophila*, the role of CPEs has not been addressed, and the polyadenylation signal is dispensable in some cases, since embryonic cytoplasmic polyadenylation occurs on a *bicoid* engineered mRNA deleted for this element (Salles *et al*., 1994). Although genes encoding the four subunits of CPSF are present in the *Drosophila* genome (Mount and Salz, 2000), their role in cytoplasmic polyadenylation has not been determined. The *Drosophila* homologue of CPEB is the Orb protein. *orb* encodes germline-specific proteins different in male and female, and its function has been determined in the female germline (Lantz *et al*., 1992, 1994; Christerson and McKearing, 1994). Strong *orb* mutants arrest oogenesis early, before the formation of the 16-cell cyst that would normally differentiate into nurse cells and one oocyte. Using a weaker allele, orb^{mel}, Orb was shown to be required for anchoring of *oskar* mRNA at the posterior pole of the oocyte (Christerson and McKearing, 1994). However, this could result from a failure in *oskar* mRNA translation as Oskar protein is required for anchoring its own mRNA at the posterior pole. A more recent study suggests that Orb could have a function analogous to that of CPEB in cytoplasmic polyadenylation. In *orb* mutant egg chambers, the level of Oskar protein is decreased and poly(A) tails of *oskar* mRNAs are shortened (Chang *et al*., 1999).

Another key component required in a functional cytoplasmic polyadenylation complex is a PAP. In vertebrates, multiple PAP isoforms have been identified. Initially, two PAP isoforms were described, PAP I (70 kDa) and PAP II (83 kDa), that differ in their C-terminus (Raabe *et al*., 1991; Wahle *et al*., 1991). Analysis of PAP mRNAs in mouse revealed that these two PAP isoforms are generated by alternative splicing (Zhao and Manley, 1996). Truncated forms of PAP RNAs corresponding to the 5′ half of the gene have also been identified in several species (Wahle *et al*., 1991; Ballantyne *et al*., 1995; Gebauer and Richter, 1995; Zhao and Manley, 1996). However, these truncated RNAs are thought not to be translated *in vivo*, and the corresponding proteins produced in baculovirus or in *Escherichia coli* are inactive *in vitro* (Wahle *et al*., 1991; Martin and Keller, 1996; Zhao and Manley, 1996). In addition to the *PAP* gene, two new PAP-encoding genes have been identified recently in mammals, *neo-PAP* (or *PAP*γ) and *TPAP*. The *neo-PAP* gene encodes a single protein that shows 60% identity to human PAP II and has identical properties to those of PAP II in *in vitro* assays (Kyriakopoulou *et al*., 2001; Perumal *et al*., 2001; Topalian *et al*., 2001). TPAP is encoded by an intronless gene (Kashiwabara *et al*., 2000; Lee *et al*., 2000). Interestingly, *TPAP* is expressed specifically in testis, and the protein is specifically cytoplasmic in spermatogenic cells where cytoplasmic polyadenylation is active (Kashiwabara *et al*., 2000). TPAP was therefore proposed to be responsible for cytoplasmic polyadenylation in mouse testis, although this has not been addressed directly. Although the function of these four PAP isoforms has not been investigated *in vivo*, it seems plausible that they have specific functions. Results on TPAP suggest that different PAPs are responsible for nuclear and cytoplasmic polyadenylation in vertebrates.

Here, we address the function of *Drosophila* PAP *in vivo*. We found that a single PAP isoform is produced in *Drosophila* and that this single protein is responsible for both types of polyadenylation: nuclear and cytoplasmic. An increase in the level of PAP *in vivo* does not affect poly(A) tail length during nuclear polyadenylation. In contrast, such an increase during cytoplasmic polyadenylation leads to very long poly(A) tails and embryonic lethality, showing that a regulated level of PAP is essential for the control of cytoplasmic polyadenylation.

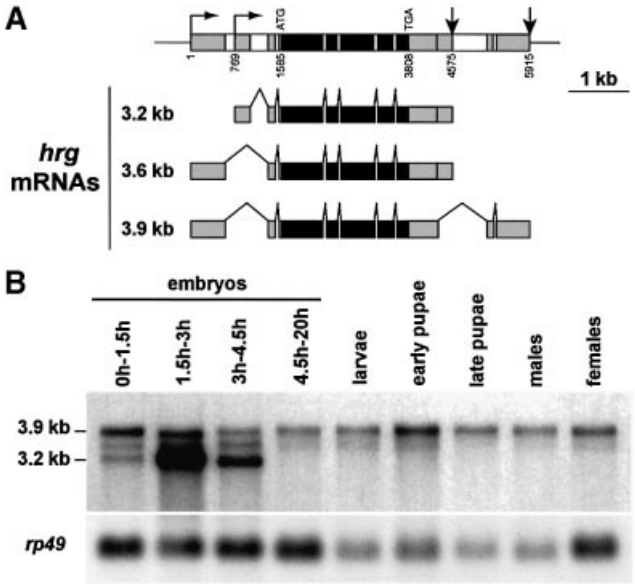

Fig. 1. Structure and expression pattern of *hrg*. (**A**) Structure of the *hrg* gene and mRNAs. Grey boxes are exons in 5′- and 3′-UTRs, black boxes are coding exons and white boxes are introns. Horizontal arrows indicate transcription start sites, and vertical arrows indicate poly(A) sites. The longest cDNA we isolated, NB61, was from a 12–24 h embryo cDNA library. Its sequence was deposited in DDBJ/EMBL/GenBank with accession No. AF231704. Coordinates are from the genomic sequence with the first transcription site starting at 1. (**B**) mRNA pattern of *hrg* during *Drosophila* development. Northern blot of poly(A)$^+$ RNAs hybridized with an RNA probe spanning the 5′ half of the *hrg* coding sequence and reprobed with the *rp49* clone as a loading control.

Results

Drosophila PAP is encoded by the hiiragi gene

We identified the *Drosophila* PAP-encoding gene by screening a *Drosophila* genomic library with a bovine PAP cDNA (Wahle *et al*., 1991). One positive phage was isolated. Several subclones of this phage were used to screen cDNA libraries from 0–3 h and 12–24 h embryos, and ovaries. Restriction mapping and partial sequencing of 69 positive cDNAs as well as two expressed sequence tags (ESTs) from the Berkeley *Drosophila* Genome Project (LD11853 and LD05439) allowed us to determine that the PAP-encoding gene produces three different mRNAs that arise from utilization of two alternative transcription start sites and two alternative poly(A) sites (Figure 1A). No alternative splicing was found in the coding sequence and this gene was found to encode a single protein (see below). In addition, no paralogous gene was found in the *Drosophila* genome either by Southern blot hybridization (not shown) or by examination of the *Drosophila* genome sequence (Mount and Salz, 2000). During this study, the

hiiragi (*hrg*) gene, which was first identified from its role in formation of the wing margin, was cloned and found to encode the same protein as is described herein (Murata *et al*., 2001). Therefore, a single PAP, which is encoded by the *hrg* gene, is produced in *Drosophila*. Northern analysis shows that the two largest mRNAs (3.9 and 3.6 kb) are present at all development stages (Figure 1B). The shortest mRNA (3.2 kb) arising from utilization of a downstream transcription start site is specific to early embryogenesis and strongly accumulates in 1.5–3 h embryos (Figure 1B). Visualization of *hrg* mRNAs in embryos by *in situ* hybridization correlates with a burst of transcription of an embryo-specific transcript before cellularized blastoderm (not shown).

Structure and in vitro activity of Drosophila PAP

The longest cDNA we isolated (NB61) was sequenced and is predicted to encode a protein of 659 residues that is overall 56% identical and 70% similar to bovine PAP. Comparison between *Drosophila* PAP and human PAP II, neo-PAP and TPAP is schematized in Figure 2A. The overall identity between *Drosophila* PAP and each of the three human proteins is similar (45–50%). The N-terminal two-thirds of PAP that contain the catalytic core (67–190) are well conserved between *Drosophila* and human. The C-terminal region is more divergent, except for a short well-conserved domain (465–537) that contains a primer-binding domain and a nuclear localization signal (NLS) (Martin and Keller, 1996).

The activity of *Drosophila* His_6-tagged PAP produced in *E.coli* was assayed in reconstituted polyadenylation reactions. Mammalian PAP requires CPSF and PABP2 *in vitro* to polyadenylate specifically a pre-cleaved AAUAAA-containing RNA. The ability of *Drosophila* PAP to carry out polyadenylation was tested in the presence or absence of bovine CPSF and *Drosophila* PABP2. The RNA substrate, L3pre, was derived from the adenovirus L3 polyadenylation site. It ended at the natural cleavage site and carried a tail of ~10 A residues so that it could be bound directly by PABP2. We found that *Drosophila* PAP is almost inactive on its own (Figure 2B, lanes 2–7). PAP activity is slightly enhanced by the presence of *Drosophila* PABP2 (Figure 2B, lanes 8–13). Bovine CPSF stimulates PAP activity more strongly (Figure 2B, lanes 14–19). This stimulation probably occurs as a result of CPSF tethering *Drosophila* PAP to the mRNA as described for mammalian PAP. In the presence of both CPSF and PABP2, *Drosophila* PAP generates poly(A) tails of 200–250 nucleotides within the first minute of the reaction (Figure 2B, lanes 20–25). This increased efficiency is probably due to enhanced processivity. After this burst, poly(A) tail extension slows as described for the bovine PAP (Wahle, 1995). These results show that *Drosophila* PAP produced in *E.coli* is active and behaves as its bovine homologue *in vitro*.

Expression profile of PAP in Drosophila

We examined the expression profile of PAP during *Drosophila* development, using a polyclonal antibody. During oogenesis, PAP is detected in both the nucleus and cytoplasm of nurse cells and follicle cells (Figure 3B). PAP is also present at a low level in oocyte nucleus and cytoplasm. We overexpressed PAP in the female germline

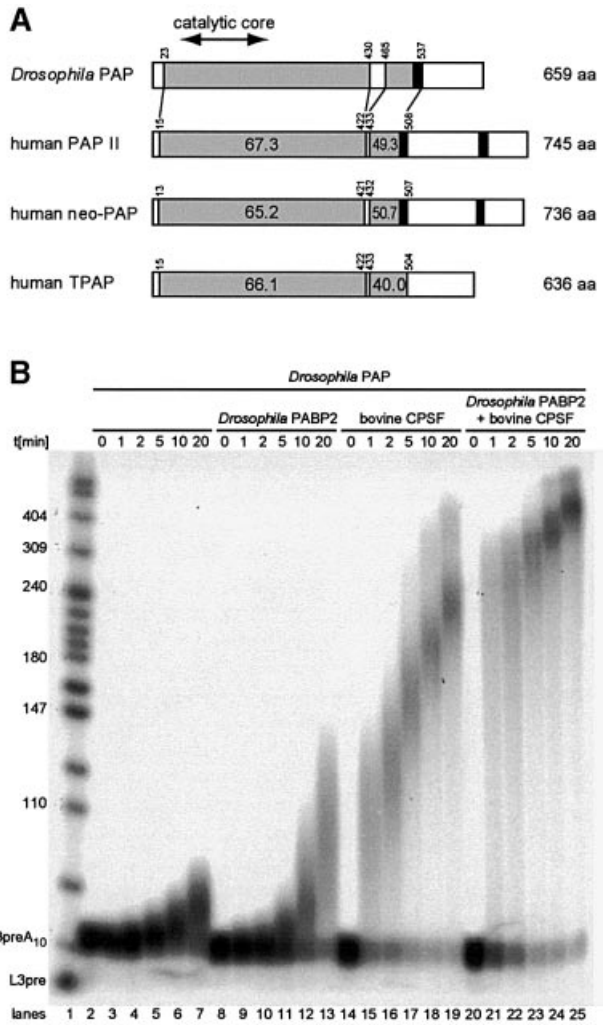

Fig. 2. Comparison of *Drosophila* and human PAPs and *in vitro* activity of *Drosophila* PAP. (**A**) Sequence comparison between *Drosophila* and human PAPs. Accession Nos are P51003, human PAP II; 15080911, neo-PAP; and 18203318, TPAP. Grey boxes are the conserved regions between *Drosophila* and human PAPs. Black boxes are the NLS. The percentages of identity with *Drosophila* PAP are indicated. (**B**) *In vitro* activity of *Drosophila* PAP. *In vitro* specific polyadenylation assays of a pre-cleaved AAUAAA-containing RNA, in the presence or absence of bovine purified CPSF and *Drosophila* PABP2. Reactions were started by the addition of ATP and stopped after the indicated times in minutes.

using a *UASp-hrg* transgene under the control of the female germline-specific driver *nanos-Gal4:VP16* (*nos-Gal4*) (Rorth, 1998). In *UASp-hrg*; *nos-Gal4* females, PAP accumulates to a high level in nuclei of nurse cells and oocyte and to a lesser extent in oocyte cytoplasm (Figure 3D). Maternally provided PAP is detected in just laid embryos where the protein is distributed uniformly (Figure 3E). During embryogenesis, the amount of PAP increases until cellularized blastoderm stage (Figure 3F) and remains stable during gastrulation (Figure 3G). The subcellular distribution of PAP was analysed in cellularized blastoderm embryos (Figure 3L–N). PAP accumulates in nuclei and is present at a lower level in the cytoplasm, as was reported for PAP II in human somatic cells (Schul *et al*., 1998; Kyriakopoulou *et al*., 2001). A high level of PAP accumulates in early embryos coming

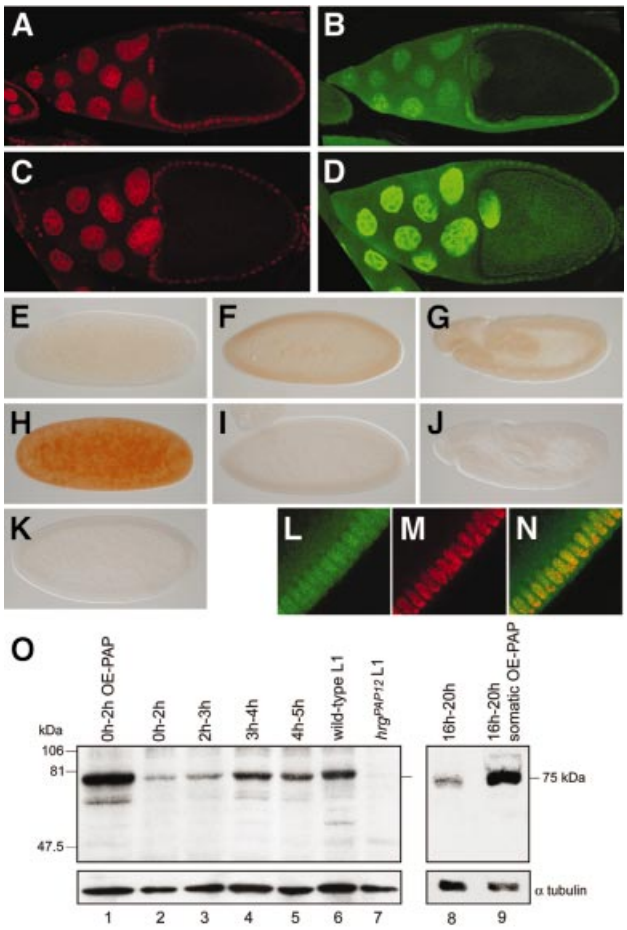

Fig. 3. PAP expression during *Drosophila* development. (A–D) Immunodetection of PAP in ovaries. Confocal images of stage 10 egg chambers from wild-type (**A** and **B**) and *UASp-hrg*; *nos-Gal4* (**C** and **D**) females, double stained with propidium iodide that stains DNA (A and C in red) and anti-PAP (B and D in green). Posterior is to the right. (E–N) Immunodetection of PAP in wild-type embryos (**E–G** and **L–N**), embryos from *UASp-hrg*; *nos-Gal4* mothers (**H**) or hrg^{PAP12} mutant embryos (**I** and **J**). (**K**) Embryo stained with the pre-immune serum. (L–N) Confocal images of double staining with anti-PAP (green) and propidium iodide (red) showing that PAP is nuclear and cytoplasmic. (E and H) Just laid embryos. (F, I, K and L–N) Cellularized blastoderm stage. (G and J) Gastrulation. Posterior is to the right. (**O**) Western blot revealed with anti-PAP. Protein extracts are from 0–2 h embryos coming from *UASp-hrg*; *nos-Gal4* mothers (OE-PAP: PAP overexpression), 0–2 h, 2–3 h, 3–4 h and 4–5 h wild-type embryos, wild-type and hrg^{PAP12} first instar larvae, and 16–20 h wild-type and *UASp-hrg*; *da-Gal4* embryos (somatic OE-PAP). Extract from 15 embryos or larvae is loaded per lane, the blot was revealed with anti-α-tubulin as a loading control.

from females where PAP is overexpressed in the germline (Figure 3H). In contrast, PAP is not detected in hrg^{PAP12} mutant embryos (see below) that show no *hrg* early zygotic transcription (not shown) (Figure 3I and J). This shows that the antibody we generated is specific for PAP. A major protein is detected in *Drosophila* extracts with this antibody by western blot (Figure 3O). This protein has a mol. wt of 75 kDa, which is the expected molecular weight for *Drosophila* PAP. Its level increases during the first hour of embryogenesis (Figure 3O, lanes 2–5); it is very abundant in embryos from females overexpressing PAP in the germline (Figure 3O, lane 1) and in late embryos overexpressing PAP ubiquitously (Figure 3O, lanes 8 and 9), and is absent in hrg^{PAP12} mutant larvae (Figure 3O, lane 7).

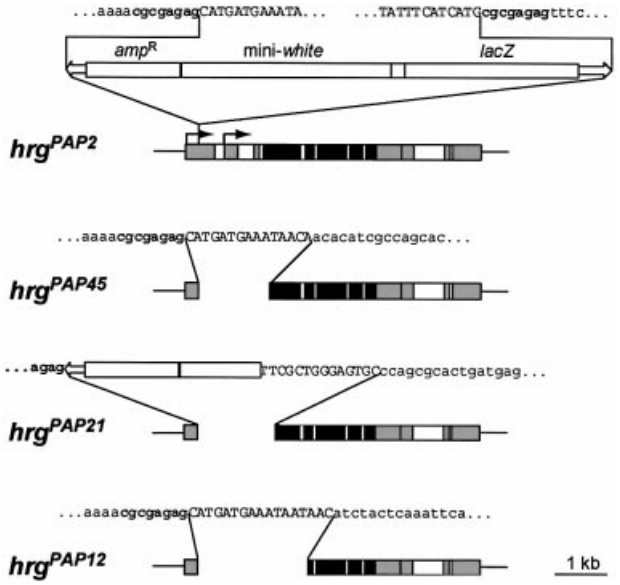

Fig. 4. Structure of *hrg* mutants. Schematic representation of the *hrg* locus; legend is as in Figure 1. The *P*-element insertion in hrg^{PAP2} is represented. The *P*-element sequence is in uppercase, and the genomic sequence surrounding the *P*-element is in lowercase. The sequence duplicated upon insertion of *P* is in bold. For each deletion mutant, the deletion and the remaining sequence are indicated.

These data show that *hrg* encodes a single form of PAP. This protein is mostly nuclear in somatic cells and is present in the cytoplasm of oocytes and early embryos where cytoplasmic polyadenylation takes place.

Characterization of hrg mutants

We mapped the *hrg* gene by *in situ* hybridization to position 56E5–6 on chromosome II. We screened a collection of 21 *P{lacW}*-induced lethal mutants on chromosome II (Torok *et al.*, 1993), containing the *P* insertion in the vicinity of region 56E5–6, by Southern hybridization. In three of these stocks, *l(2)k07618*, *l(2)k07609* and *l(2)k07626*, the *P* element was found to be inserted in the 5′-untranslated region (UTR) of *hrg*. DNA from these three stocks shows the same restriction profile, suggesting that it contains the same *P*-element insertion. The insertion was mapped in the *l(2)k07618* stock and is located 261 bp downstream of the *hrg* 5′-most transcription start site. Although this insertion was isolated in a *P*-induced lethal mutant collection, it does not cause lethality. The mutation inducing lethality in the *l(2)k07618* stock was removed by recombination. This stock was named hrg^{PAP2}. hrg^{PAP2} is viable and fertile, although ~50% of homozygous hrg^{PAP2} individuals die as first instar larvae. New *hrg* mutants were generated by imprecise excision of the *P*-element in hrg^{PAP2}. Three homozygous lethal mutants, hrg^{PAP45}, hrg^{PAP21} and hrg^{PAP12}, that show a deletion in the coding sequence were used in further studies (Figure 4). The first six residues and the first 44 residues are deleted in hrg^{PAP45} and in hrg^{PAP21}, respectively. In hrg^{PAP12}, more than the N-terminal third of PAP (243 residues), including the catalytic core, is deleted. In all three mutants, most of the 5′-UTR of the longest mRNAs as well as the embryonic transcription start site

are missing. The three mutants are lethal from late embryonic to second instar larval stages, with hrg^{PAP45} showing the weakest phenotype and hrg^{PAP12} the strongest. They do not complement each other and are, therefore, alleles of the same gene. Late embryos of all three mutants show no strong phenotype; however, they present a slight defect in head skeleton (distortion of the dorsal bridge, not shown). Lethality of hrg^{PAP45} and hrg^{PAP21} is rescued with a *hrg* genomic transgene. However, only 20% for hrg^{PAP45} and 5% for hrg^{PAP21} of the expected rescued progeny survive to adulthood, suggesting that *hrg* in the transgene is not fully expressed. Lethality of the strongest allele hrg^{PAP12} is not rescued with the genomic transgene, but is rescued with the transgene *UASp-hrg* expressed ubiquitously with the driver *daughterless-Gal4* (*da-Gal4*). We verified that *hrg* mutants described earlier (Murata *et al*., 2001) are alleles of the gene described in this study. hrg^{P1} is not lethal but shows a notched wing phenotype. hrg^{PAP12} does not complement hrg^{P1}, as hrg^{P1}/hg^{PAP12} adults have a pronounced notched wing phenotype.

Taken together, these data demonstrate that we have induced strong alleles of the *hrg* gene that encodes *Drosophila* PAP and that the lack of PAP in *Drosophila* is lethal.

Role of PAP in pre-mRNA cleavage and polyadenylation in vivo

During the mammalian 3′-end processing reaction, PAP has been reported to be required for both the cleavage and polyadenylation steps *in vitro*. However, in these assays, PAP is not involved in the cleavage of all pre-mRNAs (Takagaki *et al*., 1989). We determined whether PAP is involved in cleavage and polyadenylation of pre-mRNAs *in vivo* using *hrg* mutants. To analyse the cleavage step, we looked, by RT–PCR, for RNA molecules that had not been cleaved at poly(A) sites (Benoit *et al*., 2002; and references therein). PCR primers were selected on each side of the poly(A) sites of *rp49* and *sop*, two ubiquitously expressed genes that encode ribosomal proteins (Figure 5A), such that if cleavage occurs normally, no or a very low amount of PCR product is expected. Total RNA was prepared from wild-type and hrg^{PAP12} first instar larvae and controlled by an RT–PCR with primers located in the coding region of *pgk*, another gene expressed ubiquitously. Figure 5B shows that in hrg^{PAP12}, cleavage occurs normally at the poly(A) sites of *rp49* and *sop*, as in the wild-type (Figure 5B, lanes 1 and 2). As a positive control, we used RNA from *suppressor of forked* [*su(f)*] mutant larvae. *su(f)* encodes the *Drosophila* homologue of human CstF-77, which we have shown to be required for the cleavage step of the mRNA 3′-end processing reaction *in vivo* (Benoit *et al*., 2002). In the *su(f)* mutant, uncleaved pre-mRNAs accumulate that can be amplified by RT-PCR for both *rp49* and *sop* (Figure 5B, lane 3). These data indicate that, *in vivo*, PAP is dispensable for the cleavage step of the mRNA 3′-end processing reaction.

We next measured poly(A) tail length of *rp49* and *sop* in *hrg* mutants by poly(A) test (PAT) assays, a PCR-based technique that allows amplification of poly(A) tails (Salles and Strickland, 1999). In wild-type first instar larvae, the longest poly(A) tails of both *rp49* and *sop* mRNAs were found to be up to 140 residues (Figure 5C, lanes 1 and 4). In the weak hrg^{PAP45} mutant, poly(A) tails of *rp49* mRNA

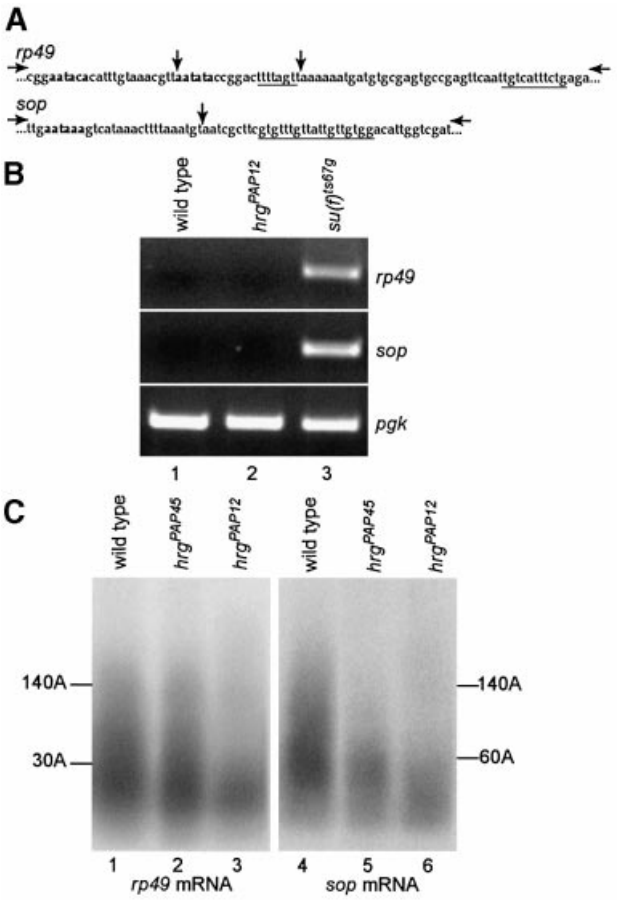

Fig. 5. Role of PAP in the cleavage/polyadenylation reaction *in vivo*. (**A**) Sequences of *rp49* and *sop* poly(A) site regions. Potential poly(A) signals are in bold. GU/U-rich sequences are underlined. Vertical arrows indicate poly(A) sites determined from cDNA sequences in databases. Horizontal arrows indicate primers used in the PCR. (**B**) RT–PCR assays. Total RNAs from larvae [first instar at 25°C for wild-type and hrg^{PAP12}, third instar at 29°C for the $su(f)^{ts67g}$ ts mutant] were used for reverse transcription. The control *pgk* PCR fragment is generated with primers on each side of intron 2 of *pgk*. The size of the *pgk* PCR product (434 bp) is the expected size for amplification of *pgk* RNA after splicing of intron 2. *rp49* (369 bp) and *sop* (316 bp) PCR fragments are obtained with the primers indicated in (A), only if no cleavage occurs at the poly(A) site. (**C**) PAT assays measuring poly(A) tail length of *rp49* and *sop* mRNAs. Total RNAs were prepared from wild-type or mutant first instar larvae. The lengths of poly(A) tails are indicated.

are not reduced and those of *sop* mRNA are reduced to 50% of their length in the wild-type (Figure 5C, lanes 2 and 5). In hrg^{PAP12} mutant larvae, poly(A) tails of both *rp49* and *sop* are strongly reduced and reach a maximal length of 30 and 60 residues for *rp49* and *sop* mRNAs, respectively (Figure 5C, lanes 3 and 6). This suggests that poly(A) tail synthesis is affected in this mutant.

Therefore, utilization of *hrg* mutants allowed us to conclude that *in vivo* PAP is dispensable for the cleavage step, but is required for poly(A) tail elongation during the mRNA 3′-end processing reaction.

Genetic interaction between hrg and orb

To address a possible role for *hrg* in cytoplasmic polyadenylation during early development, we induced germline clones homozygous for hrg^{PAP45}, hrg^{PAP21} or

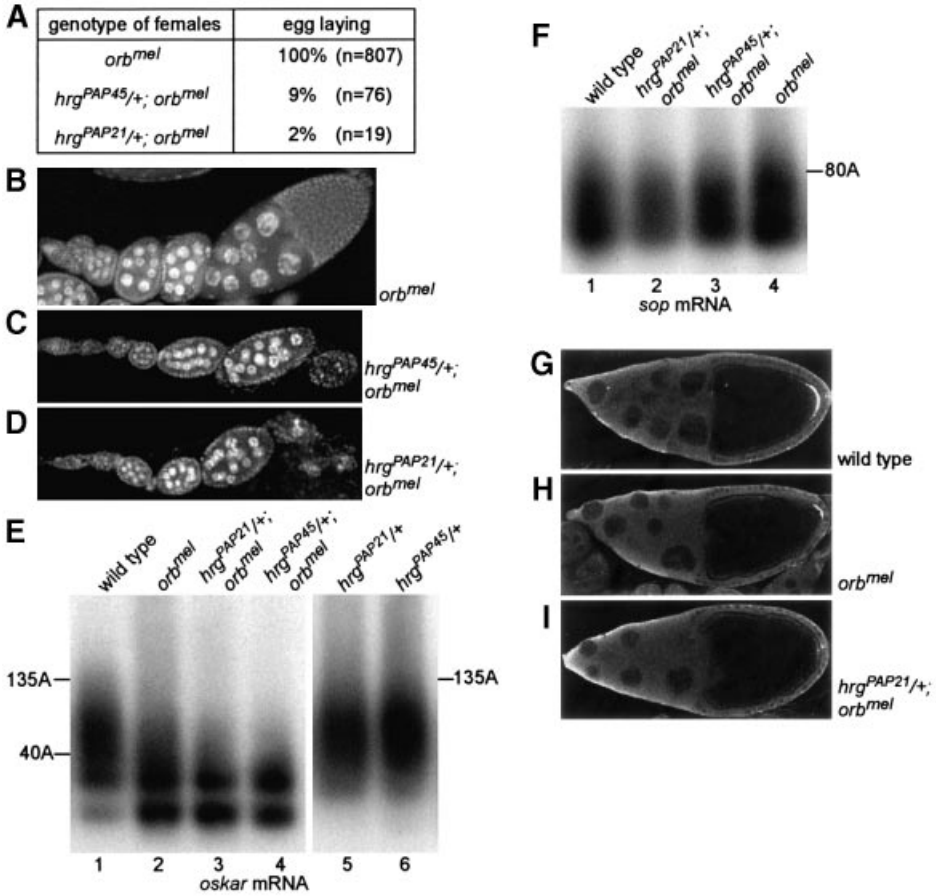

Fig. 6. Role of *hrg* in cytoplasmic polyadenylation. (**A**) Phenotype of *hrg*$^{-}$/+; *orb*^{mel} mutant females. The number of eggs laid by 50 females for 3 days is indicated. (**B**–**D**) DAPI staining of ovaries showing that the ovarian phenotype is strongly enhanced when *orb*^{mel} is combined with *hrg*$^{-}$/+. The genotype of females is indicated. (**E** and **F**) PAT assays measuring poly(A) tail lengths of *oskar* (E) or *sop* (F) mRNAs in wild-type and mutant ovaries. The genotype of females and the lengths of poly(A) tails are indicated. (**G**–**I**) Immunostaining of stage 10 egg chambers with anti-Oskar antibody showing that virtually no Oskar accumulates in *hrg*$^{-}$/+; *orb*^{mel} mutant oocytes. Oskar accumulates as a cap at the posterior of the wild-type oocyte. Posterior is to the right. The genotype of females is indicated.

hrg^{PAP12}. No germline clones were obtained for any of these mutants, possibly as a result of a requirement of PAP for cell viability. We therefore studied genetic interactions between *hrg* and *orb*, which is known to be involved in cytoplasmic polyadenylation. Females homozygous for the weak *orb*^{mel} allele produce egg chambers at all stages and lay eggs, 30% of which show a ventralized phenotype (Christerson and McKearing, 1994). We found that *hrg* lethal mutants act as dominant enhancers of the *orb*^{mel} phenotype, as *hrg*^{PAP45}/+; *orb*^{mel} and *hrg*^{PAP21}/+; *orb*^{mel} females lay almost no eggs (Figure 6A). In these females, oogenesis stops most frequently at stage 7/8, after which egg chambers degenerate (Figure 6C and D), even though one or two stage 14 oocytes per ovary can be observed. Poly(A) tails of *oskar* mRNA were shown previously to be shortened in *orb* mutant ovaries (Chang *et al*., 1999). We analysed the defect of these poly(A) tails in *hrg*$^{-}$/+; *orb*^{mel} mutants by PAT assays. We measured *oskar* mRNA poly(A) tails to be up to 135 residues in wild-type ovaries (Figure 6E, lane 1). These poly(A) tails are weakly reduced in *orb*^{mel} (Figure 6E, lane 2), but severely reduced in *hrg*$^{-}$/+; *orb*^{mel} ovaries, their maximal length reaching 40–50 residues (Figure 6E, lanes 3 and 4). These short poly(A) tails do not result from the oogenesis defect in *hrg*$^{-}$/+; *orb*^{mel} females, as unrelated mutants that stop oogenesis early show wild-type poly(A) tails of *oskar* mRNA (Chang *et al*., 1999). These poly(A) tails were also found to be of wild-type length in *hrg*^{PAP21}/+ and *hrg*^{PAP45}/+ ovaries (Figure 6E, lanes 5 and 6). This shows that the strong shortening of *oskar* poly(A) tails in *hrg*$^{-}$/+; *orb*^{mel} mutants does not result from an additive effect of two phenotypes, but from a synergistic effect of the two mutants due to a simultaneous decrease in PAP and Orb protein levels. This strongly suggests that *hrg* and *orb* are involved together in cytoplasmic polyadenylation. This was confirmed by measurements of poly(A) tails of a control mRNA, *sop*, which is thought not to be regulated by cytoplasmic polyadenylation. Poly(A) tails of *sop* mRNAs are unaffected in *orb*^{mel} as well as in *hrg*$^{-}$/+; *orb*^{mel} mutant ovaries (Figure 6F). We verified that shortening of *oskar* mRNA poly(A) tails in *hrg*$^{-}$/+; *orb*^{mel} mutants leads to a reduction of Oskar protein level, by immunostaining of ovaries with anti-Oskar. Oskar accumulates at the posterior of the oocyte from stage 9 onwards. As described before, the amount of Oskar decreases in *orb*^{mel} oocytes (Figure 6G and H). This amount decreases again in *hrg*$^{-}$/+; *orb*^{mel} oocytes to a barely detectable level (Figure 6I).

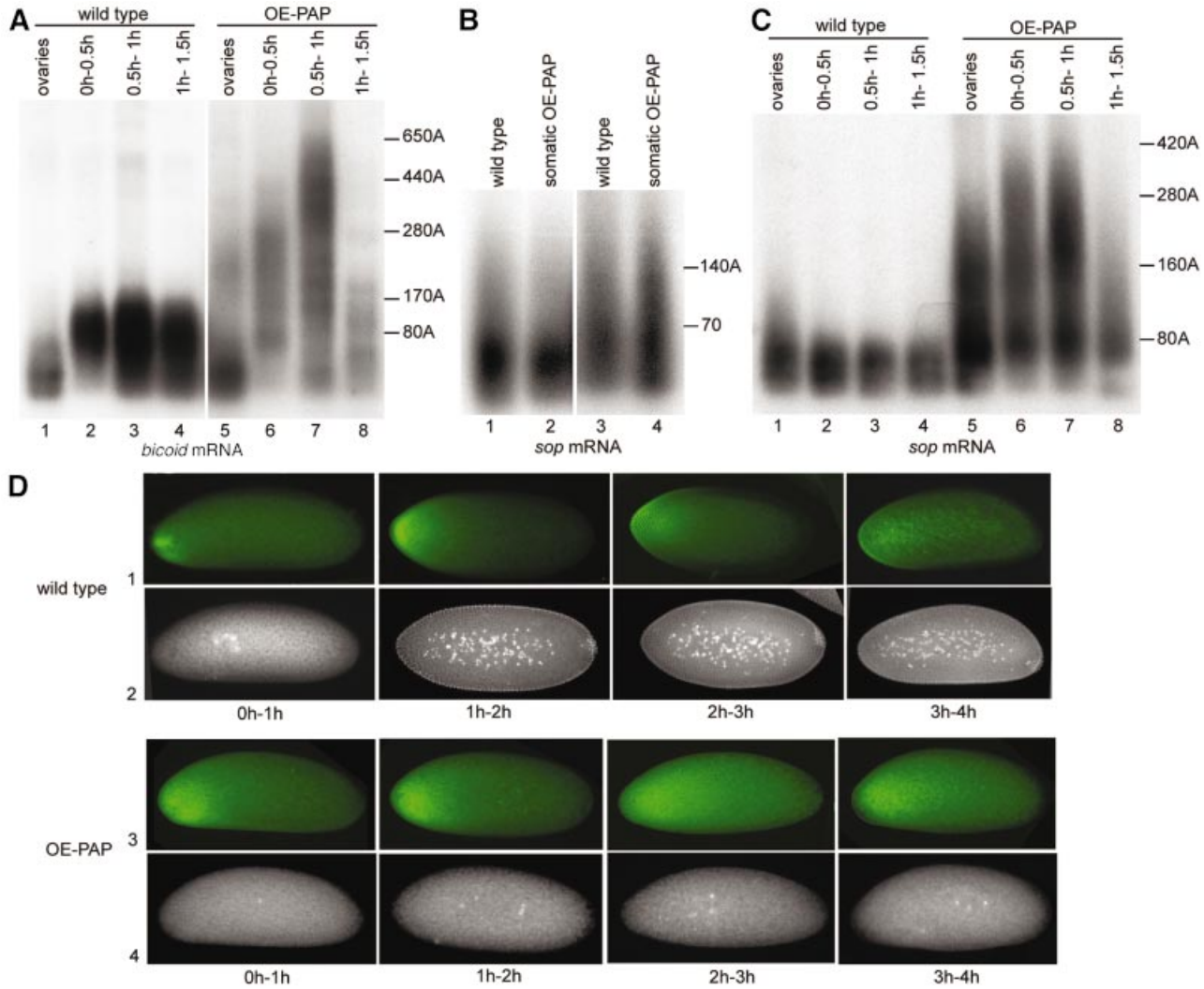

Fig. 7. Overexpression of PAP in the female germline enhances cytoplasmic polyadenylation. (A–C) PAT assays measuring poly(A) tail lengths of *bicoid* (A) and *sop* (B and C) mRNAs. (**A** and **C**) Total RNAs were prepared from ovaries or 0–0.5 h, 0.5–1 h and 1–1.5 h embryos from wild-type or *UASp-hrg*; *nos-Gal4* (OE-PAP: PAP overexpression) females crossed with wild-type males. (**B**) Total RNAs were prepared from wild-type or *UASp-hrg*; *da-Gal4* (somatic OE-PAP) 16–20 h embryos (lanes 1 and 2) or early pupae (lanes 3 and 4). Poly(A) tail lengths are indicated. (**D**) Double staining with anti-Bicoid antibody (rows 1 and 3) and DAPI (rows 2 and 4) of 0–1 h, 1–2 h, 2–3 h and 3–4 h embryos from wild-type or *UASp-hrg*; *nos-Gal4* (OE-PAP) females crossed with wild-type males. Posterior is to the right. Embryos where PAP is overexpressed show up to a 2-fold increase in Bicoid level (row 3) (quantification was with MetaMorph). These embryos contain one to a few nuclei only (row 4).

Taken together, these results show that *hrg* and *orb* cooperate in poly(A) tail lengthening during cytoplasmic polyadenylation and that alteration of this process affects protein accumulation and oogenesis.

Overexpression of PAP in the female germline causes embryonic lethality of the progeny

To address whether cytoplasmic polyadenylation could be affected by the level of PAP, we overexpressed PAP in the female germline using the *UASp-hrg* transgene and *nos-Gal4* (Figure 3D). This overexpression does not cause gross alteration of oogenesis, but is extremely detrimental to embryogenesis, leading to 99% lethality of the progeny (n = 5947). These embryos stop development early, before cleavage of nuclei (Figure 7D, row 4). We analysed cytoplasmic polyadenylation in these embryos by measuring poly(A) tails of *bicoid* mRNA that is regulated by this process during embryogenesis (Salles *et al*., 1994). In the wild-type, poly(A) tails of *bicoid* mRNA lengthen from 80 residues in oocytes to 170 residues in 1 h embryos (Figure 7A). This elongation of the poly(A) tails induces Bicoid protein synthesis in early embryos (Figure 7D, row 1) (Salles *et al*., 1994). Following overexpression of PAP, poly(A) tails of *bicoid* mRNA strongly increase in length, with a pool of mRNAs bearing a 250 residue poly(A) tail in oocytes and most mRNAs having a poly(A) tail between 300 and 600 residues in 1 h embryos (Figure 7A). The fact that *bicoid* mRNA poly(A) tails lengthen in 0–1 h embryos, at a stage when there is no transcription, shows that the process affected by PAP overexpression is cytoplasmic polyadenylation. This was confirmed by showing that when PAP is overexpressed ubiquitously in somatic cells with the *da-Gal4* driver, poly(A) tails of *sop* mRNA are not affected (Figure 7B). Therefore, poly(A) tail length control during nuclear polyadenylation is not altered by PAP overexpression, although the level of

somatic overexpression is in the same range as that of germline overexpression (Figure 3O, lanes 1 and 2, and 8 and 9). Surprisingly, although *sop* mRNA does not undergo cytoplasmic polyadenylation in wild-type embryos (Figure 7C, wild-type panel), overexpression of PAP in the female germline leads to a strong lengthening of *sop* mRNA poly(A) tails by cytoplasmic polyadenylation (Figure 7C). Similar results were found for *rp49* mRNA (not shown). This indicates that the increasing PAP level affects both poly(A) tail length control and specificity during cytoplasmic polyadenylation. We correlated Bicoid protein accumulation and *bicoid* mRNA poly(A) tail length by immunostaining of ovaries and embryos with anti-Bicoid. Poly(A) tail elongation of *bicoid* mRNA in oocytes, following PAP overexpression, does not induce translation as no Bicoid is detected in *UASp-hrg*; *nos-Gal4* oocytes (not shown). Therefore, in oocytes, long poly(A) tails are not sufficient to induce *bicoid* mRNA translation. In embryos where PAP is overexpressed, poly(A) tail lengthening correlates with a precocious accumulation of Bicoid and with an increase in Bicoid protein level (Figure 7D, row 3).

These data demonstrate that a tight regulation of PAP level is essential to control cytoplasmic polyadenylation and to early development.

Discussion

Data in this article allow us to draw two important conclusions. (i) A single isoform of PAP is able to perform both reactions of nuclear and cytoplasmic polyadenylation *in vivo*. (ii) A controlled level of PAP is essential for specificity of cytoplasmic polyadenylation and for poly(A) tail length control during cytoplasmic polyadenylation.

A single PAP in Drosophila

We and others found that there is a single PAP-encoding gene, *hrg*, in the *Drosophila* genome (Mount and Salz, 2000; Murata *et al*., 2001; this study). Although *hrg* produces three mRNAs, they all encode the same protein. Western blots on *Drosophila* extracts confirm the presence of a single PAP isoform in *Drosophila*. As expected for a gene responsible for such a fundamental process as polyadenylation, *hrg* is essential for viability. Strong *hrg* mutants are lethal at late embryonic and larval stages. *hrg* is also probably essential to cell viability as strong *hrg* mutant germline clones do not survive. We showed that PAP encoded by this gene is involved in both nuclear polyadenylation of *rp49* and *sop* mRNAs in somatic tissues and cytoplasmic polyadenylation of *oskar* mRNA in oocytes. This indicates that although the reactions of nuclear and cytoplasmic polyadenylation are not identical, a single PAP is responsible for both in *Drosophila*.

Recently, a new class of cytoplasmic PAPs was discovered that is not related in sequence to conventional PAPs (Wang *et al*., 2002). These proteins are widespread in eukaryotes and three homologues exist in *Drosophila*. A member in nematodes functions in germline and embryonic development; therefore, this class of proteins was proposed to play a role in cytoplasmic polyadenylation during development, in addition to conventional PAPs.

Role of PAP in nuclear polyadenylation

We found that *Drosophila* PAP indeed has a poly(A) polymerase activity *in vitro*, in reconstituted specific polyadenylation assays. Stimulation of *Drosophila* PAP activity by bovine CPSF indicates that *Drosophila* PAP and bovine CPSF interact. In mammalian PAP, the region thought to be involved in interaction with CPSF overlaps the first NLS (Thuresson *et al*., 1994) and this domain is conserved in *Drosophila*.

We showed that *Drosophila* PAP has a role *in vivo* in nuclear polyadenylation. In *hrg* mutant larvae, poly(A) tails of *rp49* and *sop* mRNAs are short. These short poly(A) tails probably result from the decay of *rp49* and *sop* transcript pools and a lack of newly polyadenylated mRNAs. *In vitro* studies led to the general belief that PAP is required for the cleavage of pre-mRNAs at poly(A) sites (Zhao *et al*., 1999). However, we found that it is not the case *in vivo*, at least for two different pre-mRNAs. Overexpression of PAP in somatic tissues does not alter poly(A) tail length of *sop* mRNAs. This indicates that the amount of PAP is not limiting *in vivo* for nuclear polyadenylation. This is not unexpected as, during the polyadenylation reaction, PABP2 plays an important role in stimulating PAP. Once PABP2 is bound to the newly synthesized 10 residue poly(A) tail, a single PAP molecule is required to polymerize the complete poly(A) tail (Bienroth *et al*., 1993). PABP2 also controls the length of the poly(A) tail, and this function of PABP2 may prevent the recruitment of new PAP molecules in the complex, and the poly(A) tail to lengthen even if more PAP is present.

Role of PAP level in cytoplasmic polyadenylation

We showed that *hrg*, in conjunction with *orb*, is involved in cytoplasmic polyadenylation of *oskar* mRNA during oogenesis. The control of *oskar* mRNA translation is very complex. *oskar* mRNA is transported to the posterior pole of the oocyte and translation does not start before this posterior localization (Kim-Ha *et al*., 1995; Markussen *et al*., 1995; Rongo *et al*., 1995). An essential determinant in translational repression during *oskar* mRNA transport is the Bruno protein (Kim-Ha *et al*., 1995; Webster *et al*., 1997). Although the mechanism underlying translational repression by Bruno currently is unknown, it has been shown, in a cell-free system, to be independent of poly(A) tail length (Lie and Macdonald, 1999). Therefore, although *oskar* mRNA was shown to undergo cytoplasmic polyadenylation (Chang *et al*., 1999), the role of this regulation in the control of *oskar* mRNA translation is unclear. Our data provide further evidence that cytoplasmic polyadenylation has a role in Oskar expression, since, when this process is impaired, Oskar does not accumulate at the posterior pole of the oocyte. Regulation of *oskar* mRNA poly(A) tail length probably represents an additional level of control of Oskar expression. We found that in hrg^{PAP21}/+; orb^{mel} mid-oocytes, the amount of *oskar* mRNA is low (not shown). This suggests that cytoplasmic polyadenylation could be required to unbalance rapid deadenylation and decay of *oskar* mRNA.

Other mRNAs regulated by cytoplasmic polyadenylation in *Drosophila* oogenesis have not been identified, but many are to be expected. Strong alleles of *orb* stop

oogenesis early, and a recent study indicates that *orb* is required for oocyte determination (Huynh and St Johnston, 2000). This suggests that Orb regulates translation of mRNAs that have a function very early during oogenesis. In agreement with this, we found that hrg^{-}/+; orb^{mel} females stop oogenesis at an earlier stage than orb^{mel} females. mRNAs regulated by cytoplasmic polyadenylation during early oogenesis were identified in mouse. They encode two proteins of the synaptonemal complex, a complex required for recombination during meiosis, and these proteins are not produced in CPEB knockout mouse oocytes (Tay and Richter, 2001).

A crucial conclusion from our data is that a tightly regulated level of PAP has a major role in cytoplasmic polyadenylation. Overexpressing PAP in the female germline results in a strong elongation of poly(A) tails of *bicoid* and *sop*, mRNAs that are and are not regulated by cytoplasmic polyadenylation, respectively. Therefore, increasing the level of PAP alters both poly(A) tail length control and the specificity of cytoplasmic polyadenylation for certain mRNAs. This deregulation leads to early embryonic lethality. That a low level of PAP is important for cytoplasmic polyadenylation regulation correlates with the repression of PAP activity by phosphorylation in *Xenopus* oocytes during meiotic maturation, when cytoplasmic polyadenylation occurs (Ballantyne *et al*., 1995; Colgan *et al*., 1998). In contrast, overexpression of PAP in somatic tissues does not affect poly(A) tail length control during nuclear polyadenylation. This difference has mechanistic implications and suggests that if PABP2 is involved at some step in cytoplasmic polyadenylation, as we found to be the case (B.Benoit and M.Simonelig, in preparation), its role is different from that during nuclear polyadenylation. That an increase of PAP level leads to unregulated very long poly(A) tails suggests that poly(A) tail synthesis during cytoplasmic polyadenylation mainly depends on the ability of PAP to interact with mRNAs and that cytoplasmic polyadenylation never enters a processive state where a single PAP molecule would be sufficient to complete a poly(A) tail in one event. This correlates with the slowness of the reaction during embryogenesis where elongation of *bicoid* mRNA poly(A) tail extends for 1–1.5 h of development (Salles *et al*., 1994; this study).

In *Xenopus* oocytes, CPEB makes the cytoplasmic polyadenylation reaction specific to some mRNAs, and it is probable that Orb has the same role in *Drosophila*. The loss of specificity of the reaction following PAP overexpression indicates that the PAP level also has an active role in this specificity. In this context, cytoplasmic poly(A) tail elongation of *sop* and other mRNAs that do not normally undergo this reaction probably does not require Orb, nor another protein that would recognize these mRNAs specifically. This suggests that an active cytoplasmic polyadenylation complex can form in the absence of Orb/CPEB, that would contain CPSF and PAP only. *In vitro* studies have also led to this conclusion (Dickson *et al*., 2001). However, whether or not such a complex is actually responsible for cytoplasmic polyadenylation of some mRNAs *in vivo* under normal conditions, and in that case what makes the reaction specific, represent a challenge for further studies.

Materials and methods

Drosophila stocks and genetics

The w^{1118} stock was used as a control. Homozygous *hrg* mutants were selected using the balancer chromosome *CyO-pAct-GFP*. We used the *da-Gal4* (Wodarz *et al*., 1995) and *nos-Gal4* (Rorth, 1998) driver lines, that mediate ubiquitous expression and expression in the female germline, respectively. *P*-element transformation was performed using a standard method. *hrg* deletion mutants were screened using the loss of the $white^{+}$ marker in the *P*-element. A total of 83 lines were recovered after mobilization of *P{lacW}* in hrg^{PAP2}, in the presence of transposase. Twenty-three lines are homozygous viable, 22 homozygous lethal and 34 homozygous sublethal. Germline clones were induced by using the FLP/FRT technique (Chou and Perrimon, 1996). No hrg^{PAP45}, hrg^{PAP21} or hrg^{PAP12} mutant germline clone was obtained, whereas control wild-type germline clones were obtained.

DNA constructs

The genomic *hrg* transgene *pCaSpeR-hrg* was constructed as follows. A *Pst*I–*Sal*I 7.9 kb genomic fragment containing the *hrg* gene with 0.9 kb upstream of the 5′-most transcription start site and 1.1 kb downstream of the 3′-most poly(A) site was cloned into pBluescript digested with *Pst*I and *Sal*I. The genomic fragment was then digested with *Xho*I and *Not*I and cloned into pCaSpeR4 digested by *Xho*I and *Not*I. The *UASp-hrg* transgene was generated as follows. A cDNA fragment containing the complete *hrg* coding sequence was digested from NB61 with *Hae*II and *Nde*I, repaired with Klenow and cloned into pBluescript digested with *Eco*RV. The cDNA fragment was then digested with *Not*I and *Kpn*I and cloned into pUASp (Rorth, 1998) digested with *Not*I and *Kpn*I.

RNA manipulation

Poly(A)$^{+}$ RNAs for northern blots were purified using the Oligotex mRNA kit (Qiagen). PAT assays were performed as described (Salles and Strickland, 1999) and repeated at least four times with two independent RNA preparations for each experiment. The specific primers were 5′-CTGCCCACCGGATTCAAGAAGT for *rp49*, 5′-GGATTGCTACAC-CTCGGGCCGT for *sop*, 5′-GGCGCAGTGGGCGTGGTACG for *oskar*, and 5′-CATTTGCGCATTCTTTGACC for *bicoid*. RT–PCR to assay cleavage of pre-mRNAs were performed as described (Benoit *et al*., 2002).

Expression and purification of His_6-PAP and polyadenylation assays

A His-tagged *Drosophila* PAP was expressed in *E.coli* as follows. A 2.6 kb *Afl*III fragment from NB61 was cloned in the pET30a vector (Novagen) digested by *Nco*I. The resulting construct contains the whole PAP-coding sequence fused in-frame with a His_6 tag at its N-terminus. His_6-PAP was expressed in the *E.coli* BL21 strain and purified as described (Martin and Keller, 1996). Polyadenylation assays were performed as described (Wahle, 1995) except that *Drosophila* PABP2 was substituted for bovine PABP2 (Benoit *et al*., 1999).

Antibody preparation, immunostaining and western blots

Anti-PAP polyclonal antibody was raised in rat by Eurogentec against the purified bacterially expressed His_6-PAP. Immunostaining and western blots were performed as described (Benoit *et al*., 1999). Antibody dilutions were: anti-PAP (1/500 or 1/1000), anti-Oskar (1/500) (Kim-Ha *et al*., 1995), anti-Bicoid (1/200) (Kosman *et al*., 1998) and anti-α-tubulin (1/1000) (Sigma).

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