Downloaded from www.rnajournal.org on February 14, 2006

RNA

Redundant RNA recognition events in bicoid mRNA localization

P. M. Macdonald and K. Kerr

RNA 1997 3: 1413-1420

References Article cited in:
http://www.rnajournal.org/cgi/content/abstract/3/12/1413#otherarticles

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here**

Notes

To subscribe to *RNA* go to:
http://www.rnajournal.org/subscriptions/

© 1997 RNA Society

Downloaded from www.rnajournal.org on February 14, 2006

RNA (1997), 3:1413–1420. Cambridge University Press. Printed in the USA.
Copyright © 1997 RNA Society.

Redundant RNA recognition events in *bicoid* mRNA localization

PAUL M. MACDONALD and KAREN KERR
Department of Biological Sciences, Stanford University, Stanford, California 94305, USA

ABSTRACT

A *cis*-acting signal in the 3′ UTR of the *Drosophila bicoid* mRNA directs both the transport of the mRNA from the nurse cells to the oocyte and its anterior localization within the oocyte. Here we demonstrate that the signal mediates redundant RNA recognition events, A and B, that initiate largely overlapping programs of mRNA localization during oogenesis. Recognition event A requires a region encompassing stem-loops IV/V of the predicted secondary structure, and can be eliminated by a single nucleotide mutation. Localization initiated through event B begins slightly later in oogenesis, and requires sequences that have not been narrowly defined. Using forms of the 3′ UTR lacking this RNA recognition redundancy, we reexamine the roles of the *swallow*, *staufen*, and *exuperantia* genes, which are all required for normal *bicoid* mRNA localization. Our results reveal that *exuperantia* first becomes essential for localization at a time when well-defined microtubule tracks between the nurse cells and oocyte disappear. Thus, *exuperantia* may specifically facilitate a form of nurse cell-to-oocyte mRNA transport not dependent on the microtubule tracks.

Keywords: ***Drosophila*; oogenesis; posttranscriptional control; 3′ UTR**

INTRODUCTION

RNA recognition events underlie various stages of gene expression. Nuclear processing of pre-mRNAs requires the accurate recognition of splice sites and the RNA signals that direct cleavage and polyadenylation. Once in the cytoplasm, additional RNA recognition events mediate other processes, including initiation of translation, translational regulation, and programmed degradation. Current understanding of the mechanisms responsible for each of these processes has depended in large part on the characterization of the accompanying RNA recognition events.

A further stage in the use of some mRNAs involves localization to specific regions within the cytoplasm. The phenomenon of mRNA localization is widespread: examples have been described in a variety of cell types (reviewed by St Johnston, 1995). Not surprisingly, such mRNAs harbor *cis*-acting signals that specify where and how they are to be localized (reviewed by Macdonald, 1992). Each localization signal presumably mediates one or more RNA recognition events, whereby the RNA is associated with some component of the localization machinery. We expect that detailed characterization of the RNA recognition events will reveal much about the mechanisms of localization.

The *Drosophila bicoid* (*bcd*) mRNA is representative of an important class of localized maternal mRNAs. Proteins encoded by these mRNAs act as body patterning determinants, and their activities must be restricted to certain regions of the oocyte or embryo. Prelocalization of the mRNAs is an essential step in limiting the distribution of the determinants; if mRNA localization fails, expression of the determinants is altered dramatically, and the resulting body patterning defects are lethal (reviewed in St Johnston, 1995).

Localization of *bcd* mRNA occurs during oogenesis and early embryogenesis (Berleth et al., 1988; St Johnston et al., 1989). The mRNA is synthesized in ovarian nurse cells, but moves to the oocyte. Within the oocyte, *bcd* transcripts become restricted to the anterior margin, and are maintained there into embryogenesis. This localization program is directed by a signal within the 3′ UTR of the *bcd* mRNA (Macdonald & Struhl, 1988). Efforts to determine how the signal is recognized by the localization machinery have followed two general approaches. In one, candidate recognition factors, identified as mutants defective in *bcd* mRNA localization, *exuperantia* (*exu*), *swallow* (*sww*), and *staufen* (*stau*), have been characterized. The exu protein has RNA binding activity, but the binding is nonspecific and therefore unlikely to act in recognition

Reprint requests to: Paul M. Macdonald, Department of Biological Sciences, Stanford University, Stanford, California 94305, USA; e-mail: pmac@leland.stanford.edu.

Downloaded from www.rnajournal.org on February 14, 2006

of the localization signal (Wang & Hazelrigg, 1994; Macdonald et al., 1995). Although sww protein has weak homology to an RNA binding motif, there is no evidence of RNA binding (Chao et al., 1991). In contrast, *stau* does appear to act in RNA recognition. The stau protein contains multiple copies of a double-stranded RNA binding domain (St Johnston et al., 1992), and the results of an indirect assay suggest that stau binds to an array of several helical regions within the *bcd* 3′ UTR (Ferrandon et al., 1994, 1997). In the absence of *stau* activity, *bcd* mRNA is localized normally during oogenesis, but becomes partially delocalized in the embryo, and so the stau RNA recognition event apparently serves to tether the mRNA after its initial localization (St Johnston et al., 1989). Thus, none of the mutants provides a strong candidate for a protein that first recognizes *bcd* mRNA to initiate localization.

A second approach to understanding recognition of the *bcd* mRNA localization signal has been to identify the essential parts, and both direct and indirect strategies have been used. In the indirect approach, features of the *bcd* 3′ UTR conserved during evolution were identified (Macdonald, 1990; Seeger & Kaufman, 1990), with the expectation that some or all of these features will be important for *bcd* mRNA localization. The most striking conserved feature was a stereotypical secondary structure that can be formed by the 3′ UTR. Thus far, it has proven difficult to test the role of the hypothesized structure, largely because of functional redundancy in the *bcd* 3′ UTR (see below). However, in their analysis of stau protein interaction with the *bcd* 3′ UTR, Ferrandon and coworkers (1994, 1997) provided evidence indicating that at least a modest subset of the predicted base pairing interactions do occur. Although the overall structure remains largely hypothetical, it still serves as a useful starting point for exploring the organization of the *bcd* localization signal.

In the direct strategy for identifying essential parts of the *bcd* localization signal, the 3′ UTR has been systematically mutated (Macdonald et al., 1993). Although a single region essential for localization (and *bcd* function) could be defined using deletion mutants of about 50 nt, even that region proved to be nonessential (for both localization and *bcd* function) if deleted in parts. Thus, the *bcd* localization signal contains redundant localization information. This poses a significant problem in identifying and characterizing relevant RNA recognition events, which are likely to involve specific protein–RNA interactions. In particular, we can predict that mutating any single protein binding site in the signal will not severely affect localization, making it difficult to prove that a particular binding protein acts in that process. This problem could be overcome by defining nonredundant yet functional forms of the localization signal, which would then be useful for detailed characterization of the RNA recognition events of mRNA localization.

Here we show how RNA recognition redundancy can be stripped from the localization signal. Our results demonstrate that two redundant recognition events initiate largely overlapping programs of *bcd* mRNA localization during oogenesis, and that these events are distinct from the later *stau*-dependent recognition event. Further, we show that a minimal localization signal competent for only one recognition event can be inactivated by a single point mutation. This RNA therefore provides an ideal substrate for biochemical identification of RNA binding proteins that associate the mRNA with the localization machinery. Finally, we resolve ambiguity in the time when *exu* activity is first required; these results suggest a specific model for *exu* action.

RESULTS

In a previous deletion analysis of the *bcd* mRNA 3′ UTR, one small deletion (Δ14S) was found to have a novel property: the earliest wild-type movement to the oocyte during stages 4–6 of oogenesis did not occur, although all later phases of localization proceeded normally (Macdonald et al., 1993). To determine if a more subtle mutation could have the same effect, we created a *bcd* transgene bearing a single nucleotide mutation (4496 G>U) in the same region. As shown in Figure 1B, 4496 G>U mRNA behaves just like Δ14S mRNA, preventing localization only during the earliest stages of the process. This mutation therefore disrupts one recognition event, in which the *bcd* mRNA is recognized by the localization machinery; we refer to this as recognition event A. Recognition event A presumably entails the binding of one or more proteins to the site disrupted by mutation 4496 G>U. There are no good candidates for a gene encoding such a binding protein, because mutations in genes known to affect *bcd* localization cause defects only at later stages. Clearly, this recognition event is not a prerequisite for later events in localization, because most of the localization program occurs normally (Fig. 1B) and the Δ14S mutant provides full *bcd* function for embryonic development (Macdonald et al., 1993).

A second recognition event (B), unaffected by the 4496 G>U mutation, must begin during stage 6, when recognition event A is becoming dispensable. It is possible that the programs of localization directed by the two recognition events are not extensively overlapping, and that event A-dependent localization ceases as event B-dependent localization begins. Alternatively, event A may continue to support localization even after the second recognition event occurs. If so, the two events could be redundant, each independently promoting association with the localization machinery. To test the latter option, we set out to strip RNA recognition redundancy from the *bcd* 3′ UTR; a localization signal lacking this redundancy might then

Downloaded from www.rnajournal.org on February 14, 2006

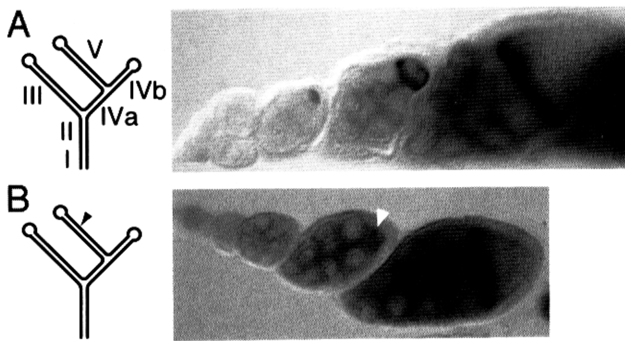

FIGURE 1. A single nucleotide change in the *bcd* 3′ UTR disrupts early mRNA localization to the oocyte. Diagrams at left show the predicted structure (Macdonald, 1990) of the 3′ UTR (in simplified outline form). In later figures, deleted portions of the 3′ UTR are grey. The 4496 G>U mutation is indicated by an arrowhead. **A:** Localization patterns of the *bcd+lacZ* transgene mRNA as revealed by in situ hybridization to whole-mount ovary preparations. In this and all other figures, stages of oogenesis progress from left to right. The mRNA is concentrated in the oocyte from stage 4–5 onward and lines the anterior margin of the oocyte beginning at stage 9. **B:** Localization of the *bcd+lacZ* 4496 G>U transgene mRNA. Early localization to the oocyte in stages 4–5 is absent, although the mRNA can be detected in the nurse cells. Beginning in stage 6, traces of oocyte localization appear (arrowhead). By stage 8, localization to the oocyte is strong. The subsequent concentration of the mRNA at the anterior margin of the oocyte is indistinguishable from wild type. To best show the earliest time of oocyte localization, the sample shown in B was more extensively stained than that shown in A. Consequently, some background staining appears throughout the early-stage egg chambers, both in the germ line cells (where *bcd* mRNA is transcribed) and the somatic cells (where *bcd* transcription does not occur).

display a prolonged dependence on event A. Three progressively smaller parts of the 3′ UTR were tested for localization activity. These parts correspond to domains of the predicted structure (Macdonald, 1990; Seeger & Kaufman, 1990) of the complete 3′ UTR: stem loops IV and V (IV/V), stem loops IVb and V (IVb/V), and stem loop V alone (V) (see Figs. 1, 4). Stem loop V was retained in each version because it includes the sites of the Δ14S and 4496 G>U mutations and is thus implicated in recognition event A. When placed into a *bcdΔ21+lacZ* transgene (which by itself lacks almost all of the *bcd* 3′ UTR and has no localization activity; Macdonald et al., 1993), neither IVb/V nor V directed any aspect of *bcd* mRNA localization; the mRNAs appeared at the normal time, but remained dispersed throughout the nurse cells (data not shown). In contrast, IV/V directed localization to the oocyte beginning at stage 4–5, and the mRNA became highly concentrated at the anterior margin of the oocyte from stage 8–9 onward (Fig. 2A). Much later, in early embryos, the IV/V-localized mRNA was not correctly restricted to the anterior pole. This embryonic localization defect is similar to that found in embryos from *stau* mutant mothers (St Johnston et al., 1989), and is consistent with evidence suggesting that stau protein interacts with multiple different regions of the *bcd* mRNA 3′ UTR, some of which lie outside of the IV/V region (Ferrandon et al., 1994, 1997). The IV/V localization activity—wild type in oogenesis and *stau*-like defects in embryos—was retained when tested in another reporter transgene with a completely different sequence context (see Materials and Methods). This consistent activity of the IV/V localization signal in different contexts indicates that it is robust, and should not be impaired by subtle sequence changes that do not specifically affect an RNA recognition element (see legend to Fig. 2).

We next addressed the role of recognition event A in IV/V localization, using the 4496 G>U point mutation to eliminate event A. In the context of the complete *bcd* 3′ UTR, this mutation affects localization only during stages 4–6 (Fig. 1B). In striking contrast, the point-mutated IV/V region is almost completely defective in all phases of localization (Fig. 2B). Thus, recognition event A is important for the entire program of localization directed by IV/V. We conclude that the IV/V region comprises a localization signal stripped of recognition redundancy: recognition event A is retained, but event B is lost. Furthermore, we conclude that, during most of oogenesis, from stage 6 onward, *bcd* mRNA localization is directed by redundant recognition events, requiring either event A or event B, but not both.

Genetics of RNA recognition in *bcd* mRNA localization

Redundancy in RNA recognition may involve either multiple binding sites recognized by the same factor, or multiple binding sites bound by different factors. In the latter scenario, loss of a single recognition factor is unlikely to have a dramatic effect on localization. This could explain why genetic approaches have failed to reveal how *bcd* mRNA is initially recognized by the localization machinery during oogenesis: none of the genes known to affect *bcd* mRNA localization alter the earliest step in the process (Stephenson et al., 1988; St Johnston et al., 1989). If there are indeed different recognition factors with redundant functions, forms of the *bcd* localization signal lacking recognition redundancy provide the tools to identify the missing recognition factors genetically. Specifically, the IV/V region supports only recognition event A, and mutations in a gene required for that event should abolish all IV/V-directed localization. Similarly, the intact *bcd* 3′ UTR bearing the 4496 G>U mutation does not support recognition event A, and thus provides a way to identify genes required for recognition event B (assuming there are only two redundant recognition events during oogenesis).

As an immediate application of this general approach, we have reexamined the roles of genes already known to act in localization of *bcd* mRNA—*sww*, *exu*, and *stau*. The *sww* and *exu* mutants disrupt localiza-

Downloaded from www.rnajournal.org on February 14, 2006

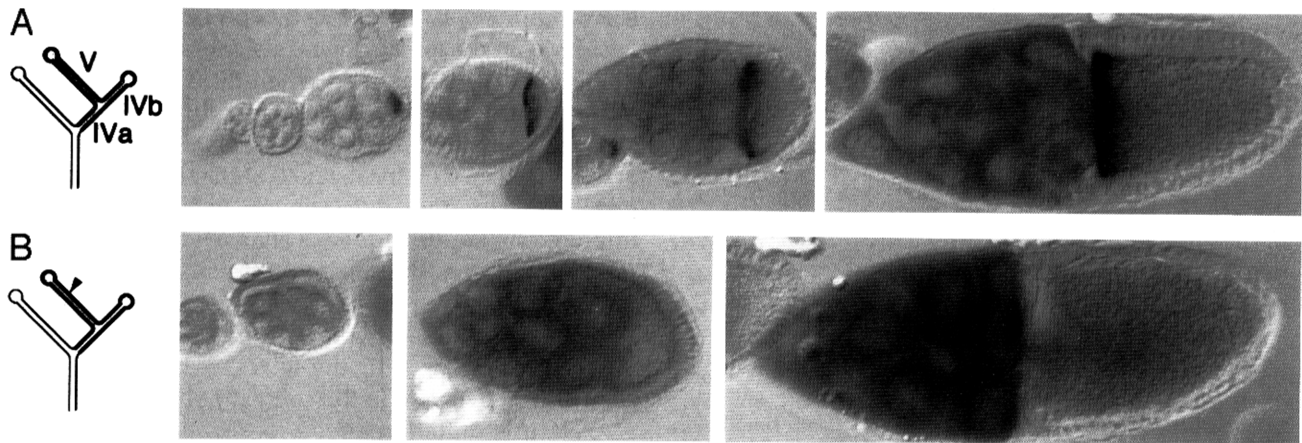

FIGURE 2. Stripping RNA recognition redundancy from the *bcd* mRNA localization signal. Sequences represented by the structures at left were inserted into the *bcd*Δ*21*+*lacZ* reporter construct. **A:** Localization directed by the IV/V subdomain of the *bcd* 3′ UTR. Movement of the mRNA to the oocyte and the subsequent localization at its anterior margin mimic that of the endogenous *bcd* mRNA. As the oocytes mature into embryos, the mRNA becomes spread out over the anterior third of the embryo (data not shown), just as observed for endogenous *bcd* mRNA in embryos from *stau*$^-$ mothers (St Johnston et al., 1989). Transgenes bearing smaller portions of this subdomain, either IVb/V or V, displayed no localization (data not shown). The ability of IV/V to direct localization is unexpected, because larger fragments of the 3′ UTR were previously found to lack localization activity (Macdonald & Struhl, 1988). The explanation for this difference is not known, although two factors may be involved. First, in the earlier experiments, localization was measured in embryos, not ovaries, and a less-sensitive assay was used. Second, the earlier deletions do not cleanly remove domains of the predicted structure, and may disrupt proper folding (but note that the structure is predicted, not proven). **B:** Localization activity of the IV/V subdomain bearing the 4496 G>U mutation. Using standard in situ hybridization procedures, no localized mRNA is detected, although the mRNA can be detected in the nurse cells (RNase protection analysis indicates that the mRNA is present at a level similar to that of the IV/V mRNA; data not shown). Extensive overdevelopment of the in situ hybridization signal revealed some localization in stage 9, but only in a minor fraction of the egg chambers (data not shown). Disruption of localization is specific to the 4496 G>U mutation, because mutations 4504-5 AU>UG had no effect on localization (data not shown).

tion at stages when both recognition events A and B are active, and the wild-type proteins presumably act in some aspect of localization not unique to one event. Nevertheless, the mutants might have different effects on localization directed by the different recognition events. In *sww* and *exu* mutant backgrounds, we monitored the activities of localization signals competent only for recognition event A (IV/V) or recognition event B (*bcd* 3′ UTR 4496 G>U). In the *sww* mutant flies, both localization signals were defective in localization beginning at stage 10B (data not shown), just as observed for localization of endogenous *bcd* mRNA (Stephenson et al., 1988; St Johnston et al., 1989). Thus, *sww* acts only in a process shared by both recognition events.

The results were quite different in *exu* mutant flies. Localization directed by IV/V can be detected until as late as stage 9 or early 10A, when the mRNA fails to remain concentrated at the anterior of the oocyte (Fig. 3A). In contrast, the *bcd* 3′ UTR 4496 G>U localization signal supports only traces of localization in *exu* mutants (Fig. 3B), despite the fact that this signal normally initiates localization during stage 6 (see Fig. 1B). The initial description of *bcd* mRNA localization in *exu* mutants is consistent with these results; localization is completely defective from stage 10 onward, but minor defects are reported to appear earlier (St Johnston et al., 1989). Interpretations of the seemingly different requirements of recognition events A and B for *exu* are considered in the Discussion.

Mutants lacking *stau* activity have normal *bcd* mRNA localization during oogenesis, but fail to properly tether the mRNA in the embryo (St Johnston et al., 1989). An earlier role for *stau* could be obscured by the redundant A and B RNA recognition events, and so we also tested the A- and B-specific localization signals in *stau* mutant flies. No ovarian localization defects were found, supporting the conclusion that *stau* acts only late in *bcd* mRNA localization (data not shown).

DISCUSSION

We can now identify two RNA recognition events sufficient to initiate the localization of *bcd* mRNA. Event A directs localization from stage 4–5, and can be eliminated by a single nucleotide substitution mutation within the stem-loop V portion of the *bcd* 3′ UTR. Event B directs localization beginning at stage 6, and does not have the earlier event A-dependent localization as a prerequisite. As currently defined, the minimal RNA that mediates recognition event A corresponds to stem-loops IV and V of the predicted structure of the *bcd* mRNA 3′ UTR. Although the stem-loop V region is necessary for event A, it is not sufficient, because RNAs

Downloaded from www.rnajournal.org on February 14, 2006

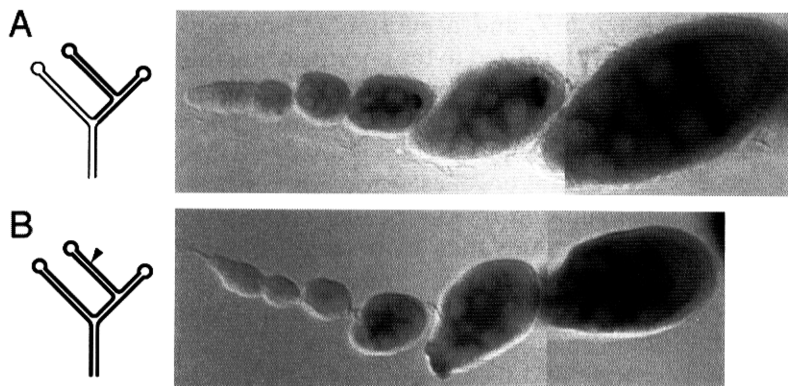

FIGURE 3. Localization directed by RNA recognition events A and B in *exu*^{4} mutant ovaries. **A:** Localization directed by the IV/V subdomain in *exu*^{4} mutant ovaries. As in wild type, the mRNA is concentrated in the oocyte beginning at stage 4–5. Localization to the anterior margin of the oocyte can be detected until stage 9 or 10A (right-most egg chamber). The intensity of anterior staining is less than in wild-type ovaries (compare to Fig. 2A), and appears to diminish as oogenesis proceeds. Note that *exu*^{4} is a null, bearing a nonsense codon early in the reading frame and making no detectable protein (Macdonald et al., 1991; Marcey et al., 1991). Thus, the observed anterior localization is not due to residual *exu* activity. **B:** Localization directed by the *bcd* 3′ UTR bearing the 4496 G>U mutation in *exu*^{4} mutant ovaries. The mRNA can be detected in the nurse cells, but no localized mRNA appears in the oocyte. We occasionally observe traces of mRNA localized to the anterior margin of the oocyte in stage 9 egg chambers.

corresponding to IVb and V, or V alone, lack localization activity. Thus, recognition event A may involve the binding of a localization factor or factors to multiple sites, or to a binding site made up of sequences that are dispersed in the primary structure. Alternatively, parts of the IV/V region may act only indirectly by contributing to an RNA structure required to present the binding site properly. In either case, this minimal region is significantly smaller than that defined previously (273 nt versus 628 nt) (Macdonald & Struhl, 1988) and thus more amenable to structural analysis. Moreover, our finding that a point mutation can disrupt event A-dependent localization demonstrates that the site or sites bound by recognition factors can be defined by mutational analysis. Our understanding of the RNA sequences or structures involved in recognition event B is much less complete.

A primary goal of the analysis of the RNA recognition events of *bcd* mRNA localization is to identify and characterize the binding factors; knowledge of their identities and interactions with other proteins should provide insights into the mechanisms of localization. Factors involved in recognition events A and B have not been identified, although the IV/V RNA now provides an ideal substrate to identify and isolate some of these factors. It is possible that the same binding factor acts in both events. If so, differences in binding affinity, or requirements for cofactors, could explain the different times at which the two events begin to direct localization. Alternatively, events A and B may involve different binding factors. This option is attractive because it suggests why genetic analyses have failed to reveal the identities of the binding factors: loss of either factor would eliminate only one of the two redundant recognition events, not causing an easily detectable phenotype.

Analysis of other localized mRNAs suggests that redundancy within localization signals may be common, but probably not universal. For the *Drosophila* K10 mRNA, the transport and localization signal has been narrowly mapped to a single short region, and lacks redundancy (Serano & Cohen, 1995). In contrast, multiple portions of the 3′ UTRs from some other localized mRNAs support localization, although efficiency may be compromised (Davis & Ish-Horowicz, 1991; Kislauskis et al., 1994; Lantz & Schedl, 1994; Gavis et al., 1996). In no case is it known if the redundancy represents multiple redundant binding sites for the same localization factor or factors, or if redundancy is achieved through the use of qualitatively different binding sites and factors.

Additional RNA recognition events in *bcd* mRNA localization

Information about RNA recognition events involved in localization of *bcd* mRNA has also come from analysis of BLE1, a 53-nt region of the 3′ UTR taken from stem-loop V. Two copies of BLE1 (2×BLE1) support apparently normal localization from stage 4–5 through stage 10A of oogenesis, although a single copy of BLE1 has no localization activity (Macdonald et al., 1993). Because 2×BLE1 and IV/V initiate localization at the same stage and have sequences in common, it seems likely that 2×BLE1 supports recognition event A. Nevertheless, we are not yet able to make instructive comparisons between IV/V and 2×BLE1 to learn more about event A. One complication in such a comparison is that the localization programs directed by 2×BLE1 and IV/V may well encompass more that just event A, and are clearly not identical; 2×BLE1-directed localization ends by stage 10A, whereas IV/V-directed localization continues through oogenesis. Another complication is the dependence on two copies of BLE1 for localization activity, whereas IV/V (and *bcd* itself) does not require such a duplication. The basis for this dependence remains unknown, and therefore introduces uncertainty in comparing 2×BLE1 to IV/V.

Downloaded from www.rnajournal.org on February 14, 2006

Other RNA recognition events are required for later stages in localization. One has been suggested by analysis of 2×BLE1. Certain point mutations in 2×BLE1 specifically affect perdurance of mRNA localization at the anterior margin of the oocyte in stage 10A, and the same mutations greatly reduce binding of an ovarian protein, exl, to 2×BLE1 RNA in vitro (Macdonald et al., 1995). This step also appears to involve functional redundancy, because inactivation of the exl binding site in the context of the complete *bcd* 3′ UTR or IV/V alone does not have a significant effect on localization (Macdonald et al., 1993; our unpubl. results). A further recognition event occurs late in oogenesis or early in embryogenesis and involves the stau protein. Stau includes multiple copies of a double-stranded RNA binding domain (St Johnston et al., 1992), and the protein appears to interact with helical stem regions of stem-loops III, IV, and V of the predicted structure of the *bcd* 3′ UTR (Ferrandon et al., 1994, 1997). The exl and stau binding sites do not seem to overlap with one another, in that exl binds to sequences not predicted to form helices in either 2×BLE1 or stem-loop V (Macdonald et al., 1995; our unpubl. data). However, because the binding site or sites involved in recognition event A have not yet been narrowly defined, overlap with the binding sites for exl or stau is possible, and progress through the stages of localization may require restructuring of ribonucleoprotein complexes.

exu Function

Delineation and separation of RNA recognition events A and B has also provided new insight into the requirement for *exu* in *bcd* mRNA localization. Notably, in *exu* mutants, localization directed by event A can extend until stage 9 or 10A, whereas event B-directed localization is almost undetectable. There are two simple interpretations of these results. In one model, *exu* has different roles for localization directed by the different events. Specifically, *exu* would perform an early event B-specific function beginning at stage 6–7. A later function, either common to both events or limited to event A, would begin at stage 9–10A. Because exu protein has RNA binding activity (Wang & Hazelrigg, 1994; Macdonald et al., 1995), the event B-specific early function could be in RNA recognition. However, exu RNA binding activity is nonspecific (Wang & Hazelrigg, 1994; Macdonald et al., 1995), and the low levels of correctly localized mRNA that persist in *exu* null mutants (Fig. 3A) would argue against such a role for *exu*. In the second and simpler model, which we favor, *exu* performs a single function for localization initiated by both recognition events. The *exu*-dependent step can now be shown to begin at stage 6–7, when event B-initiated localization is eliminated in *exu* mutants. Why is there a delay in the elimination of event A-initiated localization (as well as loss of endogenous *bcd* mRNA localization)? In *exu* mutants, localization initiated by event A remains active from stage 4–5 to stage 6–7, and a substantial amount of mRNA becomes localized to the oocyte during this interval. Continued localization until stage 9–10A could simply reflect perdurance of the previously localized mRNA. The ratio of localized to unlocalized mRNA appears to decrease as oogenesis proceeds, consistent with a gradual dilution or loss of the localized mRNA coupled with accumulation of newly synthesized and unlocalized mRNA.

Previous models for *exu* function have included both movement of *bcd* mRNA from the nurse cells to the oocyte and its anterior localization within the oocyte (Berleth et al., 1988; Macdonald et al., 1991; Marcey et al., 1991; Wang & Hazelrigg, 1994). Particular emphasis has been placed on the latter role, because exu protein is transiently concentrated at the anterior of the oocyte (Wang & Hazelrigg, 1994). However, this accumulation could be a byproduct of *bcd* localization, rather than a contributing factor. Indeed, one aspect of our results supports this interpretation. Specifically, we find that, in *exu* mutants, mRNAs transported to the oocyte by recognition event A proceed to be localized to the anterior margin of the oocyte (Fig. 3A); thus, *exu* is not absolutely required for anterior localization in the oocyte. We suggest a more restricted model of *exu* function, in which *exu* acts only in movement of *bcd* mRNA from nurse cells to oocyte beginning during stage 6. Other factors are then required for anterior localization. Such a role for *exu* is especially attractive in light of studies on the organization of the microtubule cytoskeleton during oogenesis (Theurkauf et al., 1992), taken in conjunction with our demonstration that *exu* function is initially required at stage 6–7. Up to stage 6, microtubules extend into the nurse cells from a microtubule-organizing center in the oocyte. These microtubles have been suggested to provide tracks for directed movement of mRNAs from the nurse cells into the oocyte (Theurkauf et al., 1992), and indeed *bcd* mRNA localization is sensitive to microtubule disruption (Pokrywka & Stephenson, 1991). The initial phase of recognition event A-dependent localization is likely to occur by this mechanism. At stage 7, cytoskeletal organization undergoes a dramatic change, and microtubules adopt an anterior to posterior density gradient in the oocyte (Theurkauf et al., 1992). This form of polarity is intriguing and often discussed, because it suggests that the selective use of minus- and plus-end directed motors can explain how some factors are localized to the anterior of the oocyte, and other factors to the posterior (Theurkauf et al., 1992; Clark et al., 1994, 1997; Li et al., 1994; McGrail et al., 1995; Karlin-McGinness et al., 1996). Another important consequence of the cytoskeletal reorganization, not so widely discussed, is the loss of obvious microtubule pathways for movement of mRNAs from nurse

Downloaded from www.rnajournal.org on February 14, 2006

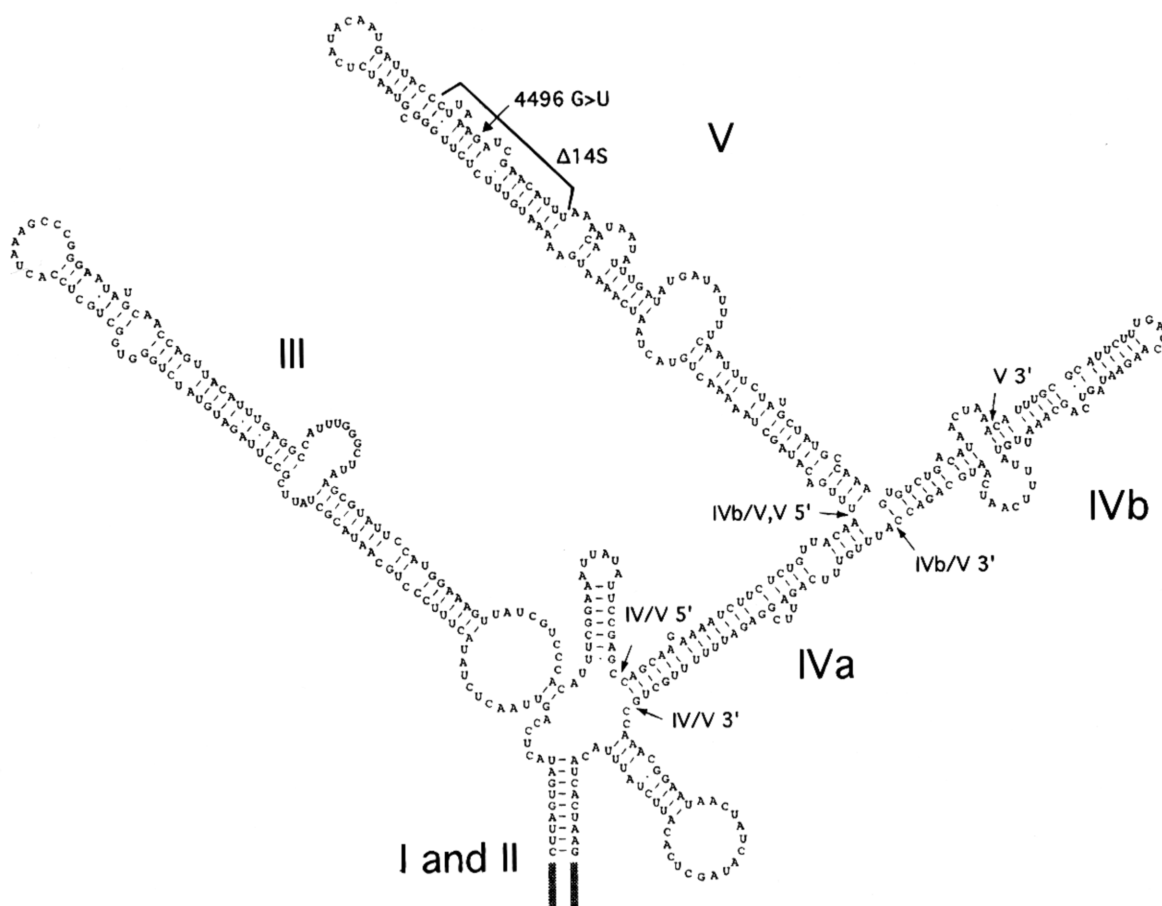

FIGURE 4. Forms of the *bcd* mRNA 3′ UTR used in this analysis. A portion of the 3′ UTR is shown, presented in the form of the structure predicted in 1990 (Macdonald, 1990) by a combination of computer folding (Zuker, 1989) and phylogenetic comparison. The major stem loops (III, IV, and V) are shown in their entirety, with most of stems I and II indicated by shaded bars. Endpoints of the truncated versions (IV/V, IVb/V, and V) are indicated with arrows and labeled accordingly. The sequences removed in the Δ14S deletion are indicated with a bracket, and the site of the 4496 G>U mutation is indicated by a labeled arrow. Refolding of the RNA using the current Zuker and Turner mfold 2.3 program and parameters (http://www.ibc.wustl.edu/~zuker/rna/form1.cgi) generates similar structures, with the most variation occurring throughout stem loop IV and the proximal part of stem loop V.

cells to oocyte (Theurkauf et al., 1992; Karlin-McGinness et al., 1996). Nevertheless, mRNAs continue to be transported from nurse cells to oocyte after the reorganization (Pokrywka & Stephenson, 1991; Karlin-McGinness et al., 1996; data presented here). We suggest that the specific and possibly sole function of *exu* is to facilitate this transport in the absence of well-organized microtubule tracks.

MATERIALS AND METHODS

Transgenes

The *bcd* mRNA transgene (*bcd+lacZ*) tagged by addition of *lacZ* sequences near the 3′ end of the 3′ UTR was that used previously (Macdonald et al., 1993). A transgene (*bcd*Δ3′ *UTR+lacZ*) lacking most of the 3′ UTR, but otherwise identical to *bcd+lacZ*, was used to test subdomains of the 3′ UTR for localization activity (Macdonald et al., 1995). The subdomains were amplified by PCR and had the following endpoints: IV/V, 4389–4663; IVb/V, 4413–4633; V, 4413–4573 (nucleotide coordinates from GenBank accession no. X51741). Each subdomain was amplified with 5′ *Bam*H I and 3′ *Bgl* II sites to facilitate cloning. The IV/V region was also shown to be active in another reporter, *osk/gfp*. The *osk/gfp* transgene includes the *osk* promoter and 5′ UTR, a coding region consisting of the first 37 amino acids of osk^{SHORT} protein (Markussen et al., 1995; Rongo et al., 1995) fused to green fluorescent protein (Chalfie et al., 1994), and a short 3′ UTR made of synthetic linker sequences (including a unique *Xba* I site) and the final 32 nt of the *nanos* mRNA [which provide the polyadenylation signal (Wang & Lehmann, 1991)]. The *osk/gfp* reporter transgene includes none of the sequences involved in either *osk* or *nos* mRNA localization (Kim-Ha et al., 1993; Gavis et al., 1996). For inclusion of IV/V in the *osk/gfp* reporter transgene, *Xba* I and *Spe* I sites were added to the 5′ and 3′ ends, respectively, and used for cloning into the reporter *Xba* I site. Point mutations were introduced into the *bcd* 3′ UTR or the IV/V region by PCR and were confirmed by DNA sequencing. All *bcd* 3′ UTR mutants described here are presented in graphic form in Figure 4.

Downloaded from www.rnajournal.org on February 14, 2006

Flies

Transgenic stocks were created by P element-mediated transformation. Multiple independent lines were obtained and analyzed for each transgene, all in the w^{1118} genetic background. For transgenes to be tested in different genetic backgrounds, third-chromosome insertions were identified by segregation tests, and the flies were balanced on the second and third chromosomes. The balanced stocks were then crossed to sww^{1497}, exu^4, and $stau^{D3}$ stocks for analysis.

mRNA analysis

Localization patterns were determined by in situ hybridization using probes specific for *lacZ* or *gfp* sequence tags (Kim-Ha et al., 1993, 1995). RNA levels were determined by RNAse protection assays (Macdonald et al., 1986).

ACKNOWLEDGMENTS

We thank Howard Lipshitz and Robin Wharton for flies, Michael Zuker for his online RNA folding service, Don Gilbert for his loopdloop computer program for display of RNA secondary structures, and Bob Cohen and members of the Macdonald laboratory for comments on the manuscript. This work was supported by NIH grant GM42612.

Received July 31, 1997; returned for revision August 26, 1997; revised manuscript received September 10, 1997

REFERENCES

Berleth T, Burri M, Thoma G, Bopp D, Richstein S, Frigerio G, Noll M, Nüsslein-Volhard C. 1988. The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J 7*:1749–1756.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. 1994. Green fluorescent protein as a marker for gene expression. *Science 263*:802–805.

Chao YC, Donahue KM, Pokrywka NJ, Stephenson EC. 1991. Sequence of *swallow*, a gene required for the localization of *bicoid* message in *Drosophila* eggs. *Dev Genet 12*:333–341.

Clark I, Giniger E, Ruohola-Baker H, Jan LY, Jan YN. 1994. Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr Biol 4*:289–300.

Clark IE, Jan LY, Jan YN. 1997. Reciprocal localization of Nod and kinesin fusion proteins indicates microtubule polarity in the *Drosophila* oocyte, epithelium, neuron and muscle. *Development 124*:461–470.

Davis I, Ish-Horowicz D. 1991. Apical localization of pair-rule transcripts requires 3′ sequences and limits protein diffusion in the *Drosophila* bastoderm embryo. *Cell 67*:927–940.

Ferrandon D, Elphick L, Nüsslein-Volhard C, St Johnston D. 1994. Staufen protein associates with the 3′UTR of *bicoid* mRNA to form particles that move in a microtubule-dependent manner. *Cell 79*:1221–1232.

Ferrandon D, Koch I, Westhof E, Nüsslein-Volhard C. 1997. RNA–RNA interaction is required for the formation of specific *bicoid* mRNA 3′ UTR-STAUFEN ribonucleoprotein particles. *EMBO J 16*:1751–1758.

Gavis ER, Curtis D, Lehmann R. 1996. Identification of *cis*-acting sequences that control *nanos* RNA localization. *Dev Biol 176*:36–50.

Karlin-McGinness M, Serano TL, Cohen RS. 1996. Comparative analysis of the kinetics and dynamics of *K10*, *bicoid*, and *oskar* mRNA localization in the *Drosophila* oocyte. *Dev Genetics 19*:238–248.

Kim-Ha J, Kerr K, Macdonald PM. 1995. Translational regulation of *oskar* mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell 81*:403–412.

Kim-Ha J, Webster PJ, Smith JL, Macdonald PM. 1993. Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development 119*:169–178.

Kislauskis EH, Zhu X, Singer RH. 1994. Sequences responsible for intracellular localization of β-actin messenger RNA also affect cell phenotype. *J Cell Biol 127*:441–451.

Lantz V, Schedl P. 1994. Multiple *cis*-acting targeting sequences are required for *orb* mRNA localization during *Drosophila* oogenesis. *Mol Cell Biol 14*:2235–2242.

Li Mg, McGrail M, Serr M, Hays TS. 1994. *Drosophila* cytoplasmic dynein, a microtubule motor that is asymmetrically localized in the oocyte. *J Cell Biol 126*:1475–1494.

Macdonald PM. 1990. *bicoid* mRNA localization signal: Phylogenetic conservation of function and RNA secondary structure. *Development 110*:161–171.

Macdonald PM. 1992. The means to the ends: Localization of maternal messenger RNAs. *Dev Biol 3*:413–424.

Macdonald PM, Ingham P, Struhl G. 1986. Isolation, structure and expression of *even-skipped*: A second pair-rule gene of *Drosophila* containing a homeo box. *Cell 47*:721–734.

Macdonald PM, Kerr K, Smith JL, Leask A. 1993. RNA regulatory element BLE1 directs the early steps of *bicoid* mRNA localization. *Development 118*:1233–1243.

Macdonald PM, Leask A, Kerr K. 1995. exl protein specifically binds BLE1, a bicoid mRNA localization element, and is required for one phase of its activity. *Proc Natl Acad Sci USA 92*:10787–10791.

Macdonald PM, Luk SKS, Kilpatrick M. 1991. Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes & Dev 5*:2455–2466.

Macdonald PM, Struhl G. 1988. *Cis*-acting sequences responsible for anterior localization of *bicoid* mRNA in *Drosophila* embryos. *Nature 336*:595–598.

Marcey D, Watkins WS, Hazelrigg T. 1991. The temporal and spatial distribution pattern of maternal *exuperantia* protein: Evidence for a role in establishment but not maintenance of *bicoid* mRNA localization. *EMBO J 10*:4259–4266.

Markussen FH, Michon AM, Breitwieser W, Ephrussi A. 1995. Translational control of *oskar* generates Short OSK, the isoform that induces pole plasm assembly. *Development 121*:3723–3732.

McGrail M, Gepner J, Silvanovich A, Ludmann S, Serr M, Hays TS. 1995. Regulation of cytoplasmic dynein function in vivo by the *Drosophila* glued complex. *J Cell Biol 131*:411–425.

Pokrywka N, Stephenson EC. 1991. Microtubules mediate the localization of *bicoid* mRNA during *Drosophila* oogenesis. *Development 113*:55–66.

Rongo C, Gavis ER, Lehmann R. 1995. Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development 121*:2737–2746.

Seeger MA, Kaufman TC. 1990. Molecular analysis of the *bicoid* gene from *Drosophila pseudoobscura*: Identification of conserved domains within coding and noncoding regions of the *bicoid* mRNA. *EMBO J 9*:2977–2987.

Serano TL, Cohen RS. 1995. A small predicted stem-loop structure mediates oocyte localization of *Drosophila* K10 mRNA. *Development 121*:3809–3818.

St Johnston D. 1995. The intracellular localization of messenger RNAs. *Cell 81*:161–170.

St Johnston D, Brown NH, Gall JG, Jantsch M. 1992. A conserved double-stranded RNA-binding domain. *Proc Natl Acad Sci USA 89*:10979–10983.

St Johnston D, Driever W, Berleth T, Richstein S, Nüsslein-Volhard C. 1989. Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Development 107*(Suppl):13–19.

Stephenson EC, Chao Y, Fackenthal JD. 1988. Molecular analysis of the *swallow* gene of *Drosophila melanogaster*. *Genes & Dev 2*:1655–1665.

Theurkauf WE, Smiley S, Wong ML, Alberts BM. 1992. Reorganization of the cytoskeleton during *Drosophila* oogenesis: Implications for axis specifications and intercellular transport. *Development 115*:923–936.

Wang C, Lehmann R. 1991. Nanos is the localized posterior determinant in *Drosophila*. *Cell 66*:637–647.

Wang S, Hazelrigg T. 1994. Implications for *bcd* mRNA localization from spatial distribution of *exu* protein in *Drosophila* oogenesis. *Nature 369*:400–403.

Zuker M. 1989. On finding all suboptimal foldings of an RNA molecule. *Science 244*:48–52.