Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10787–10791, November 1995
Developmental Biology

exl protein specifically binds BLE1, a bicoid mRNA localization element, and is required for one phase of its activity

PAUL M. MACDONALD*, ANDREW LEASK, AND KAREN KERR

Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020

Communicated by Bruce Baker, Stanford University, Stanford, CA, August 16, 1995 (received for review May 16, 1995)

ABSTRACT **Localization of mRNAs, a crucial step in the early development of some animals, has been shown to be directed by cis-acting elements that presumably interact with localization factors. Here we identify a protein, exl, that binds to BLE1, an RNA localization element from the *Drosophila* bicoid mRNA. Using mutations in BLE1, we demonstrate a correlation between *in vitro* exl binding and one phase of *in vivo* localization directed by BLE1, implicating exl in that localization event. Furthermore, the same phase of localization is disrupted in exuperantia mutants, suggesting that exl and exuperantia proteins interact. Identification of a protein that binds specifically to an mRNA localization element and acts in mRNA localization opens the way for a biochemical analysis of this process.**

Localized determinants are often used in the regional specification of early embryos (1). One mechanism to achieve the asymmetric distributions of such proteins is the prelocalization of their mRNAs (2). A strategy of mRNA localization is also recognized to operate in establishing local concentrations of cytoskeletal proteins in a variety of cell types (3). Although many localized mRNAs have now been found to contain cis-acting localization signals (4–10), identification of the proteins (or other factors) that bind specifically to these signals and direct localization has been difficult. Two related experimental limitations have contributed to this problem. (*i*) Most localization signals are defined only as large regions; many proteins may bind to such regions, but which act in localization? (*ii*) Within the better defined localization elements, substantial functional redundancy exists. For example, most of a 630-nt region both necessary and sufficient for bicoid (*bcd*) mRNA localization in *Drosophila* (4) can be made dispensable when removed in deletions of ≈50 nt, and even the single essential region defined in this fashion is itself dispensable if removed in parts (11). Consequently, testing the role of a binding protein will be difficult, as mutation of a single binding site in the context of an otherwise intact localization signal should not abolish localization.

A solution to these problems is offered by the identification of functional subdomains of localization signals, such as BLE1 from the bcd mRNA (11). Two tandem copies of this 53-nt element (2× BLE1) can restore some localization steps to an unlocalized bcd mRNA lacking its 3′ untranslated region (UTR), although the late steps do not occur. 2× BLE1-driven localization reflects the endogenous *bcd* localization process in (*i*) the mRNA distributions achieved and (*ii*) the dependence on the exuperantia (*exu*) gene, indicating that the same mechanisms are used. The advantages offered by BLE1 in identifying localization factors are its small size, which should limit the number of proteins that bind, and the absence of functional redundancy of the isolated element, making it possible to test the roles of binding proteins through the use of mutations that interfere with binding. Here we take this approach to identify an ovarian protein that binds specifically to 2× BLE1 RNA and appears responsible for one phase of 2× BLE1-directed localization *in vivo*.

MATERIALS AND METHODS

Extracts for RNA binding were made by homogenizing dissected ovaries in 150 mM KCl/50 mM Hepes, pH 7.9/1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride. Homogenates were cleared by centrifugation at 13,000 × *g*, and glycerol was added to 20%. Binding reactions (12, 13) contained 5 μl of extract (equivalent to about one ovary pair), 10 μg of tRNA, 30 mM Hepes (pH 7.9), 100 mM KCl, 2 mM $MgCl_2$, and 10^6 cpm of RNA probe (prepared by *in vitro* transcription in the presence of ^{32}P-labeled UTP) in a total volume of 11 μl. Protein–RNA complexes were formed at room temperature in microtiter plates. For competition binding experiments, the probe and competitor RNAs were added simultaneously to the binding mixture. UV crosslinking was done on ice in a Stratalinker (Stratagene), and then RNase A (30 μg) was added for 15 min at 37°C. The products of the binding reaction were subjected to SDS/PAGE and visualized by autoradiography. All binding experiments were done multiple times, with essentially no variation seen for binding of exl protein. The nonspecific binding of other proteins was less consistent.

Immunoprecipitations were done in Nonidet P-40 buffer according to ref. 14, using protein G-agarose beads (Boehringer Mannheim). Five standard RNA binding reactions were taken through the RNase step and pooled for a single immunoprecipitation. Rat anti-exu and anti-oskar antibodies have been described (15, 16). The preimmune serum was from an unrelated rat.

Point mutations in BLE1 DNA were generated by error-prone PCR (17). Singly mutated DNAs were recloned as dimers, using flanking 5′ *Bam*HI and 3′ *Bgl* II restriction sites. Deletion mutants were constructed by PCR, using oligonucleotides that introduced (or retained) *Bam*HI and *Bgl* II restriction sites at the 5′ and 3′ ends, respectively, of the truncated BLE1 DNAs. Mutant forms of 2× BLE1 DNA were introduced into a reporter transgene as described (11). Standard procedures were used to establish transgenic fly lines (18) and to monitor transgene mRNA distributions by *in situ* hybridization to whole-mount ovary preparations (19).

RESULTS

Using an ultraviolet (UV) crosslinking assay, we tested ovarian protein extracts for binding to 1× and 2× BLE1 RNAs. Because isolated 2× BLE1 directs mRNA localization, whereas 1× BLE1 does not, we expected that a localization factor might bind preferentially to 2× BLE1 RNA. We detect several proteins that bind to both RNAs, and a single protein (migrating as ≈115 kDa in denaturing gel electrophoresis) that binds exclusively to 2× BLE1 RNA (Fig. 1*A*; upper arrow-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: UTR, untranslated region.

*To whom reprint requests should be addressed.

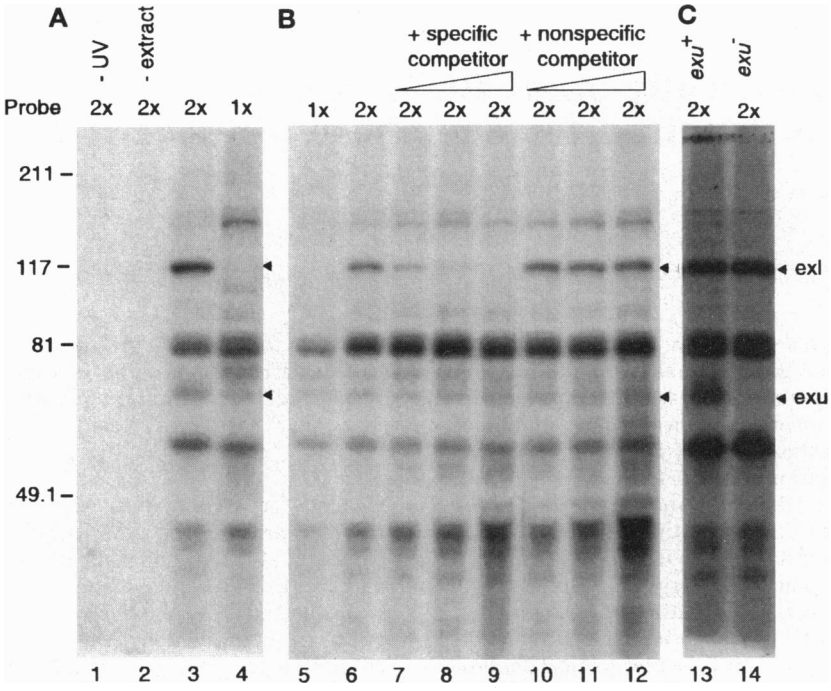

FIG. 1. Characterization of proteins that bind to BLE1 RNA. *Drosophila* ovarian protein extracts were tested by a UV crosslinking assay for binding to radioactive RNA probes corresponding to 1× or 2× BLE1 (11). Bound proteins appear as radioactive adducts; their sizes can be estimated by comparison to standards (given in kDa and indicated at left). (*A*) No proteins bind when the UV irradiation (− UV) or protein extract (− extract) is omitted from the assay. Comparison of proteins bound to 1× or 2× BLE1 probes shows one consistent difference invariably observed in hundreds of assays using dozens of independent extracts; a protein of ≈115 kDa, exl, binds only the 2× BLE1 RNA (lane 3; indicated by the upper arrowhead in all panels and identified at right). A slower migrating protein binds preferentially to the 1× BLE1 RNA (lane 4) but is not consistently detected (see lane 5). (*B*) Specificity of protein binding was tested by competition assays. Binding to 2× BLE1 RNA was challenged by addition of 10-, 30-, or 100-fold excess unlabeled RNA (lanes 7 and 10, 8 and 11, or 9 and 12, respectively). The specific competitor was 2× BLE1, and the nonspecific competitor was from a flanking region in the *bcd* 3′ UTR (*Ssp* I–*Stu* I, nt 4518–4733 of ref. 20). Another nonspecific competitor transcribed from the *bcd* 3′ UTR (*Hpa* I–*Sma* I, nt 4226–4294 of ref. 20) gave similar results. Binding to exl is specific, being reduced only by addition of the specific competitor RNA. (*C*) Comparison of BLE1 binding proteins from exu^+ and exu^4 ovaries, showing that exl is not encoded by *exu*. exu^4 is a nonsense mutant that makes no detectable exu antigen (15, 21). A long film exposure is presented to better display the absence of a different RNA-binding protein encoded by *exu* (see Fig. 2).

head). Binding of this protein to 2× BLE1 RNA is specific, as addition of unlabeled 2× BLE1 competitor RNA substantially reduces binding (Fig. 1*B*, lanes 7–9), whereas similar or greater amounts of nonspecific competitor RNAs do not (Fig. 1*B*, lanes 10–12). For reasons outlined below, we call this protein exl. The only known gene required for 2× BLE1 activity and, therefore, a candidate to encode exl is *exu* (11). However, the exu protein is much smaller (15, 21), and the exl binding activity persists in extracts from exu^- ovaries (Fig. 1*C*, lane 14).

One BLE1-binding protein is nevertheless absent in the exu^- extracts, and its apparent molecular mass (≈70 kDa) is slightly greater than that of exu protein alone [60 kDa (15, 21), as would be expected for an RNA/protein adduct]. To determine if this binding protein is exu, products of similar binding reactions were immunoprecipitated (Fig. 2) with exu or control antibodies. The exu antibodies precipitate the binding protein (Fig. 2, lane 1), while the control antibodies do not (lanes 2 and 3), confirming that the protein is, indeed, exu. Several other binding proteins, including exl, are precipitated nonspecifically (Fig. 2, lanes 1–3), even in the absence of antibodies (data not shown). This nonspecific precipitation prevents us from asking whether exu and exl proteins can be coimmunoprecipitated. In the UV crosslinking assay exu protein binds to 1× and 2× BLE1 (Fig. 1*A*), as well as other unrelated RNAs (data not shown), and is not affected by addition of competitor RNAs (Fig. 1*B*). This lack of specificity mirrors that observed for exu protein made in bacteria (P. Webster, A.L., and P.M.M., unpublished data; T. Hazelrigg and S. Wang, personal communication) and suggests that exu is not involved in specific recognition of RNA localization elements.

To test for possible correlations between 2× BLE1-dependent *in vitro* protein binding and *in vivo* mRNA localization, we first created a series of BLE1 deletion and point mutations and used the dimerized (2×) mutant RNAs as probes in the UV crosslinking assay (Fig. 3). A deletion mutant retaining only the 3′ 23 nt has substantial exl binding (lane 3), whereas loss of the 3′-most 10 nt abrogates detectable exl binding (lane 4). Three point mutants in the 3′ region fail to bind exl (lanes 11, 12, 14), whereas the remaining mutants have either normal or reduced but detectable exl binding. None of the deletion or point mutants eliminates exu binding, as expected given the lack of specificity noted above.

Localization directed by the wild-type 2× BLE1 transgene can be divided into two stages. The early stage is independent

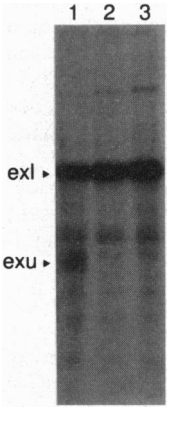

FIG. 2. Identification of exu by immunoprecipitation. Binding reactions with 2× BLE1 RNA probes were modified by an immunoprecipitation step before SDS/PAGE. The different immunoprecipitation reactions included the following antibodies: lane 1, anti-exu; lane 2, anti-oskar; lane 3, preimmune serum. The binding protein missing in exu mutant extracts is precipitated in the presence of anti-exu antibodies (lane 1) but not when other antibodies were used (lanes 2 and 3). The nonspecific precipitation of other binding proteins, including exl, occurs even without antibodies, using either protein A- or protein G-agarose beads (data not shown).

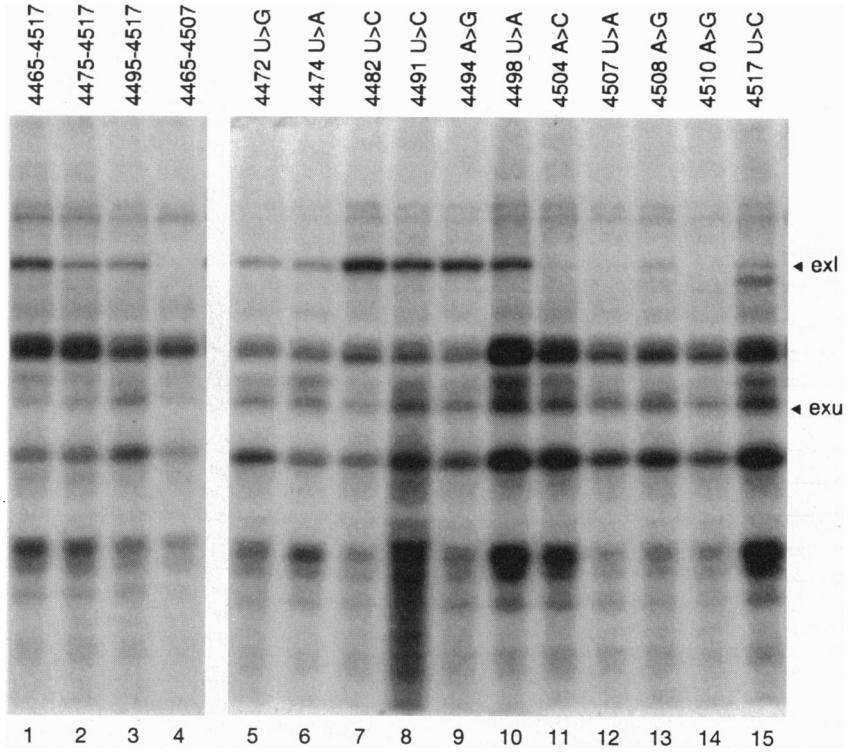

FIG. 3. Binding properties of mutant 2× BLE1 RNAs. Equal amounts of radiolabeled mutant RNA probes were used in individual binding assays, corresponding to individual lanes. Each lane is identified by the mutant RNA used. All probes consist of dimerized versions of BLE1, as schematized in Fig. 4. The full-length BLE1 corresponds to nt 4465–4517 (sequence coordinates from ref. 20) and is so labeled; deletion mutants are identified by the same nomenclature. Point mutations are labeled to indicate the nucleotide position involved and the change (e.g., 4466 G>U indicates a $G^{4466} \rightarrow U$ substitution); each change is present in both copies of the dimerized BLE1. Of the deletion mutants (left) only one, 4465–4507 fails to bind exl. Three point mutants (4504 A>C, 4507 U>A, and 4510 A>G) do not detectably bind exl, four have reduced binding (4472 U>G, 4474 U>A, 4508 A>G, and 4517 U>C), and the remaining four are strongly bound. The deletion and point mutants both indicate that sequences critical for exl binding are located near the 3′ end of BLE1. The binding results for the point mutants in the 3′ portion of BLE1 were confirmed in competition binding experiments (data not shown). None of the mutants eliminates binding to exu, although binding to deletion mutant 4465–4507 is reduced. Additional mutants tested the importance of nonbicoid sequences that appear in the 2× BLE1 RNA used for all binding experiments. Vector sequences flanking 2× BLE1 were altered and reduced by recloning 2× BLE1 between the *Pvu* II and *Bgl* II sites of pSP72, and the linker that joins the two copies of BLE1 was changed from GAAGATCC to GATCTG. Neither type of change altered exl binding (data not shown).

of any gene known to act in mRNA localization and includes the initial concentration in the oocyte (the mRNA is synthesized in the adjacent and interconnected nurse cells) and the subsequent localization at the anterior margin of the oocyte until stage 9 of oogenesis. A late stage, defined by its dependence on *exu*, entails the maintenance of anterior mRNA localization until stage 10A of oogenesis, when localization is lost. To determine whether exl binding correlated with either localization stage, a subset of the deletion and point mutants was tested for localization *in vivo*. DNA templates of each dimerized mutant BLE1 were introduced into a *lacZ*-tagged version of the *bcd* gene that lacks most of its 3′ UTR and is therefore unlocalized (the *lacZ* tag allows detection of the transgenic mRNA by *in situ* hybridization) (Fig. 4). Transgenic fly strains were established and tested for localization of the various mRNAs, using as a positive control a described wild-type 2× BLE1 transgene (11). Three point mutants, those that abolish exl binding *in vitro*, display a specific localization defect; anterior mRNA localization in the oocyte disappears during stage 9 and is absent by stage 10A (Figs. 4 and 5*B*). Although no other point or deletion mutant displays such a phenotype, it is indistinguishable from that seen for wild-type 2× BLE1-directed localization in *exu* mutants (11). In contrast, three 2× BLE1 point mutants that retain substantial exl binding are wild type in their ability to support localization (Figs. 4 and 5*A*). Finally, all of the deletion mutants and the single remaining point mutant support neither early nor late stages of localization (Figs. 4 and 5*C*), suggesting that for 2× BLE1-directed localization the early stage is a prerequisite for subsequent events.

DISCUSSION

Our mutational analysis of 2× BLE1 defines two stages of its activity, the same stages suggested by the behavior of the wild-type 2× BLE1 in wild-type or *exu* mutant ovaries. The early stage cannot yet be correlated with either a gene or a binding protein, or even a more narrowly defined binding site, as all of the 2× BLE1 deletion mutants are defective in this stage. In comparison, none of the point mutations in the deleted regions affects the early stage, even though the phenotype of the $U^{4498} \rightarrow A$ mutation (which lies outside the deleted regions) shows that the earliest localization can be disrupted by a single nucleotide change. These results suggest that interaction with the relevant localization factor is sensitive to RNA structure.

Our experiments are substantially more revealing about the late stage of localization directed by 2× BLE1, the primary focus of this report. We find a direct correlation between the ability of 2× BLE1 point mutants to direct this stage of localization and to bind exl, a protein we have identified that binds specifically to 2× BLE1 RNA. This correlation argues strongly that exl is responsible for the second stage of localization. exl is therefore an example of a predicted class of localization factors, those involved in the specific recognition of localization elements in the mRNAs.

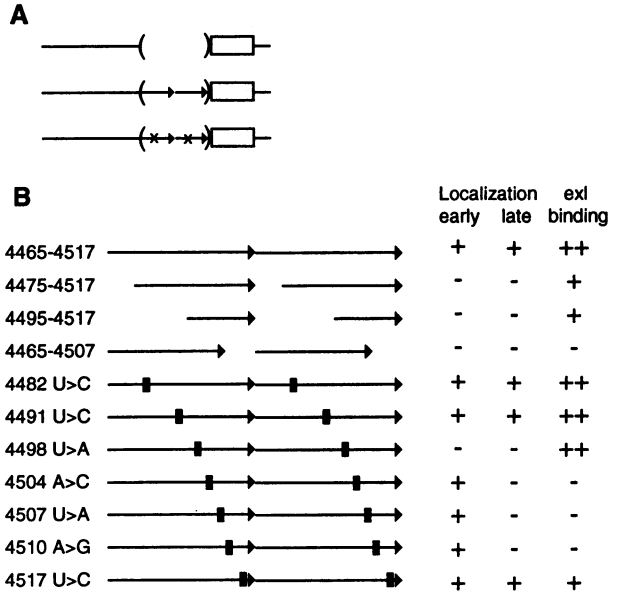

FIG. 4. Testing BLE1 mutants for defects in mRNA localization. (*A*) Schematic diagram of the transgenic mRNAs used in the *in vivo* localization assays. (*Top*) A *bcd* gene modified by deletion of most of its 3′ UTR and addition of *lacZ* sequences (box); mRNAs produced by this construct are unlocalized. (*Middle*) Addition of two tandem copies of BLE1 restores the early stages of *bcd* localization (data for these two constructs are from ref. 11. (*Bottom*) Generic structure of the mutants tested for localization; both copies of BLE1 carry the same mutation. (*B*) Localization directed by transgenes. Each version of 2× BLE1 tested is presented diagramatically, named as in Fig. 3. The ability of each version to direct the two stages of 2× BLE1-dependent localization is indicated at right, together with a summary of *in vitro* binding to exl protein (++, strong binding; +, weaker binding; −, no detectable binding).

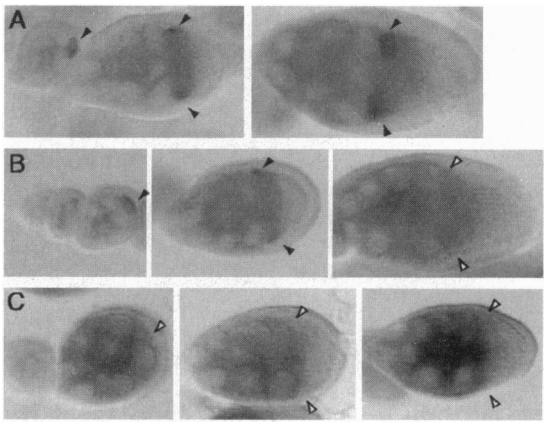

FIG. 5. 2× BLE1-directed mRNA localization in transgenic *Drosophila* ovaries. Distributions of transgenic mRNAs from 11 different 2× BLE1 mutants were examined by *in situ* hybridization to whole-mount ovary preparations. Three patterns were found. (*A*) The wild-type pattern directed by 2× BLE1 and three point mutants (Fig. 4). In early egg chambers (*Left*) (stages 5 and 6; stages according to ref. 22), the mRNA is concentrated in the oocyte (this and other sites of localization are indicated by filled arrowheads, while similar sites lacking obvious localization of some mutant RNAs are indicated by open arrowheads). In stages 8 and 9 (*Left* center) the mRNA is localized to the anterior margin of the oocyte, where it is concentrated near the cortex. Localization persists into stage 10A but becomes dispersed along the lateral cortex (*Right*), and is absent by stage 10B–11. (*B*) The pattern directed by all point mutants tested that fail to bind exl protein *in vitro* (Fig. 4). The early stages of localization are normal. Transcripts become concentrated in early-stage oocytes (*Left*) and adopt the anterior position within stage-8 and -9 oocytes (*Center*). This anterior localization is, however, lost precociously during stage 9, and no localized mRNA is detected by stage 10A (*Right*), even though substantial amounts of the mRNA are present in the nurse cells. This phenotype was previously observed for 2× BLE1-directed mRNA localization in exu mutants (11). (*C*) The pattern directed by all deletion mutants and one point mutant, 4498 U>A (Fig. 4). No localization is detected at any stage.

As the defects in 2× BLE1-directed localization caused by loss of *exu* activity or by mutations that prevent exl binding are strikingly similar (the exl phenotype is *exu*-like, hence the name), *exu* may mediate the action of exl. The specificity of *exu* action on bcd mRNA could be mediated by protein–protein contacts between exu and exl, rather than specific binding of exu protein to bcd mRNA. Such a model would account for our inability to find specificity in the RNA-binding activity of exu. More importantly, this model is consistent with the finding that an exu protein lacking the ability to bind RNA *in vitro* is still competent for bcd mRNA localization *in vivo* (T. Hazelrigg and S. Wang, personal communication). The RNA-binding activity of exu could instead promote the formation or contribute to the stability of an exl–exu–bcd complex but not be absolutely required to do so. Our attempts to detect an exl–exu protein interaction by biochemical methods have been unsuccessful (data not shown) but have used a bacterially produced exu protein that lacks the multiple phosphates of the *Drosophila* protein (15); S.K.-S. Luk and P.M.M., unpublished work).

BLE1 occurs only once in bcd mRNA, and we do not know why the isolated BLE1 is only active as a dimer. Perhaps BLE1 acts in concert with another type (or types) of localization element in the bcd mRNA and cannot normally act alone. If so, a second copy of BLE1 could substitute for the hypothetical element. Alternatively, additional copies of the binding sites in BLE1 may exist elsewhere in the bcd mRNA but as yet be unrecognizable, given our incomplete understanding of the structure of the binding sites. These additional sites could be dispersed in the linear bcd mRNA sequence but consolidated in a folded structure that forms *in vivo* (23, 24). Further definition of the exl binding site in 2× BLE1 RNA should facilitate identification of such sites, if they exist. Moreover, other mRNAs can also be examined for potential exl-binding sites. exl-binding activity is present throughout much of embryogenesis (data not shown) and may, therefore, act on other RNAs. An essential embryonic function would account for the failure to find exl mutants among collections of maternal-effect mutants that alter body patterning (25–27). As mRNA localization signals have been suggested to be assembled from combinations of individual elements not restricted in use to a single mRNA (8, 10), the use of exl more than once during development would not be surprising.

We thank J. Kim-Ha for advice on RNA-binding assays and J. Kim-Ha, Craig Smibert, Philippa Webster, and Joan Wilson for discussions and critical review of the manuscript. This work was supported by a grant from the National Institutes of Health. A.L. was a Medical Research Council of Canada Postdoctoral Fellow. P.M.M. is the recipient of a Packard Fellowship and was a Pew Scholar.

1. Davidson, E. H. (1986) *Gene Activity in Early Development* (Academic Press, Orlando, FL).
2. Macdonald, P. M. (1992) *Dev. Biol.* **3,** 413–424.
3. Singer, R. H. (1993) *Curr. Biol.* **3,** 719–721.
4. Macdonald, P. M. & Struhl, G. (1988) *Nature (London)* **336,** 595–598.
5. Mowry, K. L. & Melton, D. A. (1992) *Science* **255,** 991–994.
6. Cheung, H.-K., Serano, T. L. & Cohen, R. S. (1992) *Development (Cambridge, U.K.)* **114,** 653–661.
7. Gavis, E. R. & Lehmann, R. (1992) *Cell* **71,** 301–313.
8. Kim-Ha, J., Webster, P. J., Smith, J. L. & Macdonald, P. M. (1993) *Development (Cambridge, U.K.)* **119,** 169–178.
9. Kislauskis, E. H., Zhu, X. & Singer, R. H. (1994) *J. Cell Biol.* **127,** 441–451.
10. Lantz, V. & Schedl, P. (1994) *Mol. Cell. Biol.* **14,** 2235–2242.

11. Macdonald, P. M., Kerr, K., Smith, J. L. & Leask, A. (1993) *Development (Cambridge, U.K.)* **118,** 1233–1243.
12. Wilusz, J. & Shenk, T. (1988) *Cell* **52,** 221–228.
13. Kim-Ha, J., Kerr, K. & Macdonald, P. M. (1995) *Cell* **81,** 403–412.
14. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
15. Macdonald, P. M., Luk, S. K.-S. & Kilpatrick, M. (1991) *Genes Dev.* **5,** 2455–2466.
16. Smith, J. L., Wilson, J. E. & Macdonald, P. M. (1992) *Cell* **70,** 849–859.
17. Cadwell, R. C. & Joyce, G. F. (1992) *PCR Methods Appl.* **2,** 28–33.
18. Rubin, G. M. & Spradling, A. C. (1982) *Science* **218,** 348–353.
19. Tautz, D. & Pfeifle, C. (1989) *Chromosoma* **98,** 81–85.
20. Nüsslein-Volhard, C., Frohnhöfer, H. G. & Lehmann, R. (1987) *Science* **238,** 1675–1681.
21. Marcey, D., Watkins, W. S. & Hazelrigg, T. (1991) *EMBO J.* **10,** 4259–4266.
22. Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. & Nüsslein-Volhard, C. (1988) *EMBO J.* **7,** 1749–1756.
23. Mahowald, A. P. & Kambysellis, M. P. (1978) in *Oogenesis*, eds. Ashburner, M. & Wright, T. R. F. (Academic, New York), Vol. 2, pp. 141–224.
24. Macdonald, P. M. (1990) *Development (Cambridge, U.K.)* **110,** 161–171.
25. Seeger, M. A. & Kaufman, T. C. (1990) *EMBO J.* **9,** 2977–2987.
26. Schüpbach, T. & Wieschaus, E. (1986) *Roux's Arch. Dev. Biol.* **195,** 302–317.
27. Schüpbach, T. & Wieschaus, E. (1989) *Genetics* **121,** 101–117.