Localization of bicoid mRNA in Late Oocytes Is Maintained by Continual Active Transport

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Summary

Localization of bicoid mRNA to the anterior of the Drosophila oocyte is essential to produce the Bicoid protein gradient that patterns the anterior-posterior axis of the embryo. Previous studies have characterized a microtubule-dependent pathway for bicoid mRNA localization during midoogenesis, when bicoid first accumulates at the anterior. We show that the majority of bicoid is actually localized later in oogenesis, when the only known mechanism for mRNA localization is based on passive trapping. Through live imaging of fluorescently tagged endogenous bicoid mRNA, we identify a temporally distinct pathway for bicoid localization in late oocytes that utilizes a specialized subpopulation of anterior microtubules and dynein. The directional movement of bicoid RNA particles within the oocyte observed here is consistent with dynein-mediated transport. Furthermore, our results indicate that association of bicoid with the anterior oocyte cortex is dynamic and support a model for maintenance of bicoid localization by continual active transport on microtubules.

Introduction

mRNA localization provides an important mechanism for the spatial regulation of gene expression by targeting the synthesis of cytoplasmic proteins to specific subcellular domains. Polarized cellular functions such as motility in fibroblasts and synaptic plasticity in dendrites, asymmetric division of budding yeast, and embryonic axis formation in Xenopus and Drosophila all require localized mRNAs (Kloc and Etkin, 2005; St Johnston, 2005). Axial polarity of the Drosophila embryo is established through the localization of maternal determinant mRNAs during oogenesis. Localization of gurken mRNA to a position on the future anterodorsal side of the oocyte breaks the radial symmetry of the oocyte, establishing dorsal-ventral polarity (Neuman-Silberberg and Schüpbach, 1993). Localization of oskar (osk) to the posterior of the oocyte initiates the assembly of the germ plasm, which, in turn, is essential for the posterior localization of nanos (nos) mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). Localization of bicoid (bcd) mRNA to the oocyte anterior, together with posterior localization of nos, endows the developing embryo with sources for opposing gradients of Bcd and Nos proteins that pattern the anterior-posterior body axis (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988; Gavis and Lehmann, 1992; Wang and Lehmann, 1991).

mRNA localization is generally thought to proceed in two phases: transport and anchoring. In the transport phase, mRNAs that have been packaged into ribonucleoprotein (RNP) complexes travel to their destinations, with the particular set of proteins in the RNP complex conferring specificity on the transport process. Upon arrival at their target sites, these RNP complexes presumably become anchored to the cytoskeleton. Studies of localized mRNAs in a variety of organisms suggest that the majority of mRNAs are transported on cytoskeletal frameworks by motor proteins, although the specific cytoskeletal requirements differ depending on cell type (reviewed by Kloc and Etkin [2005]; Lopez de Heredia and Jansen [2004]; and St Johnston [2005]). Analysis of ASH1 mRNA localization in yeast has provided the best evidence for transport on actin filaments by myosin motors. By contrast, microtubule-based transport appears to be the prevailing mode in Drosophila and Xenopus oocytes and embryos as well as in mammalian neurons and oligodendrocytes. Mechanisms for anchoring or retention of mRNAs at their destinations are less well characterized. Actin and actin binding proteins have been implicated in the retention of mRNAs whose transport is actin dependent, including ASH1 and chicken fibroblast -actin mRNAs, and for cortical anchoring of mRNAs like osk and Xenopus Vg1, whose transport depends on microtubules and kinesin (reviewed by Lopez de Heredia and Jansen [2004] and St Johnston [2005]). In contrast, dynein has been implicated in static anchoring of its own mRNA cargo (Delanoue and Davis, 2005).

bcd mRNA has provided a longstanding model for the investigation of microtubule-dependent mRNA localization. Synthesized in the ovarian nurse cells, bcd mRNA enters the oocyte through cytoplasmic bridges that connect the nurse cells to the oocyte at its anterior end. Accumulation at the anterior margin of the oocyte begins during midoogenesis (stages 8–10 of 14 morphologically defined stages) (Berleth et al., 1988), when the microtubule cytoskeleton is thought to be oriented with microtubule minus ends concentrated at the anterior and lateral oocyte cortex and plus ends directed toward the posterior (Clark et al., 1994; Januschke et al., 2006; Theurkauf et al., 1992). Injection experiments with fluorescently labeled bcd transcripts indicate that formation of a complex containing bcd mRNA and Exuperentia (Exu) protein in the nurse cells is required to target bcd specifically to microtubules nucleated at the anterior cortex of the stage 8 to stage 10 oocyte (Cha et al., 2001). Both pharmacological ablation of the microtubule cytoskeleton and inactivation of the dynein/dynactin complex disrupt bcd localization at these stages, suggesting that bcd is transported along these microtubules by dynein (Duncan and Warrior, 2002; Januschke et al., 2002; Pokrzywa and Stephenson, 1991). Furthermore, microtubule disruption experiments indicate that microtubules play an additional role in retention of bcd mRNA at the anterior cortex, although the mechanism of this
anchoring function is unknown (Pokrywka and Stephenson, 1991).

Analysis of the bcd localization pathway has focused primarily on events occurring during midoogenesis. However, it is likely that additional bcd mRNA enters the oocyte after stage 10, when the nurse cells initiate apoptosis and extrude or “dump” their contents into the oocyte. Using a strategy for in vivo imaging of bcd mRNA described below, we find that, in fact, the majority of bcd enters the oocyte and is localized during this later period of oogenesis. A late phase of bcd localization is indeed consistent with the behavior of particular exu mutants, in which bcd localization is disrupted during midoogenesis but partially recovers at later stages (Riechmann and Ephrussi, 2004). However, the mechanism responsible for the late phase of bcd localization is unclear, since polarization of the oocyte microtubule cytoskeleton is lost around the time of nurse cell dumping, when microtubules reorganize to form parallel arrays beneath the oocyte cortex (Theurkauf et al., 1992). These subcortical microtubules drive the movements of the oocyte cytoplasm (ooplasmic streaming) that mix the incoming nurse cell contents with the ooplasm (Gutzeit, 1986). Our previous analysis of nos mRNA, whose localization occurs after nurse cell dumping, revealed that translocation of nos mRNA from the nurse cells to the posterior of the oocyte occurs by a diffusion-based mechanism, rather than by microtubule-dependent transport (Forrest and Gavis, 2003). Ooplasmic streaming increases access of nos to the germ plasm at the posterior pole, where it becomes entrapped and anchored to the actin cytoskeleton (Forrest and Gavis, 2003). Posterior localization of osk transcripts injected into late-stage oocytes occurs similarly (Glotzer et al., 1997), suggesting that diffusion/trapping may be a general mechanism for localization of mRNAs in late-stage oocytes that lack a polarized cytoskeleton.

Analysis of the γ-tubulin ring complex components, γ-tubulin37C (γ-Tub37C) and γ-tubulin ring complex protein 75 (Dgrip-75), suggests that microtubules could play a more direct role in bcd localization in late-stage oocytes. γ-Tub37C and Dgrip-75 are enriched at the anterior margin of the oocyte, and mutations in γ-Tub37C and Dgrip-75 cause loss of bcd from the anterior cortex during or after stage 10 without affecting ooplasmic streaming (Schnorrer et al., 2002). These results suggest that bcd is maintained at the anterior cortex of late-stage oocytes by a subpopulation of microtubules nucleated from an anterior microtubule organizing center (MTOC). Direct evidence for such microtubules and a potential role in localization of bcd during and after nurse cell dumping have been lacking, however. Also unclear is the role of Staufen (Stau), a double-stranded RNA binding protein that is thought to participate in anchoring of bcd at the anterior cortex during egg activation and mediates association of bcd with astral microtubules in an embryo injection assay (Ferrandon et al., 1994; St Johnston et al., 1989).

We have now investigated bcd localization in late-stage oocytes through live imaging of bcd mRNA labeled in vivo with either GFP or RFP. This noninvasive method, previously used for analysis of nos mRNA localization (Forrest and Gavis, 2003), is advantageous both because it permits analysis of RNA synthesized endogenously under physiological conditions and because it facilitates visualization of RNA in late-stage oocytes, which are largely inaccessible to molecular probes. Our results reveal a distinct Staufen-dependent pathway for localization of bcd mRNA that enters the oocyte after stage 10. Localization of particles containing bcd and Staun requires a discrete population of microtubules anchored at the anterior cortex by the actin cytoskeleton. For the first time, to our knowledge, we observe anteriorly directed movement of bcd RNA particles within the oocyte that is consistent with microtubule-dependent transport. We provide evidence that maintenance of bcd at the anterior cortex requires continual active transport by dynein.

Results

Fluorescent Labeling of bcd mRNA In Vivo
To visualize bcd mRNA in living oocytes, we have followed the strategy used previously to generate GFP-labeled nos mRNA (Forrest and Gavis, 2003). In this scheme, a bcd-(ms2)_6 transgene was generated that contains six stem-loop binding sites for bacteriophage MS2 coat protein in conjunction with the hsp83-MCP-GFP transgene, which produces a MS2 coat protein-GFP fusion (MCP-GFP) (Forrest and Gavis, 2003). Binding of MCP-GFP to its cognate stem-loops labels bcd-(ms2)_6 mRNA with GFP. Moreover, by generating a hsp83-MCP-RFP transgene, we have expanded the capacity of the system to allow simultaneous detection of mRNA labeled with GFP and RFP fusion proteins. The bcd-(ms2)_6 transgene, alone or in combination with hsp83-MCP-GFP/RFP, completely rescues the bcd mutant phenotype. In our initial characterization (this section) as well as in pharmacological disruption experiments (see below), RFP- and GFP-labeled bcd mRNA behaved identically. For technical reasons (see Experimental Procedures), we have used GFP-labeled bcd mRNA (designated as bcd*GFP) for analysis of mutants that affect bcd mRNA localization and RFP-labeled bcd mRNA (designated as bcd*RFP) for colocalization studies.

The distributions of bcd*GFP and bcd*RFP were visualized in live egg chambers by laser scanning confocal microscopy. Through stage 10 of oogenesis, the Droso phila oocyte develops within an egg chamber, where it is connected to 15 germline nurse cells and is surrounded by a somatic follicular epithelium (Spradling, 1993). During midoogenesis (stages 5–10a), bcd*GFP and bcd*RFP become localized to the anterior margin of the oocyte. Recapitulating the distribution of native bcd mRNA previously revealed by in situ hybridization, fluorescent bcd mRNA is localized first in a ring around the anterior margin of the oocyte (Figures 1A and 1D; best seen in projection, Movie 1S; see the Supplemental Data available with this article online) and then shifts to a disc-like pattern at the oocyte anterior during stage 10 (Figures 1B and 1E). Fluorescence from MCP-GFP or MCP-RFP is never detected at the anterior in the absence of the bcd-(ms2)_6 transgene (Figures 1G–11 and data not shown). At the end of stage 10 (stage 10b), the oocyte initiates microtubule-dependent ooplasmic streaming that continues through stage 12. Immediately after the onset of ooplasmic streaming, the nurse cells undergo an actin-dependent contraction (nurse cell dumping) that transfers their cytoplasm rapidly into the
streaming oocyte and are subsequently eliminated. During late stages of oogenesis (stages 11–14), \(bcd\text*\text{GFP}\) and \(bcd\text*\text{RFP}\) appear to increase substantially at the oocyte anterior (Figures 1C and 1F).

A Late Phase of \(bcd\) Localization that Initiates with Nurse Cell Dumping

The apparent increase in \(bcd\) mRNA at the anterior of the oocyte after stage 10 suggests that additional \(bcd\) enters the oocyte during nurse cell dumping and is subsequently localized. Consistent with such a late phase of \(bcd\) localization, time-lapse confocal imaging of \(bcd\text*\text{GFP}\) and \(bcd\text*\text{RFP}\) from stage 10 (predumping) to stage 13 (postdumping) shows dramatic accumulation of \(bcd\) at the anterior as the nurse cells empty their contents into the oocyte (Movies S2 and S3). Fluorescence intensity measurements indicate that the amount of \(bcd\) mRNA increases at least 10-fold between stage 10b, when nurse cell cytoplasm first enters the oocyte, and stage 13, when dumping and streaming have ceased. Northern blot analysis confirms that this increase is not due to preferential stabilization of fluorescent \(bcd\text{mRNA}\) at late stages of oogenesis (Figure S1).

The ability to selectively disrupt nurse cell dumping with low doses of latrunculin A (latA) (Forrest and Gavis, 2003) enabled us to test whether dumping is required for accumulation of \(bcd\) at the anterior cortex in late-stage oocytes. Egg chambers expressing \(bcd\text*\text{RFP}\) were dissected at stage 10b, cultured in medium containing latA, and monitored by time-lapse imaging. Although localization of \(bcd\text*\text{RFP}\) is initiated normally, the RNA ceases to accumulate at the anterior cortex of the oocyte after nurse cell dumping arrests (Figures 2A and 2B). By contrast, \(bcd\text*\text{RFP}\) continues to accumulate at the anterior cortex in mock-treated egg chambers during an equivalent time period (Figures 2C and 2D). Neither ooplasmic streaming nor the oocyte cortical actin filaments are disrupted in the latA-treated egg chambers (data not shown; Forrest and Gavis, 2003). Moreover, latA treatment of oocytes at stage 12 does not disrupt localization of \(bcd\text*\text{RFP}\) that has already accumulated at the anterior (Figures 2E and 2F). Together, these results provide direct evidence for a distinct late phase of \(bcd\) localization that initiates with and requires nurse cell dumping. Moreover, they show that the majority of \(bcd\text{mRNA}\) present at the anterior of the embryo becomes localized during this phase.

Stau Protein Colocalizes with \(bcd\) mRNA and Is Required for Late Phase \(bcd\text{mRNA}\) Localization

Genetic analysis has suggested that stau acts during the transition from oogenesis to embryogenesis, when
Previous studies have shown that microtubule depolymerization results in loss of bcd localization in both mid and late stages of oogenesis (Pokrywka and Stephens, 1991). We therefore examined the effect of microtubule depolymerization on localization of bcd/Stau particles in late-stage oocytes. Egg chambers expressing bcd*RFP and GFP-Stau were dissected at stages 11–12 and were cultured in the presence of colcemid under conditions previously shown to abolish oocyte microtubules and ooplasmic streaming but permit progression through oogenesis (Forrest and Gavis, 2003). Although nurse cell dumping is unaffected by colcemid treatment, bcd*RFP and GFP-Stau entering the oocyte from the nurse cells fail to accumulate at the anterior and remain unlocalized throughout the remainder of oogenesis. Furthermore, bcd*RFP and GFP-Stau that have accumulated at the anterior prior to colcemid addition are released (Figures 4A–4C and Movie S4). No effect on either bcd*RFP or GFP-Stau was detected in mock-treated controls (Movie S5). This result, together with results presented above, suggest that association of bcd with Stau is essential for the microtubule-dependent localization of bcd mRNA that enters the oocyte during nurse cell dumping. Moreover, our in vivo analysis of bcd*RFP and GFP-Stau reveals a role for microtubules in transport as well as in retention or maintenance of bcd/Stau complexes at the anterior.

From stage 10b onward, egg chambers dissected at stages 11–12 results in loss of bcd mRNA from the anterior cortex of the oocyte but do not affect oocyte viability and actin-independent events like ooplasmic streaming (see below) (Forrest and Gavis, 2003). CytoD treatment of egg chambers dissected at stages 11–12 results in loss of bcd*RFP from the anterior cortex of the oocyte and the formation of RNA-containing aggregates that
are swept away from the cortex by ooplasmic streaming (Movie S6). By contrast, bcd*RFP localization is unaffected in mock-treated egg chambers (Movie S5). This requirement for the actin cytoskeleton is specific to bcd localization in late-stage oocytes because treatment of mid-stage egg chambers (stages 8–10) with cytoD does not disrupt the association of bcd*RFP with the anterior cortex (data not shown). Time-lapse imaging of GFP-Stau and bcd*RFP in egg chambers treated with cytoD shows that GFP-Stau remains associated with bcd mRNA when anterior localization is lost, colocalizing with bcd*RFP aggregates (Figures 4E–4H).

To further investigate the role of the actin cytoskeleton in bcd localization, we compared the distributions of bcd and actin in egg chambers expressing bcd*RFP and GFP-actin. While a portion of bcd*RFP is coincident with GFP-actin at the anterior cortex of the oocyte, the remainder lies more interiorly in a nonoverlapping domain (Figure 4I). In addition, aggregates of bcd that form upon cytoD treatment appear to contain actin (Figures 4J and 4K). Although we cannot rule out the possibility that fragmented actin filaments become associated with bcd aggregates after cytoD treatment, our results suggest that bcd is linked to the actin cytoskeleton at the anterior cortex through an intermediary component and remains similarly associated with fragmented actin filaments produced by cytoD treatment.

Interdependence of the Actin and Microtubule Cytoskeletons
Results from the studies described above show that the maintenance of bcd localization during late stages of oogenesis depends on both the microtubule and actin cytoskeletons. However, they do not distinguish whether this maintenance function is provided by two distinct mechanisms, one microtubule-based and the other actin-based, or by a single mechanism that integrates the activities of the two cytoskeletal systems. To distinguish between these possibilities, we assayed the effect of disrupting one type of cytoskeletal element on the behavior of the other in egg chambers expressing bcd*RFP and either GFP-actin or Tau-GFP. Colcemid treatment of late-stage oocytes has no detectable effect on the cortical actin cytoskeleton (data not shown). By contrast,
cytoD treatment of stage 11 and stage 12 egg chambers results in release of the anterior subpopulation of oocyte microtubules and bcd*RFP from the cortex (Movie S7). This effect of cytoD treatment is specific to the anterior microtubules, as ooplasmic streaming powered by cortical microtubule bundles continues. Notably, the oocyte nucleus is released from its anterodorsal position and, along with aggregates of bcd mRNA, is swept away by the vigorous movement of the ooplasm (Movie S7).

Microtubules released from the anterior cortex upon cytoD treatment appear as densities around bcd RNA aggregates (Figures 5A–5C). To elucidate the relationship between actin filaments and microtubules in the formation of bcd aggregates, we treated dissected egg chambers expressing bcd*RFP first with cytoD to produce aggregates (Figures 5D–5F; Movie S8) and subsequently with colcemid (Figures 5G–5I; Movie S8). Upon addition of colcemid to the culture medium, the aggregates of bcd*RFP quickly dissociate into fine particles that disperse within the ooplasm. A confocal Z-series was used to confirm that the bcd aggregates have indeed dissociated and have not simply moved out of the imaging plane (Movie S8).

Taken together, these results provide evidence for a single mechanism involving both microtubules and the actin cytoskeleton. While we cannot rule out an independent function of the actin cytoskeleton in bcd localization, evidence that the association of bcd with actin aggregates is microtubule dependent is most consistent with an indirect role for actin in anchoring or organizing a distinct population of microtubules nucleated at the anterior cortex that mediate bcd localization.

Dynamic Population of Localized bcd mRNA

Microtubules nucleated at the anterior cortex could mediate bcd localization by physically tethering bcd through stable interactions with bcd/Stau complexes. Alternatively, association of bcd with the anterior cortex might be transient such that maintenance requires continual microtubule-dependent transport of bcd to the anterior. To begin to distinguish whether microtubules function in anchoring or in transport of bcd, we first investigated whether there is turnover in the population of bcd mRNA localized at the anterior cortex by using fluorescence recovery after photobleaching (FRAP). Fluorescence of bcd*RFP was inactivated in a discrete region of the anterior cortex using high-intensity illumination, and fluorescence recovery was monitored by time-lapse imaging. These experiments targeted bcd mRNA in stage 13 egg chambers, after nurse cell dumping and ooplasmic streaming have ceased and the accumulation of bcd at the anterior appears to be complete. For comparison, we carried out similar FRAP experiments with nos mRNA, which is anchored at the posterior pole of the stage 13 oocyte by the actin cytoskeleton (Forrest and Gavis, 2003; Figure 6E). Fluorescence due to bcd recovers to ~50% of its initial value, with a half time of 9 min (Figures 6A–6D; Figure S2), indicating repopulation of bcd*RFP at the anterior cortex. By contrast, fluorescence of RFP-labeled nos mRNA (nos*RFP) does not detectably recover within a similar time period (Figures 6E–6H).

To independently confirm turnover in the population of bcd mRNA at the anterior cortex, we performed fluorescence loss in photobleaching (FLIP) analysis. A region posterior to the domain of bcd*RFP localization at the anterior cortex was repeatedly photobleached, and the fluorescence intensity of bcd*RFP at the anterior cortex, outside of the bleached region, was subsequently measured. In these experiments, fluorescence due to bcd decreased by ~24% over a 1 hr time period, consistent with a dynamic population of bcd at the anterior cortex. Incomplete exchange of bcd in both the FRAP and FLIP experiments may be due to photodamage incurred by the oocyte and/or the prolonged association of some bcd mRNA with the cortex. However, evidence for significant turnover of bcd in both FRAP and FLIP studies suggests that the association of bcd mRNA with the anterior cortex is dynamic and supports an active role for microtubules in the recycling of bcd mRNA to the anterior.

Dynein Motor Activity Is Required to Maintain Localization of bcd at the Anterior Cortex

A model for the maintenance of bcd localization by active transport on microtubules nucleated at the anterior cortex predicts that bcd localization in late-stage oocytes should depend on a minus end-directed motor. We therefore examined the effect of hypomorphic dynein heavy chain (dhc) mutants that permit oocyte development (McGrail
and Hays, 1997) on the distribution of bcd*GFP. Anterior accumulation of bcd*GFP is decreased or absent in ~50% of dhc mutant oocytes (n = 47) during stages 9–13 (Figure S3); residual bcd localization is likely due to the hypomorphic nature of these dhc mutations, which reduce, but do not eliminate, Dhc function (MacDougall et al., 2003). While this mutant analysis establishes a role for dynein in bcd localization throughout oogenesis, it does not test specifically whether maintenance of bcd localization requires dynein. Disruption of the dynein/dynactin complex by overexpression of the p50/dynamin (Dmn) subunit of dynactin blocks bcd localization during midoogenesis without perturbing microtubule organization (Duncan and Warrior, 2002; Januschke et al., 2002). We therefore took advantage of a heat shock-inducible Dmn (hs-Dmn) transgene (Duncan and Warrior, 2002) to disrupt dynein/dynactin complex activity selectively in late-stage oocytes in which bcd*RFP was already localized. After heat shock, bcd*RFP dissociates from the anterior cortex, in a similar manner as with colcemid treatment (Figures 6I and 6J). By contrast, anterior localization of bcd*RFP is unaffected in control oocytes lacking the hs-Dmn transgene that are subjected to the same heat shock regimen (Figure S4). The microtubule cytoskeleton, visualized with Tau-GFP, is not grossly altered by hs-Dmn (data not shown). Moreover, unlike colcemid treatment, Dmn overexpression does not displace the oocyte nucleus, indicating that anterior microtubules remain intact (Figure 6J). As an additional control, we monitored nos mRNA, whose anchoring at the posterior pole is microtubule independent (Forrest and Gavis, 2003). Overexpression of Dmn has no detectable effect on the retention of nos*RFP at the posterior pole (Figures 6K and 6L), further supporting a specific effect on bcd localization.

Together, these results are consistent with continual dynein-mediated transport of bcd to the anterior.
However, recent evidence indicates that dynein can act as a static anchor for pair rule transcripts that are localized apically within the blastoderm embryo (Delanoue and Davis, 2005). Since dynein motor activity is ATP dependent, we used the ATPase inhibitor sodium orthovanadate (vanadate) to test whether dynein motor function is required for retention of bcd at the anterior cortex. Vanadate has been previously used to block dynein-dependent transport of mRNAs in embryos (Delanoue and Davis, 2005). Similar treatment of oocytes with vanadate blocks two processes predicted to require ATP, actin/myosin II-dependent nurse cell dumping and kinase-dependent ooplasmic streaming (data not shown). In addition, treatment of oocytes expressing bcd*RFP and GFP-Stau results in the simultaneous loss of bcd and Stau from the anterior cortex (Figures 6M–6P). These results are consistent with a requirement for dynein motor activity in bcd localization. However, because vanadate inhibits ATPases generally, we cannot exclude the contribution of additional ATP-dependent functions in the maintenance of bcd at the anterior cortex. 

Directed Movement of Fluorescent bcd Particles

To provide further evidence that anterior localization of bcd is maintained by continual active transport, we assayed the movement of bcd*RFP particles by imaging at high magnification and time resolution (time-lapse interval of at least one frame per 1.5 s). Although we were unable to detect bcd particles entering the oocyte from the nurse cells during stages 10b–11, discrete particles of bcd*RFP that showed directed movement were observed in stage 12 and stage 13 oocytes, near the anterior cortex (Figures 7A–7C; Movie S9). We have only been able to follow the movement of individual particles for short time periods (~1 min) due in part to photo-bleaching and in part to the tendency of particles to move out of the imaging plane. A total of 52 particles (from 5 individual oocytes) that remained within the imaging plane for at least 10 s were analyzed for both direction and rate of movement (Figures 7A–7E). The majority of these particles exhibit sustained movement with anterior or anterolateral trajectories. Velocities of anteriorly directed particles range from 0.06 to 0.35 \( \mu \text{m/s} \), consistent with active transport. A smaller number move laterally (0.03–0.18 \( \mu \text{m/s} \)) or posteriorly (0.03–0.12 \( \mu \text{m/s} \)), and several show complex multidirectional trajectories. The predominately directional movement of these bcd RNA particles is distinct from the movement of other particles at or near the cortex, including yolk granules during ooplasmic streaming and Swa-GFP (Stephenson, 2004; data not shown).

Discussion

Bcd patterning activity in the early embryo depends on the efficacy of bcd mRNA localization during oogenesis (Driever and Nüsslein-Volhard, 1988; Frohnhofer and Nüsslein-Volhard, 1987). Through live imaging of bcd mRNA fluorescently labeled in vivo, we provide direct evidence for a distinct, late bcd localization pathway that initiates with nurse cell dumping and is responsible for the majority of bcd present at the anterior of the embryo. Since specification of cell fates along the anterior-posterior axis is sensitive to single changes in bcd gene dosage (Driever and Nüsslein-Volhard, 1988), the predominant late source of localized bcd mRNA most likely makes the primary contribution to the Bcd protein gradient. Thus, we propose that although bcd localization also occurs during midoogenesis, the late pathway is the relevant one for anterior-posterior patterning. We identify a new role for Stau as a component of this pathway and show that localization of bcd/Stau complexes during late stages of oogenesis depends on the integrity of a subpopulation of microtubules that are anchored at the anterior cortex by the actin cytoskeleton. Movement of bcd RNA particles within the oocyte is consistent with anteriorly directed transport along these microtubules. Moreover, our results reveal dynamic behavior of bcd that does not fit the prevailing two-step transport and anchoring paradigm for mRNA localization. Rather, they support a model for steady-state localization of bcd at the anterior cortex by continual active transport (Figure 7F).

A Distinct Microtubule-Dependent Pathway for bcd mRNA Localization in Late-Stage Oocytes

Evidence for microtubule-directed transport in late-stage oocytes, when the cytoskeletal polarity thought to underlie transport along the anterior-posterior axis is no longer apparent, has been lacking. Indeed, previous studies have shown that posterior localization of mRNAs like nos and osk after stage 10 does not depend directly on microtubules, but occurs by a diffusion/trapping mechanism that is facilitated by ooplasmic streaming (Forrest and Gavis, 2003; Glotzer et al., 1997). In contrast, results presented here suggest that microtubules emanating from the anterior cortex support transport of bcd mRNA to the anterior. Since we have not been able to follow the movement of bcd particles as they enter the oocyte during nurse cell dumping, we cannot eliminate the possibility that some bcd is simply trapped by anterior microtubules as it enters, in a manner analogous to trapping of nos mRNA by germ plasm at the posterior. However, the requirement for microtubules, dynein, and ATP to maintain bcd mRNA localization after nurse cell dumping and ooplasmic streaming, together with the anteriorly directed movement of bcd particles near the anterior cortex, implicates these microtubules in active transport of bcd. Although ooplasmic streaming is capable of distributing mRNA localization complexes to the posterior pole, we do not detect bcd particles in the posterior half of the oocyte. Thus, bcd must associate rapidly with these anterior microtubules upon entry into the oocyte. In the early embryo, nearly all bcd mRNA resides at the anterior cortex, whereas only a small fraction of nos mRNA is localized at the posterior pole (Bergsten and Gavis, 1999). The existence of a microtubule-dependent pathway specific for anterior transport in late-stage oocytes could account for the dramatic difference in the efficiencies with which bcd and nos mRNAs are localized.

Anterior microtubules that mediate bcd mRNA localization in late-stage oocytes are most likely nucleated by a MTOC formed at the anterior cortex during stage 10. Analysis of mutants \( \gamma\)-Tub37C and Dgrip-75 implicates this MTOC in the transition of bcd mRNA from its ring-like distribution in stages 8–9 to a disc-like distribution in stage 10 (Schnorrer et al., 2002). Previously
Figure 7. Movement of bcd RNA Particles

(A–C) High-power images of regions near the anterior (oriented to the left) of stage 13 oocytes expressing bcd*RFP. (C) Corresponds to Movie S9. Moving particles were tracked for (A) 65 s, (B) 36 s, or (C) 40 s, and trajectories are plotted on the initial frame from each time series. The positions of particles in the initial frame are indicated by open circles, and positions in the final frame are indicated by the tip of the arrows. Dots indicate positions at (A) 15 s, (B) 9 s, and (C) 10 s intervals. Particles with fewer time points shown entered the field later or left the field earlier than particles with more time points shown. Examples of anterior/anterolateral movement are shown in green, lateral movement in yellow, posterior movement in blue, and multidirectional movement in orange.

(D) Tabulated data for 52 particles from 5 independent oocytes. The anterior category includes particles with anterior and anterolateral trajectories.

(E) A graph plotting the velocity for each particle in the anterior, lateral, and posterior categories in (D).

(F) Model for bcd mRNA localization in late-stage oocytes. bcd RNPs are coupled to dynein motor complexes (a) and are transported to the anterior (b) on a specialized population of microtubules anchored to the actin cytoskeleton. Association of bcd RNPs with the anterior cortex (c) is transient. After dissociating from the cortex (d), bcd RNPs can be reloaded onto dynein motors (e) for additional rounds of transport (f). Kinesin may recycle dynein (g) for continued transport, as suggested by their interdependence during midoogenesis (Duncan and Warrior, 2002; Januschke et al., 2002).
localized bcd mRNA is subsequently released from the anterior cortex in \( \gamma \)-Tub37C and Drp1-75 mutants during nurse cell dumping, whereas other microtubule-dependent processes, such as ooplasmic streaming, are unaffected. These defects suggest that a specific reorganization of microtubules at the anterior cortex is responsible for maintaining bcd localization, while the majority of microtubules are reorganized for ooplasmic streaming.

Microtubules present at the anterior cortex in late-stage oocytes are distinct from microtubules that mediate bcd mRNA localization during midoogenesis and from cortical microtubules that mediate ooplasmic streaming by their dependence on the actin cytoskeleton. Our results suggest that association with the actin cytoskeleton enables microtubules nucleated from the anterior MTOC during stage 10 to survive the dramatic changes in the oocyte that occur with nurse cell dumping and ooplasmic streaming and persist to later stages. These microtubules serve multiple functions in late-stage oocytes, as their selective perturbation disrupts both bcd mRNA localization and oocyte nucleus positioning.

Localization of bcd mRNA during mid- and late oogenesis can also be distinguished by a requirement for Stau. Stau participates in both the transport and anchoring of osk mRNA during midoogenesis, and Stau homologs have been implicated in microtubule-dependent transport of mRNA in mammalian hippocampal neurons and Xenopus oocytes (Kloc and Etkin, 2005; St Johnston, 2005). This evidence, together with the presence of Stau in neuronal RNA granules (Kanai et al., 2003; Mallardo et al., 2003), suggests a common function for Stau in coupling mRNAs to motor proteins for transport. We show that Stau’s function in bcd mRNA localization is not limited to anchoring bcd during the transition from oogenesis to embryogenesis as previously thought (Ferrandon et al., 1994; St Johnston et al., 1991); rather, Stau plays an important role from the onset of nurse cell dumping. Although the stau mutant used in our experiments is a null allele (Ramos et al., 2000), bcd mRNA localization is not completely eliminated in all stau mutant oocytes. Similarly, posterior localization of osk mRNA is greatly reduced, but it is not abolished in stau null mutant oocytes (van Eeden et al., 2003). It is possible that bcd mRNA localized during midoogenesis can persist at the anterior cortex even after dumping and streaming have ended and accumulation is maximal. This turnover suggests that bcd/Stau RNPs transported to the anterior cortex are unable to make stable associations with cortical components. Upon release from dynein, bcd/Stau RNPs may interact transiently with the cortex or may be released directly into the ooplasm, where they are released onto dynein for another round of transport (Figure 7F). Continual active transport may be critical for anterior localization of mRNAs like bcd, which occurs at a time when the rapid growth and movement of the oocyte cortex may inhibit or delay the establishment of a static anchoring mechanism. It will now be of interest to determine whether other localized mRNAs, particularly those in cell types that undergo rapid morphological changes such as migrating growth cones, behave similarly to bcd.

## Experimental Procedures

### Fly Strains

The following mutants and transgenic lines were used: \( y^{w^{27}C3} \) (Lindsay and Zimm, 1992), bcd\(^{F1} \) (Frohnhöfer and Nüsslein-Volhard, 1986), stau\(^{23} \) (St Johnston et al., 1991), Dhc6-12 and Dhc6-12 (Gepner et al., 1996), hsp83-MCP-GFP and nos-(ms2)6 (Forrest and Gavis, 2003), UASp2-GFP-actin (Kelso et al., 2002), GAL4::VP16-nos::UTR (Van Doren et al., 1998), Tau-GFP (Micklem et al., 1997), GFP-Stau (Schnitzler et al., 2000), hsp70-Dmn (Duncan and Warrior, 2002).

### Construction of Transgenes and Transgenic Lines

The bcd-(ms2)6 transgene is based on pCaSbcdE, which contains an 8.7 kb EcoRI genomic bcd rescue fragment in the CaSpeR P element vector (Berleth et al., 1988). A 400 bp EcoRI-EcoRV fragment with six copies of the ms2 stem-loop binding site was isolated from pSL-MS2-6 (Bertrand et al., 1998), end filled, and inserted into an end-filled MluI site in the bcd 3'UTR sequences to generate pCaSbcd-(ms2)6. The hsp83-MCP-RFP transgene is identical to hsp83-MCP-GFP (Forrest and Gavis, 2003), except that EGFP was replaced by monomeric RFP (mRFP1) (Campbell et al., 2002).

Transgenes were introduced into the \( y^{w^{27}C3} \) strain by P element-mediated germline transformation (Spradling, 1986), and multiple independent transgenic lines were isolated. To facilitate analysis, chromosomes carrying two bcd-(ms2)6 insertions were generated by recombination or by local P element transposition; also, bcd-(ms2)6 MCP-RFP and bcd-(ms2)6 MCP-GFP recombinant chromosomes were generated. Use of MCP-RFP eliminates background autofluorescence of yolk granules in live oocytes caused by GFP excitation among bcd mRNA molecules during oogenesis would enable Stau, or another factor, to couple many bcd molecules to a single dynein motor. Concurrent transport of multiple mRNAs may contribute to the efficiency of bcd localization.

### A Dynamic Model for Maintenance of Localized mRNA by Continual Active Transport

Current models for mRNA localization invoke independent transport and anchoring steps. Evidence for distinct steps comes from the differential effects of cytoskeletal inhibitors applied during and after translocation of RNAs to their destinations. In this manner, a kinesin- and microtubule-dependent transport step is paired with an actin-dependent anchoring step for localization of Vg1 and osk mRNAs, whereas a dynein- and microtubule-dependent transport step is paired with a dynein-dependent anchoring step for Drosophila pair-rule transcripts (Delanoue and Davis, 2005; St Johnston, 2005). Our results indicate that bcd localization does not fit neatly into this two-step model. FRAP and FLIP experiments show turnover in the population of bcd mRNA at the anterior cortex even after dumping and streaming have ended and accumulation is maximal. This turnover suggests that bcd/Stau RNPs transported to the anterior cortex are unable to make stable associations with cortical components. Upon release from dynein, bcd/Stau RNPs may interact transiently with the cortex or may be released directly into the ooplasm, where they are released onto dynein for another round of transport (Figure 7F). Continual active transport may be critical for anterior localization of mRNAs like bcd, which occurs at a time when the rapid growth and movement of the oocyte cortex may inhibit or delay the establishment of a static anchoring mechanism. It will now be of interest to determine whether other localized mRNAs, particularly those in cell types that undergo rapid morphological changes such as migrating growth cones, behave similarly to bcd.

### Colocalization of Stau in particles with bcd mRNA

Colocalization of Stau in particles with bcd mRNA suggests that it is an integral component of a bcd localization RNP. Individual bcd RNA particles that exhibit directional movement range in size from 0.3 to 1 \( \mu \)m, indicating that they consist of multiple mRNA and protein molecules. These particles are similar in size to the particles that form after injection of synthetic bcd 3'UTR RNA into embryos and become associated with astral microtubules. Formation of bcd 3'UTR particles requires Stau as well as intermolecular interactions between two or more bcd 3'UTRs (Ferrandon et al., 1994, 1997). Similar assembly of large particles through interactions...
wavelengths. Thus, for inhibitor, FRAP, and particle tracking experiments as well as for colocalization studies, three copies of the bcd-(ms2)6 transgene were used in combination with one copy of MCP-RFP. Because two copies of bcd-(ms2)6 are sufficient for visualization with MCP-GFP, however, labeling with MCP-GFP simplifies genetic manipulations required for analysis of bcd in mutant backgrounds.

Manipulation of Egg Chambers and Confocal Imaging
Ovaries from well-fed females were dissected from Schneider’s insect culture medium (GIBCO-BRL), and individual egg chambers were separated from ovarioles by using tungsten needles. For immediate live imaging, egg chambers were mounted in Schneider’s medium on glass slides and were covered with a #1.5 glass coverslip (Cormack). For the analysis of bcd/GFP in stau mutant oocytes, yolk autofluorescence was reduced by fixation for 15 min in electron microscopy grade formaldehyde (Polysciences), 4% in PBS. After fixation, egg chambers were washed several times and mounted in PBS. For time-lapse imaging and inhibitor treatment, egg chambers were transfected to uncoated #1.5 glass-bottom culture dishes (MatTek) containing 200 μl Schneider’s medium and were covered with a 1 mm² coverslip cut from a #1.5 glass coverslip (Cormack). For inhibitor treatment, either colcemid or cytochalasin D, each in 100% ETOH, was added directly to the medium as described by Forrest and Gavis (2003). Latrunculin A was used at a final concentration of 0.5 μM. For mock-treated controls, an equivalent amount of 100% ETOH was added. Sodium orthovanadate (Sigma) was prepared in water and added to the medium after mounting at a final concentration of 10 mM (Delanoue and Davis, 2005). For heat shock, induction of hsp70-Dnm, egg chambers were mounted as described above, and a pre-heat shock image was collected. The culture dish was then incubated for 40 min in a covered chamber of water equilibrated to 37°C on a hot plate. Time-lapse imaging was initiated after heat shock.

All images were collected with a Zeiss LSM 510, except for those shown in Figures 3A–3D, which were collected during a demonstration of the Zeiss LSM 510 Meta system. Images were acquired by using C-Apochromat 40×/1.2W and C-Apochromat 63×/1.2W objectives. For time-lapse imaging, RFP and GFP signals were collected sequentially by channel in line mode. To quantify accumulation of bcd at the anterior cortex, individual egg chambers were cultured as described above, and fluorescence intensity at the anterior cortex was measured at the brightest optical section, first at stage 10B and then at stage 12. The relative increase in bcd at the anterior cortex was estimated by comparing the values for the two time points after correction for background fluorescence and is likely to be an underestimate because the surface area of the cortex over which bcd is localized increases during this time.

FRAP and FLIP Analysis
Individual stage 13 oocytes were mounted in glass-bottom dishes as described above. For FRAP experiments, each oocyte was imaged at 543 nm for 5 min using a 63× objective. The condenser was then closed, and a small region at the anterior (bcd) or posterior (nos) cortex was photobleached for 5 min with white light from a mercury lamp. The shutter was reopened, and the RFP signal was monitored by using settings identical to those used for prebleach collection. Similar results were obtained by photobleaching for 5–10 min with a 543 nm laser using the Zeiss LSM 510 Edit ROI tool. In either case, the mean fluorescence of the bleached region corrected for background and total loss of fluorescence during image collection and was normalized to 100% for the initial fluorescence. Data for bcd were plotted using Prism4.0 (Graphpad Software) and were fitted with a single exponential association equation (Figure S2).

For FLIP experiments, the Edit ROI tool was used to demarcate a region immediately posterior to the domain of localized bcd*RFP. The demarcated region was photobleached for 5 min, after which the anterior cortex was imaged. Photobleaching and imaging were repeated over a 1 hr period, and the mean fluorescence of bcd*RFP at the anterior cortex was determined.

Analysis of Particle Movement
Live egg chambers were dissected and mounted on glass slides in Schneider’s medium and were covered with a 22 mm² #1.5 glass coverslip (Cormack). A region near the anterior cortex was defined using the Zeiss LSM 510 Edit ROI tool, and bcd*RFP was monitored by time-lapse imaging at 1.5 s intervals. Particles that remained visible for at least 10 s without leaving the focal plane were tracked manually every 8–11 frames, using Zeiss LSM 510 software. Trajectories were mapped from the first frame where movement was detected, using the coordinates determined from individual frames. The velocity for an individual particle represents an average rate determined by dividing the time of transit between the first and last coordinates monitored by the distance traveled.

Supplemental Data
Supplemental Data include Figures S1–S3 and Movies S1–S9 and are available at http://www.devolutionalcell.com/cgi/content/full/11/2/251/DC1/.

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required for the anterior localization of bicoid.


