The dynamics of fluorescently labeled endogenous gurken mRNA in Drosophila

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Summary

During Drosophila oogenesis, the targeted localization of gurken (grk) mRNA leads to the establishment of the axis polarity of the egg. In early stages of oogenesis, grk mRNA is found at the posterior of the oocyte, whereas in the later stages grk mRNA is positioned at the dorsal anterior corner of the oocyte. In order to visualize the real-time localization and anchorage of endogenous grk mRNA in living oocytes, we have utilized the MS2-MCP system. We show that MCP-GFP-tagged endogenous grk mRNA localizes properly within wild-type oocytes and behaves aberrantly in mutant backgrounds. Fluorescence recovery after photobleaching (FRAP) experiments of labeled grk mRNA in egg chambers reveal a difference in the dynamics of grk mRNA between young and older egg chambers. grk mRNA particles, as a population, are highly dynamic molecules that steadily lose their dynamic nature as oogenesis progresses. This difference in dynamics is attenuated in K10 and sqd mutants such that mislocalized grk mRNA in older stages is much more dynamic compared with that in wild-type controls. By contrast, in flies with compromised dynein activity, properly localized grk mRNA is much more static. Taken together, we have observed the nature of localized grk mRNA in live oocytes and propose that its maintenance changes from a dynamic to a static process as oogenesis progresses.

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Introduction

Asymmetric messenger RNA localization provides a means to spatially restrict protein synthesis. In many cell types, on-site translation serves to maintain and elaborate a polarized architecture. However, the fundamental questions of how a transcript moves to its destination, how it remains translationally silent while in transit and how it is anchored at its target site remain unanswered for many mRNAs (reviewed in St Johnston, 2005). In Drosophila, several asymmetrically localized transcripts have been identified in the developing oocyte. bicoid mRNA is localized at the anterior of the oocyte, whereas oskar can be found spatially restricted at the posterior of the oocyte (Berleth et al., 1988; Ephrussi et al., 1991; Lehmann and Nusslein-Volhard, 1986). These transcripts, along with gurken (grk) mRNA, are necessary for the patterning of the Drosophila embryo. gurken encodes a transforming growth factor-α (TGFα)-like protein that is secreted by the oocyte and activates the epidermal growth factor receptor (EGFR) in the overlying somatic follicle cells. In early oogenesis, grk mRNA accumulates at the posterior of the oocyte. Gurken protein, synthesized from this mRNA, induces posterior cell fates in the overlying follicle cells. Soon thereafter, during mid-to-late oogenesis, grk mRNA and protein are localized to the dorsal anterior corner of the oocyte, establishing dorsal cell fates in lateral follicle cells (Neuman-Silberberg and Schupbach, 1996; St Johnston, 2005; Van Buskirk and Schupbach, 1999).

Mutations in several genes have been identified that result in mislocalization of grk mRNA. Some of these genes, such as squid and hrbc27c, encode heterogeneous nuclear ribonucleoproteins (hnRNPs) (Goodrich et al., 2004; Neuman-Silberberg and Schupbach, 1993). Others include genes necessary for cytoskeletal integrity, such as cappuccino, spire (an actin nucleator) and spindle-F (microtubule organizer) (Abdu et al., 2006; Mansneau and Schupbach, 1989; Neuman-Silberberg and Schupbach, 1993). In addition, it has been shown that the motor enzymes kinesin and dynein are required for correct grk mRNA localization (Brendza et al., 2002; Clark et al., 2007; Delanoue et al., 2007; Duncan and Warrior, 2002; Januschke et al., 2002). These requirements suggest a model whereby grk mRNA is assembled into an RNP complex that is transported on filaments by molecular motors. In fact, grk mRNA synthesized in vitro and injected during mid-oogenesis assembles into nonmembranous transport particles that move first to the anterior of the oocyte and then dorsally towards the nucleus, and these movements are dependent on dynein. Furthermore, microtubule depolymerization disrupts the directionality of injected grk mRNA, implying active transport along microtubules (Clark et al., 2007; Delanoue et al., 2007; MacDougall et al., 2003). However, little is known about how grk mRNA is maintained or anchored at its destination. Recent ultrastructural analysis suggest that grk mRNA is not only transported but also anchored by dynein to large cytoplasmic structures called sponge bodies at the dorsal-anterior corner (Delanoue et al., 2007).

We have used a system for fluorescently labeling mRNA in vivo to visualize the real-time localization and anchoring of endogenous grk mRNA in living oocytes. We show that GFP-labeled endogenous grk mRNA properly localizes within wild-type oocytes and behaves aberrantly in mutant backgrounds. Interestingly, pharmacological studies show that the anchoring of grk mRNA might not be dependent on an intact cytoskeleton or, alternatively, it might utilize a subpopulation of microtubules that are very drug resistant. Fluorescence recovery after photobleaching (FRAP) experiments of grk mRNA particles in live egg chambers reveal a difference in the dynamic state of localized grk mRNA between early- and mid-stage egg chambers. grk mRNA particles, as a population, are highly dynamic molecules, exhibiting high fluorescence recovery, during...
early stages. As oogenesis progresses, grk mRNA steadily loses its dynamic nature. This difference in grk mRNA mobility is attenuated in K10 and sqd mutants such that mislocalized grk mRNA in older stages remains much more dynamic. By contrast, in flies with compromised dynein activity, properly localized grk mRNA is more static. Taken together, we have observed the changing nature of localized grk mRNA in live oocytes and have demonstrated that its maintenance can be a dynamic process. We show that localized grk mRNA, as a population, can be highly dynamic or static, depending on the stage of oogenesis. Investigating the changing nature of grk mRNA allows us to gain insight into the mechanisms involved in the maintenance of localized transcripts.

Results

In vivo labeling of gurken mRNA in egg chambers

In order to visualize grk mRNA in live egg chambers, we took advantage of a system for fluorescent labeling of mRNA in vivo. We have utilized the MS2-MS2 coat protein (MS2-MCP) system pioneered by Singer and colleagues in yeast (Bertrand et al., 1998) and adapted for Drosophila by Gavis and colleagues (Forrest and Gavis, 2003; Weil et al., 2006). Twelve stem-loop binding sites for MCP were inserted into the 3'UTR of grk mRNA. The grk-(MS2)$_2$ transgene rescues the grk$_{2B}$ mutant phenotype, indicating that the stem loops do not significantly interfere with the function of the RNA. grk-(MS2)$_2$ was coexpressed with either the hsp83-MCP-GFP or the hsp83-MCP-RFP transgene, which encode MCP fused to either green fluorescent protein (GFP) or red fluorescent protein (RFP) (Weil et al., 2006). Eggs from flies expressing both transgenes, grk-(MS2)$_2$, together with hsp83-MCP-GFP or hsp83-MCP-RFP (referred to as grk*GFP and grk*RFP), show no obvious alterations in morphology. Moreover, the labeled RNAs, grk*GFP and grk*RFP, have localization patterns identical to that of wild-type grk mRNA.

As previously described, grk mRNA is expressed in early egg chambers (stages 1-7) and localized to the posterior end of the oocyte. Starting at stage 8, the oocyte nucleus moves from the center to the anterior edge of the oocyte. As the egg chamber grows, this edge will become the future dorsal anterior corner. During this stage, grk mRNA begins to accumulate around the new position of the nucleus as well as the anterior of the oocyte, creating a transient cortical ring. From stages 9 to 10B, grk mRNA remains in tight association with the dorsal anterior corner, forming a cap over the nucleus (Neuman-Silberberg and Schapbach, 1993). We used laser scanning confocal microscopy for live imaging of grk*GFP and grk*RFP during oogenesis (Fig. 1). Egg chambers were dissected and kept within a culture dish with nutrient media and then immediately imaged. Throughout early stages 4-7, grk*GFP and grk*RFP can be seen lining the posterior of the oocyte (Fig. 1A). During stage 7, when the oocyte nucleus is centered or shifting to one side, live images of grk*GFP show it to accumulate around the anterior cortex as well as continuing to line the posterior. At stage 8, the oocyte nucleus moves entirely to one side. As soon as the nucleus is asymmetric, the amount of grk*GFP at the posterior and ventral lateral sides of the oocyte decreases and, instead, grk*GFP appears to line the lateral wall posterior to the nucleus (Fig. 1B). At this stage, the dorsal-anterior cap of grk*GFP above the nucleus becomes increasingly prominent. While stage 8 progresses, grk*GFP continues to be visible anteriorly in a prominent ring, and meanwhile little to no grk*RFP can be seen at the posterior. As the egg chamber develops through stage 9, less and less grk*RFP accumulates in the ring, whereas grk*GFP in the ventral-anterior corner persists a bit longer, as previously reported by MacDougall and colleagues using fluorescent in situ hybridization (MacDougall et al., 2003). During this time, grk*RFP becomes more prominently localized to the dorsal-anterior cap above the nucleus. From late stage 9 to 10B, grk*RFP continues to be found above the nucleus and has a fainter appearance.

Actin-destabilizing drugs do not disrupt the maintenance of localized grk mRNA in live egg chambers

An intact actin cytoskeleton has been shown to be important for asymmetric localization of RNA and protein in Drosophila oocytes (reviewed by Hudson and Cooley, 2002). Treatment with actin-destabilizing drugs, such as cytochalasin D, disrupts the anchoring of nanos and bicoid mRNA (Forrest and Gavis, 2003; Weil et al., 2006). Also, elimination or reduction of actin-binding proteins, such as moesin and troponymosin, disrupts the posterior maintenance of Osk protein and mRNA (Jankovics et al., 2002; Polesello et al., 2002). In order to investigate whether grk mRNA requires an intact actin framework, we treated live egg chambers expressing grk*RFP with the actin-destabilizing drugs latrunculin A and cytochalasin D, either individually or in combination. To test the effectiveness of these drugs, we stained egg chambers with phalloidin, which revealed very reduced, barely detectable F-actin, except for prominent ring canals. The drug concentrations that we used were capable of blocking nurse cell cell dumping, which results from actin-dependent contractions of the nurse cells (Gutzeit, 1982). Time-lapse imaging of egg chambers from drug-treated GFP-actin flies show rapid depolymerization. We were therefore confident that actin was severely disrupted. Interestingly, we found that grk*RFP is well maintained during stages 6-10 (Fig. 2). grk*RFP remains tightly anchored both early at the posterior and later at the dorsal-anterior corner, and this anchoring is disrupted only when the drug
concentrations are high enough to cause the oocyte to collapse. In these cases, grk*RFP spreads along the cortex of the oocyte. Additionally, we fed adult female grk*RFP flies latrunculin A and cytochalasin D and observed that grk*RFP was properly localized (data not shown). Similarly, Saunders and Cohen (Saunders and Cohen, 1999) saw no changes in the localization of the grk transcript from flies fed cytochalasin D.

Resistant microtubules might explain why grk mRNA remains localized after using microtubule-destabilizing drugs

Microtubules are central for the localization of several mRNAs in Drosophila oocytes (St Johnston, 2005). With respect to maintenance, disruption of microtubules has been shown to release bicoid, js(1)K10, orb and Bicaudal-D from the anterior of the oocyte (Pokrywka and Stephenson, 1995). In order to investigate whether an intact microtubule network is needed to maintain grk mRNA localization, we treated live egg chambers from flies expressing grk*RFP with a combination of the microtubule-destabilizing drugs colchicine and colcemid. After drug treatments, grk*RFP localization appeared not to have been disrupted, whether positioned at the posterior or at the dorsal anterior corner during stages 6-10 (Fig. 3). Time-lapse imaging of drug-treated egg chambers over intervals of 45-60 minutes confirmed these results. These results suggest that anchoring of grk*RFP is not dependent on microtubules or that the anchoring is mediated by a population of microtubules that are resistant to these inhibitors. To ensure that certain populations of microtubules were indeed disrupted, we monitored the microtubule-dependent ooplasmic streaming that occurs before nurse cell dumping (Gutzeit, 1982). This unidirectional flow ceased within 3 minutes of applying our treatment of colcemid and colchicine, indicating that the drug treatment was very efficient at disrupting microtubules that promote cytoplasmic streaming. We also imaged drug-treated tau-GFP-expressing fly egg chambers. Tau-GFP decorates microtubules and allowed us to follow depolymerization in real time. Interestingly, while depolymerization of microtubules in the oocyte and nurse cells was plainly evident, the microtubule basket surrounding the oocyte nucleus [previously reported at stage 9 by MacDougall and colleagues (MacDougall et al., 2003)] was clearly resistant to the combined effects of colchicine and colcemid (see supplementary material Movie 1). This suggests that there might be different types of microtubules present in the oocyte that have different sensitivities to depolymerizing drugs. Whether grk*RFP is anchored to these resistant microtubules is unclear, but our data clearly show that grk*RFP is not disrupted by the treatment.

We also examined the combined effect of colcemid and colchicine on grk*RFP in egg chambers from females fed with these inhibitors. We observed a range of defects, including loss of grk*RFP, mislocalized grk*RFP in the oocyte and accumulation of grk*RFP in the nurse cells (supplementary material Fig. S1). By contrast, it has been reported that there is an accumulation of grk transcripts in the oocytes rather than the nurse cells from colchicine-fed flies (Saunders and Cohen, 1999). The combination of two inhibitors used in our experiments might account for the differences. Nevertheless, under these conditions, it is difficult to distinguish between the roles microtubules might play in mRNA transport versus anchoring within the oocyte.

grk mRNA is a dynamic molecule

In some cases, mRNAs are stably anchored at their destinations. For example, nanos mRNA diffuses to the posterior of the oocyte and becomes stably anchored in an actin-dependent manner (Forrest and Gavis, 2003). Dynein acts as a static anchor for the apical transcripts runt and fushi tarazu in Drosophila blastoderm embryos (Delanoue and Davis, 2005). However, recent work indicates that the maintenance of localized mRNAs might also be a dynamic process. bicoid mRNA is maintained at the anterior oocyte cortex by continual active transport on microtubules (Weil et al., 2006). To examine whether grk mRNA is stably anchored during different stages of oogenesis, we performed FRAP experiments. grk*GFP was photobleached, either in the region posterior to the oocyte nucleus during stages 6 and 7 or on the dorsal-lateral side of the cap above the oocyte nucleus during stages 8 and 9 (see supplementary material Movies 2-4). Our FRAP conditions cause photobleaching of a 2.5 μm² area of grk*GFP in the focal plane, as well as grk*GFP as far out of focus as 2-3 μm above and below the plane. This suggests that grk mRNA is dynamically transported in the microtubule network during stages 6-9.
This plane (supplementary material Fig. S2D-F). Quantitative analysis shows 70% recovery of fluorescence, with a half-time of ~80 seconds (t1/2 = τ*ln2, where τ is the time constant of recovery) during stage 6. By contrast, by late stage 9, recovery is reduced to 30%, with a t1/2 of >120 seconds (Fig. 4). Thus, the steady-state population of grk*GFP is highly dynamic during early stages of oogenesis, but this dynamic behavior subsides at later stages.

What is the source of fluorescence recovery shown by grk mRNA? Fluorescence recovery could result from lateral movement of grk*GFP that is already localized. To address this possibility, we measured the fluorescence intensity values of the neighboring pools of localized non-bleached grk*GFP that surround the bleached area during a FRAP experiment for a corresponding decrease in fluorescence. The fluorescence of these neighboring regions was not decreased, however, implying that recovery does not primarily involve prelocalized sources of grk*GFP present at the cortex. In addition, we performed inverse FRAP experiments on posterior localized grk*GFP. We bleached two areas of cortical grk*GFP and measured the fluorescence intensity of the localized, non-bleached grk*GFP in between the two bleached spots. In these cases, there was no appreciable loss of fluorescence to the non-bleached localized grk*GFP, while high fluorescence recovery occurred within the neighboring bleached spots (data not shown). Unless constant and rapid accumulation of new cytoplasmic grk*GFP obscures the fluorescence loss from the cortex, our results indicate that the recovery of fluorescence is due to movement of grk*GFP from the cytoplasm to the cortex. To determine whether our fast fluorescent recovery is due to newly synthesized grk*GFP from the nurse cells, we bleached the entire oocyte at stage 7. We observed partial grk*GFP recovery within 20 minutes, more than ten times longer than the recovery observed after bleaching the cortex only (data not shown). Thus, we conclude that, at stage 7, mRNA newly transported from the nurse cells cannot be the major source of cortical recovery and, instead, recovery most probably reflects exchange from the nearby cytoplasm.

Mislocalized grk mRNA in K10 and sqd1 mutant oocytes is dynamic

We considered the possibility that mislocalized grk*GFP in mutant backgrounds could manifest altered dynamics. In K10 and sqd1 mutants (Kelley, 1993; Wieschaus, 1978), grk mRNA appears to have normal localization patterns during younger stages of oogenesis, but, in mid-to-late stages, grk mRNA is mislocalized along the entire anterior circumference of the oocyte instead of being restricted to the dorsal-anterior corner (Neuman-Silberberg and Schupbach, 1993). In addition, in these mutants, grk mRNA is translated along the anterior cortex, resulting in ectopic EGFR activation. Excess receptor activation results in the induction of more dorsal cell fates, leading to expanded dorsal appendages on the egg (Kelley, 1993; Neumon-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1994; Norvell et al., 1999). grk*GFP reproducibly shows a similar pattern of localization in either K10 or sqd1 mutant backgrounds (Fig. 6B-D).

To examine the behavior of localized and mislocalized grk mRNA in the K10 and sqd1 mutant oocytes, we performed FRAP experiments. We photobleached the dorsal and ventral anterior corners of K10 and sqd1 oocytes (Fig. 6D-F; supplementary material Fig. S3). Interestingly, in mid-to-late stage 9 egg chambers, we found...
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Compromised dynein activity interferes with the recovery of gurken mRNA

Recent evidence for direct binding between gurken mRNA and the dynein light chain adds to the mounting support for the potential role of the dynein motor enzyme in gurken mRNA localization (Rom et al., 2007). Given the dynamic nature of gurken mRNA, we examined the behavior of gurken*GFP under conditions that compromise dynein motor activity. First, we overexpressed dynamin (Dmn), which is an essential component of the dynactin complex and is necessary for dynein activity (Kamal and Goldstein, 2002). Overexpression of Dmn results in an increase of anteriorly localized gurken mRNA during mid-stages of oogenesis, as seen by in situ hybridization, and mispositioning of the oocyte nucleus (Duncan and Warrior, 2002; Januschke et al., 2002). Second, we took advantage of hypomorphic dynein heavy chain alleles (dhc) alleles, which permit oogenesis but have been shown to reduce transport (Clark et al., 2007; Weil et al., 2006).

FRAP experiments were performed on localized gurken*GFP in oocytes where dynein function was reduced by either of the above methods. In both situations, we observed mislocalized gurken*GFP in the oocyte cytoplasm accompanied by large aggregates, from stages 5 to 10. These large aggregates are most clearly visible in the nurse cell cytoplasm and are very reminiscent of the aggregates we observed in colchicine-fed gurken*GFP flies (supplementary material Fig. S1B). Upon overexpression of Dmn, a significant portion of gurken*GFP is still capable of reaching its proper destination, both at the posterior and dorsal-anterior corner (Fig. 7C,D). In dhc6-10/6-12 egg chambers (Fig. 7E,F), gurken*GFP no longer accumulates in a thin crescent along the posterior cortex during stages 6 and 7 and instead is very diffuse throughout the oocyte. During mid-stages of oogenesis, there is an accumulation of gurken*GFP at the anterior cortex beginning as early as stage 7. A defined area of fluorescent gurken mRNA at the posterior and dorsal-anterior was bleached during FRAP experiments. With overexpression of Dmn, fluorescence recovery is reduced to 34% in stages 6-7 and 28% in stage late 8 to early 9 (18-e9). Similarly, in dynein mutant egg chambers, gurken*GFP recovery is reduced to 24% at stage 18-9. This is in direct contrast to control fluorescence recovery values of 67% for stages 6-7 and 46% for 18-e9 stages (Fig. 7G). Taken together, our results show that, when dynein is compromised by two different methods, we observe a significant reduction in the dynamics of gurken mRNA.

Discussion

In order to visualize gurken mRNA in vivo, we used the MS2-MCP system. Unlike in situ hybridization or FISH techniques that reveal static images of RNA, the MS2-MCP system has allowed us to observe live samples and film gurken mRNA during various stages of oogenesis. Using this new detection method, we have described the live localization patterns of fluorescent gurken mRNA. Our description is similar to those originally published (Neuman-Silberberg and Schupbach, 1993) and confirms observations recently published using FISH (MacDougall et al., 2003). We also describe gurken mRNA accumulating at the anterior before the posterior localization has

Fig. 5. gurken mRNA recovery does not specifically involve depletion of nearby localized sources. (A,B) Images of stage 7 (A) and late stage 8 (B) egg chambers from flies expressing gurken*GFP illustrating the region bleached during the FRAP experiment (black box), plus arrows pointing to the unbleached areas of gurken*GFP monitored for fluorescence loss. (C,D) Line graphs representing average relative fluorescence of gurken*GFP and two unbleached neighboring pools of gurken*GFP during FRAP experiments. In panel C, the two posterior pools that surrounded the bleached region of gurken*GFP during stage 7 show no dramatic decrease in fluorescence intensity. The pool on the right of the bleached region is represented by the dark gray line, whereas the pool on the left is the pale gray line. (D) Likewise, the surrounding sources of localized gurken*GFP found at the dorsal-anterior cap during late stage 8 also displayed no measurable decrease while recovery occurred (right, dark gray; left, pale gray). This suggests that recovery is not due to nearby localized sources of gurken*GFP.

grk*GFP at both the dorsal and ventral sides to be dynamic. In fact, grk*GFP is more dynamic (~45% recovery) compared with grk*GFP in a wild-type background (~25%) at this stage (Fig. 6E and supplementary material Movie 5). Accordingly, the recovery rates (t½) for grk*GFP during mid-to-late stage 9 egg chambers in the mutant backgrounds are much faster. The nature of grk*GFP dynamics resembles that of a younger stage with faster and higher recovery, significantly different from the control, which displayed a slow rate with little recovery (Fig. 6F). We also performed FRAP experiments on K10 and sqd mutants at stages 6 and 7, when grk mRNA is properly localized at the posterior (supplementary material Fig. S4). Surprisingly, we found a substantial amount of variability in K10 mutants and a general increase of recovery in sqd mutants.
disappeared. Moreover, in early stage 8, we observe a lateral accumulation of grk mRNA that persists on the side of the oocyte where the nucleus is found (the future dorsal side), whereas, at the same time, the opposite side (the future ventral side) no longer has grk mRNA except for a small accumulation at the anterior cortex. At this transitional stage, large amounts of grk mRNA line the entire anterior and dorsal cortex of the oocyte. This configuration eventually resolves to the well-defined dorsal-anterior cap, by first restricting grk mRNA on the dorsal side, followed by restricting grk mRNA at the anterior. The question arises as to whether grk mRNA is transported along the lateral wall from the posterior to the dorsal-anterior corner or whether the lateral RNA decays and new grk mRNA transcripts populate the corner from the nurse cells. As endogenous grk mRNA does not form particles large enough to be resolved and tracked with our imaging system, we are presently unable to address this question. Using photoswitchable fluorescent molecules in combination with the MS2-MCP system could provide some answers to these questions in the future.

The process of RNA localization is likely to involve several of the following steps: RNP assembly, nuclear export, cytoplasmic transport, anchorage, translation and decay (St Johnston, 2005). Stable anchorage versus continuous transport have been proposed as two different mechanisms for the maintenance of mRNAs before translation occurs. Possible anchors can be microfilaments, microtubules, proteins and even noncoding RNAs, such as the Xlsr1ts in Xenopus oocytes (Kloc and Etkin, 2005). Continuous transport can give the appearance of an mRNA having a static anchor because this dynamic mechanism can result in a net gain of accumulation. In fact, bicoid mRNA has recently been shown to be maintained in late oocytes by continual active transport (Weil et al., 2006). Unexpectedly, it has been demonstrated that dynein, separate from its motor function, can act as a static anchor for apical transcripts in Drosophila embryos (Delanoue and Davis, 2005). In an attempt to study whether the cytoskeleton is a necessary factor for the maintenance of already localized grk transcripts, we used cytoskeleton destabilizing drugs. Our results suggest that actin does not play a role in the anchorage. This also corresponds to data obtained by moesin-deficient flies, which have defects in localization of oskar mRNA because of a loose and detached actin network, but in which grk mRNA remains undisrupted (Janovics et al., 2002; Polesello et al., 2002). Interestingly however, Babu and colleagues (Babu et al., 2004) showed that osk mRNA has two redundant anchoring processes. They noted that latrunculin A treatments did not disrupt the maintenance of osk unless other proteins, such as Homer, were absent (Babu et al., 2004). It is therefore possible that the actin cytoskeleton is similarly involved in the maintenance of grk mRNA in a manner redundant with that of other proteins, but so far no actin-binding proteins have been found to bind directly to the grk mRNA.

Using microtubule destabilizing drugs, we see no obvious disruption of localized grk mRNA throughout stages 6–10. This agrees with injection studies using grk mRNA synthesized in vitro that show that show localized grk mRNA is not disrupted by the later injection of colcemid (MacDougall et al., 2003). Interestingly, we observed that the microtubule network around the oocyte nucleus of stage 9–10 egg chambers was resistant to our drug treatments. It is not uncommon to observe populations of microtubules to be resistant to depolymerizing drugs. Often it is a characteristic of stable versus dynamic populations of microtubules (Baas et al., 1994; Bannigan et al., 2006; Guillaud et al., 1998; Palazzo et al., 2003). These microtubules persist even when nuclear migration is disrupted in grk mutants, or when the nucleus is displaced in various other mutants (Guichet et al., 2001; Januschke et al., 2002). It is possible that grk mRNA might attach itself to these MTs. Moreover, overexpressing Dmm, a component of the dynein-dynactin complex, disrupts this oocyte nucleus microtubule scaffold, resulting in grk mRNA being dispersed in the oocyte within stage 10 egg chambers (Januschke et al., 2002), very similar to what we saw in our experiments. During stages 9 and 10, when we observed the resistant microtubule basket, our FRAP studies show little fluorescence recovery. During these later stages, grk mRNA therefore appears to have a more stable anchorage and might no longer be in transit, whereas the high percentage and fast rate of recovery during younger stages suggests a high transport phase. The t_{1/2} is remarkably similar for stages 6 to early 9, suggesting a similar mechanism might be occurring during these stages. However, recovery slows down at mid-to-late stage 9 and is likely to be indicative of a shift in an equilibrium to a more stably anchored grk mRNA. Recovery does
not appear to be from prelocalized sources of grk mRNA. It appears to occur instead from nearby cytoplasm. At least at stage 7, the recovery is not due to nurse cells. At later stages, the low amount of recovery could represent grk mRNA in transit from the nurse cells (already close to its destination) (Clark et al., 2007) or this could also be explained by continual active transport. Further studies are needed to distinguish between these possibilities.

The difference in dynamics of grk mRNA between young and old oocytes is altered in K10 and sqd mutants. In fact, during the later stages, we found an increase in recovery in the mutants, suggesting the existence of a more dynamic grk mRNA at the anterior of the oocytes. The rate of recovery also does not slow down and is more representative of grk mRNA in a younger oocyte. It might be that, in addition to a defect in the machinery needed for proper localization (Norvell et al., 1999), the transition to a more stable anchorage is impaired in these mutants. In fact, in a recent publication, Delanoue and colleagues (Delanoue et al., 2007) suggest that Squid is a necessary component for the conversion of grk transport particles to a more stable structure. In addition, we found that compromising the activity of dynein by either overexpressing Dmn or using hypomorphic alleles of the dynein heavy chain led to less fluorescence recovery in both young and mid stages. Although we could not directly distinguish the roles of dynein in either long-range transport or anchorage, we have demonstrated that, when dynein activity is reduced, localized grk mRNA is less dynamic.

Using the MS2-MCP system, we have been able to visualize grk mRNA in live egg chambers and have begun to dissect the processes involved in the maintenance of localized transcripts. We show that grk mRNA has a remarkably different set of dynamics between young and older egg chambers and we also measured differences in various mutant backgrounds, which allows us to define the various stages involved in the process.

Materials and Methods

Drosophila stocks

hsp83-MCP-Ref and hsp83-MCP-GFP transgenic flies have been described previously (Forrest and Gavis, 2003; Weil et al., 2006). We used J1(1)K10, sqd2, dhc6–20 and dhc6–12 mutant strains (Kelley, 1993; Li et al., 1994; Wieschaus, 1978). Antoine Guichet provided the UAS-Dmn flies (Guichet et al., 2001). Overexpression of Dmn was achieved by using the caubGal4 driver that begins to be active during stage 4 (mataGal4–67). For marker mutations, Gal4 lines and balancer chromosomes, see flybase@indiana.edu.

Transgene construction

A Pml site was introduced at 1603 bp of the mRNA, within the 3UTR of grk mRNA. This site is 343 bp after the stop codon. The mutagenesis was performed on a 5050 bp genomic fragment containing the complete grk locus (Queenan et al., 1999) with adjacent 5’ and 3’ sequences within the pBluescript II SK+ phagemid (Stratagene). The pSL-MS2-12 construct contains 12 MS2 stem loops (a gift from K. Forrest). We used a mutated version of the stem loop that results in a very strong affinity (Kd=10^{-15}) of MS2 coat protein to the MS2 stem loop (Johansson et al., 1998; Vlageld et al., 1997). The 693 bp RmHI-EcoRV fragment from pSL-MS2-12 was end-filled with klenow (NE Biolabs) and placed into the Pml site of grk. A Xhol-Eagl grk-MS2-12 fragment was cloned into pCasper4 using Xhol-NolI.

P-element-mediated transformation

P-element transformation was performed according to standard procedures (Spradling and Rubin, 1982) using an Eppendorf Transjector 5246 with Eppendorf Femtotips (Eppendorf). Transgene constructs were injected at a concentration of 0.4 μg/ml along with the helper plasmid pTurbo at a concentration of 0.1 μg/ml.

Live imaging and inhibitor treatment

Ovaries were dissected in Schneider’s Drosophila Medium (Gibco). Egg chambers were plated in No. 1.5 glass-bottom culture dishes (MatTek) with 200 μl of medium. A No. 1.5 micro cover glass (VWR) was cut with a diamond tip to a coverslip of about 4 mm² and placed on top of the dissected egg chambers. Egg chambers were treated with either 200 μg/ml colchicine and 100 μg/ml colcemid (Sigma) or 4.2 μg/ml nocodazole.
µg/ml latrunculin A and 100 µg/ml cytochalasin D (Sigma) for 45-65 minutes while in the glass-bottom dishes.

FRAP experiments
FRAP experiments were performed using a Zeiss LSM510 confocal microscope with a 40 1.2 NA water objective lens. Imaging was performed with a 488 nm argon laser. The following conditions gave minimal photobleaching: 70% laser output, 1.5% AOOG, zoom 4, scan speed 6, 10 seconds per frame. For each FRAP experiment, 1-3 pre-bleach images were taken. A defined region of interest (ROI), a 2.25 µm² region, was bleached at 80% power with 15 iterations (~4 seconds). After bleaching, a time series was taken every 10 seconds for a total of 10 minutes. Analysis was done using MATLAB and Imaged (NIH). In order to test for the survival and transport properties of the egg chamber, we bleached the entire oocyte of stage 8 series was taken every 10 seconds for a total of 10 minutes. Analysis was done using MATLAB and Imaged (NIH) is required to establish dorsoventral polarity of the Drosophila egg.

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