Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos

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Formation of the anteroposterior gradient of the bicoid (*bcd*) protein morphogen in *Drosophila* occurs as a consequence of the prelocalization of *bcd* mRNA to the anterior pole of the oocyte. *bcd* mRNA localization requires at least three genes, with the *exuperantia* (*exu*) gene acting earliest in the pathway. We have cloned and characterized the *exu* gene. Notably, we find that *exu* protein is colocalized with *bcd* mRNA during the early phase of localization, when *bcd* mRNA is positioned at the apical regions of the nurse cells. However, there is no colocalization at the later stage when *bcd* mRNA is transferred from the nurse cells to the anterior pole of the oocyte. There is no detectable *exu* protein in embryos. Therefore, *exu* acts only in initiating *bcd* mRNA localization and does not play a persistent role in that process.

[**Key Words:** mRNA localization; *Drosophila*; *exuperantia* gene; oogenesis; *bicoid*]

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In *Drosophila*, elaboration of the body plan in the head and thoracic regions is directed in large part by an anterior patterning system (Nüsslein-Volhard et al. 1987). Genetically identified components of this system include *bicoid* (*bcd*) (Frohnhofer and Nüsslein-Volhard 1986), which encodes a localized patterning activity, and several other genes required to localize *bcd* activity (Schüpbach and Wieschaus 1986; Frohnhofer and Nüsslein-Volhard 1987; Stephenson et al. 1988; St. Johnston et al. 1989). Bcd protein is distributed in a concentration gradient along the anteroposterior axis of the embryo, peaking at the anterior pole (Driever and Nüsslein-Volhard 1988a). This gradient provides positional information in a concentration-dependent fashion (Driever and Nüsslein-Volhard 1988b, 1989; St. Johnston et al. 1989). Formation of the gradient is made possible by the prelocalization of *bcd* transcripts to the anterior pole of the oocyte during oogenesis (Frigerio et al. 1986; Berleth et al. 1988). All other known components of the anterior system, the *exuperantia* (*exu*), *swallow* (*sww*), and *staufer* (*stau*) genes, are required for *bcd* mRNA localization (Frohnhofer and Nüsslein-Volhard 1987; Stephenson et al. 1988; St. Johnston et al. 1989).

One mechanism for localizing *bcd* mRNA to the anterior end of the oocyte was suggested by the spatial organization of the egg chamber. Each egg chamber contains a cluster of nurse cells connected by cytoplasmic bridges to the anterior pole of a single oocyte (King 1970). After synthesis in the nurse cells, *bcd* mRNA is trans-

ferred to the oocyte; trapping by a receptor at the site of entry could allow for anterior localization (Frohnhofer and Nüsslein-Volhard 1987). This simple model may be partially correct, but a careful analysis of *bcd* mRNA localization throughout oogenesis clearly demonstrated that it is incomplete (St. Johnston et al. 1989). *bcd* mRNA not only is localized within the oocyte but also to the apical regions of individual nurse cells. Therefore, the localization process begins before *bcd* mRNA enters the oocyte. Furthermore, St. Johnston et al. (1989) were able to describe four stages in the localization pathway and to identify the stages disrupted by mutations in the various anterior patterning genes. In *exu* mutants, *bcd* mRNA localization is abolished by the second stage and is somewhat aberrant even in the first stage. Mutations in the *sww* gene alter localization at a later stage, when the *bcd* transcripts are being positioned at the anterior margin of the oocyte. Finally, repositioning of *bcd* mRNA within the oocyte is defective in *stau* mutants. Among the identified genes, then, *exu* seems to act first in the localization pathway. Berleth et al. (1988) proposed previously that the *exu* product binds directly to *bcd* mRNA.

Here, we report the cloning and molecular characterization of the *exu* gene and describe the expression and localization of *exu* transcripts and protein during oogenesis. Our results do not support the notion that *exu* protein binds directly and persistently to *bcd* mRNA. Instead, they suggest a somewhat different role, in which

exu binds *bcd* mRNA transiently and perhaps indirectly, associating it in the nurse cells with components of the localization machinery.

Results

Molecular characterization of the *exu* gene

Genomic DNA from the *exu* region was cloned using a P-element-induced allele provided by T. Schüpbach (see Materials and methods). A transcription unit expressed in ovaries, as expected for *exu*, was identified by probing Northern blots of ovarian RNA with various restriction fragments from the *exu* region. Two lines of evidence prove that this gene is *exu*. First, P-element transformation with a DNA fragment containing the transcription unit rescues the maternal-effect lethality of an *exu* mutant (Fig. 1; Materials and methods). Second, sequence analysis of genomic DNA from the *exu*^{SC} mutant reveals the existence of a novel termination codon within the coding region of the gene (Fig. 2). In addition, this region has a restriction map essentially the same as that described by Hazelrigg et al. (1990) for the *exu* region.

Expression of *exu* occurs in a sex-specific fashion; one transcript of ~2.3 kb is present only in adult females and early embryos, and a male-specific transcript of ~3.0 kb first appears in late larval stages and persists into adulthood (Fig. 1). This male-specific expression was first noted by Hazelrigg et al. (1990), who demonstrated further that *exu* mutants are defective in spermatogenesis. As our analysis was undertaken to explore the role of *exu* in oogenesis, the male expression pattern is not addressed further. Nevertheless, we did wish to learn if the male and female transcripts encoded the same protein. If so, *exu* might be expected to perform the same function in the two distinct developmental processes. Toward this end, both male and female transcripts were defined further by isolation and characterization of ovarian and male cDNA clones. The DNA sequence was determined for each of the longest ovarian and male cDNAs, as well as for the corresponding genomic region (Fig. 2). Although none of the cDNAs are full length, the longest ovarian cDNA contains all of a single long open reading frame (ORF). The male cDNA is incomplete at the 5' end and therefore lacks part of the ORF. Nevertheless, the

Figure 1. Structure and temporal expression pattern of the *exu* gene. (Top) Map of the *exu* genomic region and organization of male and female *exu* mRNAs. The restriction map of genomic DNA shows all *Bgl*II and *Eco*RI sites. Indicated above the map is the *Eco*RI restriction fragment used to construct a rescuing P-element transposon. The longest female and male cDNAs are shown below the map, with the stippled region representing the long ORF. The 5' ends of the transcripts have not been mapped. (Bottom) Northern blot analysis of *exu* transcripts during development. A blot of staged samples of poly(A)⁺ RNA (provided by M. Kuroda and Y. Kim) was probed with a labeled restriction fragment from an *exu* cDNA. RNA samples were as follows: (0–3, 6–12, 12–18 hr) from embryos of the given ages after egg laying; (L1, L2, L3) from first-, second-, and third-instar larvae, male or female as indicated; (P1, P2, P3) from early, mid-, or late pupae, male or female as indicated; (adult males or females) as indicated. A male transcript of ~3.0 kb is first detected in third-instar larvae and is present throughout all later stages. A female transcript of ~2.3 kb is present in adult females. In addition, this transcript is present in early embryos, presumably through maternal contribution.

male cDNA does have a difference relative to the ovarian cDNA that can fully account for the difference in transcript size. In male transcripts one polyadenylation signal is spliced out and a downstream site is used, whereas the upstream polyadenylation site is retained and used in female transcripts. This splicing difference increases the predicted length of the transcript by ~630 nucleotides but does not alter the protein-coding region. Because male and female proteins migrate similarly in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3), it is likely that male and female proteins are identical.

Translation of the *exu* ORF predicts a 58-kD protein, assuming that the first ATG in the ORF is used for initiation. This is the case apparently, as a bacterially produced protein initiated at that ATG comigrates in SDS-PAGE with a form of the *exu* protein present in both ovaries and testes, as determined by Western blot analysis with polyclonal antisera directed against the *exu* protein (Fig. 3). Under favorable conditions, the ovarian *exu* protein is resolved into a series of distinct bands, with the fastest-migrating form comigrating with bacterially produced *exu* protein. Because there are no alternate upstream ATG codons in the same reading frame, the additional bands probably represent post-translationally modified forms of the protein.

The amino acid sequence of *exu* protein provides no clear indication of its function. Overall, *exu* protein has an amino acid composition typical of *Drosophila* proteins, except for an unusually high proportion of serine residues, 12.4% as compared with 7.6% (M. Ashburner, as cited in Smoller et al. 1990). Some of these serine residues are present in a Ser-x repeat unit (amino acids 206–235). Several RNA-binding proteins include clusters of Ser-Arg or Arg-Ser repeats (e.g., Mancebo et al. 1990). In the *exu* Ser-x repeat, x is never arginine and is most frequently glycine. Only two Ser-Arg dipeptides are found in the entire *exu* protein. As in many *Drosophila* genes (Wharton et al. 1985), *exu* has an opa-like repeat encoding a run of asparagine residues. Other regions are enriched for a single amino acid, most notably arginine between residues 419 and 427. One segment of 42 amino acids (340–381) conforms to the heptad repeat unit of coiled-coil proteins (Cohen and Parry 1986). *exu* does not appear to encode a membrane protein, having no likely signal sequence or membrane-spanning domain. Finally, comparison of the *exu* protein sequence with those in the GenBank and EMBL data bases reveals no striking similarities.

Post-transcriptional control of *exu* expression

During oogenesis, *exu* is required to localize *bcd* mRNA. To understand the role of *exu*, it would be useful to know whether *exu* gene product, either mRNA or protein, is also localized. We have therefore monitored the expression and distribution of *exu* products, first by Northern and Western blot analyses (Figs. 1 and 3). Unexpectedly, although *exu* mRNA is present in both ovaries and embryos (Fig. 1), *exu* protein can be detected only in ovaries (Fig. 3). Identical results were obtained

with antisera raised against an almost full-length *exu* protein, an amino-terminal part of *exu* protein, or a carboxy-terminal part of *exu* protein (see Materials and methods). This result contrasts sharply with what has been found for certain other proteins involved in localization of spatial cues, such as *BicaudalD* and *vasa*, which are present in both ovaries and embryos (Hay et al. 1988, 1990; Wharton and Struhl 1989; Lasko and Ashburner 1990). Therefore, it seems that *exu* function is limited to the ovary. Because *bcd* mRNA is strictly localized in both ovaries and embryos, the role of the protein encoded by *exu* must not entail persistent binding to *bcd* mRNA. Rather, the role of *exu* must necessarily be a transient one.

Concentration of *exu* protein at sites of *bcd* mRNA deposition

We also examined the spatial distribution of *exu* mRNA and protein in ovaries, in large part to determine whether there was any correspondence between sites of *bcd* mRNA localization and *exu* product distribution. Before describing the results, we review briefly the patterns of *bcd* mRNA localization during oogenesis (Frigerio et al. 1986; Berleth et al. 1988; Stephenson et al. 1988; St. Johnston et al. 1989). In stage 5–6, *bcd* mRNA is detected first in the egg chamber, being restricted to the oocyte [stages are as described by King (1970) and Mahowald and Kambyssellis (1978)]. By stage 8, *bcd* mRNA in the oocyte is restricted noticeably to the anterior margin, a situation that persists for the remainder of oogenesis. Starting in stage 9, *bcd* transcripts also appear in the nurse cells; within each nurse cell *bcd* mRNA is concentrated apically, between the outer membrane and the nucleus. At the same stage, *bcd* mRNA in the oocyte is restricted to the anterior margin. This pattern persists until stage 10B–11, when the nurse cell *bcd* mRNA is transferred to the oocyte and localized specifically at the anterior pole.

Examination of *exu* mRNA expression in ovaries revealed no comparable spatial pattern (Fig. 4). Low levels of *exu* mRNA are present in the germarium and throughout the cytoplasm of early-stage egg chambers. In stage 3 and 4 egg chambers, *exu* mRNA is concentrated in the oocyte, as is *bcd* mRNA at later stages. From stage 8–10, high levels of *exu* mRNA are present in the nurse cells. In some preparations there is a nonrandom distribution of the transcripts, with higher levels present in the posterior nurse cells relative to the more anterior nurse cells. Nevertheless, *exu* mRNA distribution clearly does not parallel that of *bcd* mRNA.

A very different result was obtained when ovaries were examined for the distribution of *exu* protein (Fig. 5). The protein appears initially in regions 2 and 3 of the germarium, where it is spread throughout the cytoplasm of all cells. In stage 1 egg chambers, *exu* protein becomes concentrated in the oocyte. This pattern persists to stage 6. By stage 7, the oocyte staining begins to be overshadowed by the accumulation of *exu* protein in the nurse cells. Within the individual nurse cells, *exu* protein be-

served in these and other egg chambers is striking. During stage 10, accumulation of exu protein in the nurse cells continues until there is a high level of protein

[illegible]

Figure 2. (See facing page for legend.)

throughout each nurse cell. In addition, a much lower level of *exu* protein remains spread evenly throughout the oocyte. This pattern persists until stage 11, when the contents of the nurse cells are transferred to the oocyte. Some residual *exu* protein remains in the now shrunk nurse cells, while most *exu* protein becomes dispersed at a low level throughout the oocyte. Notably, there is no specific accumulation of *exu* protein at the anterior of the oocyte, where *bcd* mRNA is localized. Finally, in stages 13–14, the oocyte staining disappears, as expected from our failure to detect *exu* protein in embryos by Western blot analysis (Fig. 3).

Thus, we can divide the pattern of *exu* protein distribution into three stages: two initial stages in which *exu* protein is concentrated at sites where *bcd* mRNA will accumulate, and a late stage in which there is no correlation between *exu* protein localization and *bcd* mRNA localization. We define the first stage as being when *exu* protein and *bcd* mRNA are present at high levels throughout the oocyte but are largely absent from the nurse cells. We consider this stage separate from the later period of *exu* protein and *bcd* mRNA colocalization for two reasons. First, some *bcd* mRNA does accumulate in the oocyte of *exu* mutant ovaries (St. Johnston et al. 1989); therefore, *exu* is not required absolutely for this step. Second, other mRNAs are also localized to the oocyte at the early stages of oogenesis [*fs(1)K10*, Haenlin et al. 1987; H.-K. Chung, T. Serano, and R.S. Cohen, pers. comm.; *oskar*, Kim-Ha et al. 1991; Ephrussi et al. 1991; *stau*, St. Johnston et al. 1991], suggesting the existence of a more general mechanism for this step. We define the second stage of *exu* protein distribution as the period when *exu* protein and *bcd* mRNA are colocalized at the apical regions of the nurse cells and at the anterior margin of stage 8–10A oocytes. The third stage occurs in the oocyte where *exu* protein is spread throughout and is apparently degraded uniformly. We now consider the second stage in more detail.

Colocalization of *exu* protein and *bcd* mRNA could arise in several different ways, each of which involves a somewhat different role for *exu*: (1) *Exu* protein is localized first and then directs localization of *bcd* mRNA; (2) *bcd* mRNA is localized first and then directs localization of *exu* protein; or (3) *exu* protein and *bcd* mRNA are localized simultaneously. The second possibility can be ruled out, as *bcd* mRNA does not become localized in the absence of *exu* protein. Two types of evidence are in

favor of the first explanation. First, in a careful comparison of temporal patterns, *exu* protein seems to precede *bcd* mRNA in localization to the apical regions. *exu* protein first appears concentrated at the apical regions of the nurse cells during stage 8 (Fig. 5), whereas St. Johnston et al. (1989) found that apical localization of *bcd* mRNA appears rarely in stage 8 and only consistently in stage 9. We have also obtained similar results, using flies with six copies of the *bcd* gene (Struhl et al. 1989) to increase the sensitivity of *bcd* mRNA detection. Nevertheless, a final determination of the temporal sequence of *exu* protein and *bcd* mRNA apical localization will require simultaneous detection of both molecules in the same preparations or preferably, the isolation of an RNA-null allele of *bcd* to determine whether *exu* protein is still localized. The second line of evidence comes from an examination of the effects of changing the amount of *bcd* mRNA present in the ovary. If *exu* protein and *bcd* mRNA are colocalized simultaneously as a ribonucleoprotein complex, we would expect that by changing the amount of *bcd* mRNA, the amount of localized *exu* protein would also be changed. Ovaries from flies with either one or six copies of the *bcd* gene were compared, monitoring *bcd* mRNA by in situ hybridization and *exu* protein by immunohistochemistry. Although the level of *bcd* mRNA was elevated and correct localization was maintained, there was no striking difference in *exu* protein staining (data not shown). Again, this analysis would benefit from isolation of a *bcd* allele that makes no mRNA.

exu protein patterns in mutant ovaries

We also examined *exu* protein patterns in ovaries from mutants with defects in the anterior localization pathway, *sww* and *stau*. St. Johnston et al. (1989) placed these genes downstream from *exu* in the anterior localization pathway. Not surprisingly, we find no consistent alteration in *exu* protein distribution in these mutants (data not shown).

Discussion

The asymmetric localization of mRNAs within cells is a striking phenomenon of considerable interest in cell and developmental biology. In early stages of development, such mRNAs can provide the localized determinants

Figure 2. DNA sequence of *exu*. The sequence shown is of a genomic region including most or all of the *exu* transcription unit. Introns are indicated by lowercase letters, except for the male-specific intron, which is in uppercase letters and underlined. The 5' end of the transcript has not been determined. Female and male cDNA 3' ends are each indicated by an x above the sequence (positions 2794 and 3719, respectively), and the likely polyadenylation signals are overlined. In each case, the presence of two A residues in the genomic DNA makes it impossible to determine the exact site of polyadenylation. The female 3' end lies within the male intron; the male 3' end lies at the end of the first of two copies of a nearly perfect 59-nucleotide direct repeat. The limits of each repeat are marked by carats underneath the DNA sequence (3655–3723, 3724–3782). The amino acid sequence of the long ORF is presented above the DNA sequence. Two mini-ORFs upstream from the long ORF are indicated by broken arrows above the DNA sequence, with overlining of the start and stop codons. The sequence of the female cDNA is identical to that of the corresponding sequence, except for a A → G substitution at position 1976 (no amino acid change). The codon mutated to a stop codon in the mutant *exu*^{SC} (positions 1021–1023) is underlined. Additional changes in the *exu*^{SC} gene are 317, C → A; 531, a → t; 567, a → t; deletion of two a's in the poly(A) run at 717–728; 777, T → C; 1616, G → A (Met → Ile).

Figure 3. Western blot analysis of *exu* protein expression. Samples were electrophoresed in SDS–polyacrylamide gels, transferred to nitrocellulose filters, and probed for the presence of *exu* protein using polyclonal antisera directed against a bacterially produced protein. Lanes are as indicated. (Left) The amount of bacterial protein loaded was adjusted to give a band of similar intensity to the *Drosophila* samples. Similar amounts of total protein were loaded in all *Drosophila* lanes, except for the embryo lanes that had two- to threefold less protein. (Right) The blot was overloaded with embryo protein in an attempt to detect even low levels of *exu* protein (the background bands are due to the abundant yolk proteins, which are faintly visible in the ovary samples at left). The prominent ~60-kD band of *exu* protein (at left) is present in both wild-type testes and ovaries but is absent in early embryos and in *exu*^{SC} ovaries and testes. *exu* protein in both ovaries and testes migrates as a broad band. In favorable blots, such as the one shown here, *exu* protein is clearly seen to migrate as a series of six or more narrowly separated bands. The bacterial protein comigrates with the lowest of these bands. The solid arrowheads indicate the positions of size standards: From top to bottom, these are 106, 80, 47.5, and 32.5 kD. The open arrowhead (right) indicates the position where the *exu* protein would appear if present.

that specify body patterning (Weeks and Melton 1987; Berleth et al. 1988; Wang and Lehmann 1991). Localization of mRNAs in differentiated cells may be involved in concentrating proteins at the sites where they are most needed (Lawrence and Singer 1986; Garner et al. 1988; Fontaine et al. 1988; Cheng and Bjerknes 1989; Hooek et al. 1991). One prominent example of the localization

phenomenon is the positioning of *bcd* mRNA during oogenesis in *Drosophila*. This example is well suited for a detailed analysis of the underlying mechanisms, as the *cis*-acting element responsible for localization has been defined (Macdonald and Struhl 1988) and several genes required for the process have been identified (Schüpbach and Wieschaus 1986; Frohnhofer and Nüsslein-Volhard 1987; Stephenson et al. 1988; St. Johnston et al. 1989). Furthermore, *bcd* localization involves several steps, highlighting the complexity of the process and offering insights at several levels. Taken individually, each step may provide generally useful information about how other RNA molecules are localized. Learning how the steps are connected will tell us something about how cells orchestrate elaborate intracellular movements of macromolecules.

bcd mRNA is synthesized in the nurse cells and then moves through cytoplasmic bridges into the oocyte where it is localized at the anterior pole. Because the anterior end of the oocyte abuts the nurse cells, *bcd* mRNA localization was suggested initially to occur very simply by interaction of the RNA with a receptor in the oocyte (Berleth et al. 1988; Macdonald and Struhl 1988). More recently, St. Johnston et al. (1989) monitored *bcd* mRNA throughout oogenesis and found that the situation is more complex, involving at least four steps. By extending this analysis to mutant ovaries, they were able to pinpoint the stages where the genes *exu*, *sww*, and *stau* are first required. *exu* mutants exhibited the earliest alteration in the localization pathway (St. Johnston et al. 1989), and *exu* has been suggested to encode a protein that specifically binds to the *bcd* mRNA localization signal (Berleth et al. 1988). Here, we have characterized the *exu* gene and its expression. Our results provide direct support for an interaction between *exu* protein and *bcd* mRNA but also indicate that the interaction is transient and, possibly, indirect. These results further emphasize the incompleteness of the simplest models of localization and suggest that the role of *exu* in localization is to direct association of *bcd* mRNA with other components of the localization machinery.

Evidence supporting the existence of an interaction between *exu* protein and *bcd* mRNA comes from our analysis of *exu* protein distribution during oogenesis. Specifically, we find colocalization of *exu* protein and *bcd* mRNA. Because the localization of *bcd* mRNA is *exu* dependent, we suggest that the localization of both components to the same position has functional significance. Our studies reveal further that these components are not colocalized persistently. Rather, *exu* protein and *bcd* mRNA are colocalized only at a time and place where *bcd* mRNA first acquires a distribution unlike that of any other characterized mRNA, in the apical regions of the nurse cells. At later stages, when *bcd* mRNA moves from the nurse cells to the anterior margin of the oocyte, there is no corresponding concentration of *exu* protein. These results indicate that the role of *exu* is to initiate one phase of the process by which *bcd* mRNA is localized but not to play a continuing part in the execution of that process. Nevertheless, this transient inter-

Figure 4. Spatial patterns of *exu* mRNA expression during oogenesis. A series of progressively more advanced stages of oogenesis are shown: (Top) The germarium: stage 1, stage 2, stage 3 or 4, stage 6, and stage 7 or 8; (bottom) stages 9 and 10. *exu* mRNA has been detected by in situ hybridization using a whole-mount procedure (Tautz and Pfeifle 1989). Expression of *exu* is detected initially in regions 2 and 3 of the germarium and continues for the remainder of oogenesis. At stage 3, *exu* mRNA appears somewhat concentrated in the oocyte relative to the nurse cells. This pattern continues until stage 7. In subsequent stages, *exu* mRNA is expressed at much higher levels in the nurse cells relative to the oocyte.

action is essential, because in the absence of *exu* protein *bcd* mRNA becomes spread throughout the oocyte. Thus, the movement of *bcd* mRNA from nurse cells to oocyte is not sufficient for anterior localization.

The interaction between *exu* protein and *bcd* mRNA has been suggested to involve direct binding (Berleth et al. 1988). Given our results, if the interaction is direct, then binding of *exu* to *bcd* mRNA must necessarily be followed by disassociation. Alternatively, *exu* may interact indirectly by an intermediate binding protein. At present, these possibilities cannot be distinguished. In either case, there are other important aspects of the interaction that need to be clarified. In particular, what signal or event first directs the initial association of *exu* protein and *bcd* mRNA? Similarly, what directs the subsequent release of *bcd* mRNA from the nurse cells to allow movement into the oocyte? The first step might occur only when a protein that binds to *bcd* mRNA is synthesized or activated. We cannot distinguish between this and other possibilities, however, nor can we distinguish among the many possibilities for the second step.

If *exu* protein does not bind directly to *bcd* mRNA, it will be of considerable interest to identify the protein that does perform this function. Mutations in the *sww* and *stau* genes disrupt *bcd* mRNA localization but do so only after the initial localization to the apical regions of the nurse cells. Thus, although the *sww* and *stau* proteins may at some point bind *bcd* mRNA directly (St. Johnston et al. 1991), a different protein must bind at the earlier stage. The actual *bcd* mRNA-binding protein, if not *exu* itself, may well interact with *exu* protein, suggesting a biochemical approach for its identification.

In addition to the interaction, direct or indirect, between *bcd* mRNA and *exu* protein, it is very likely that *exu* protein is involved in another interaction. This suggestion comes from the localization of *exu* protein to apical regions of the nurse cells. Because there is no equivalent concentration of *exu* mRNA, the preferential accumulation of *exu* protein presumably occurs as a result of its binding to a fixed subcellular structure in the apical regions. Candidates for such a structure are of two

general types: membranes and cytoskeletal elements. The DNA sequence of *exu* predicts neither an obvious signal sequence nor a membrane-spanning domain. Nevertheless, *exu* could be membrane bound by its association with a membrane protein. If so, apical localization could stem from polarized movement of vesicles (Matlin and Simons 1984; Rindler et al. 1984; Simons and Fuller 1985) or from a specific association with the apical plasma membrane. Alternatively, *exu* protein localization may involve the cytoskeleton. Indeed, recent studies of *bcd* mRNA localization in vivo have demonstrated a requirement for cytoskeletal elements. Pokrywka and Stephenson (1991) found that *bcd* mRNA failed to localize correctly when microtubules were destabilized but that localization occurred normally in the presence of cytochalasin D, a microfilament-destabilizing drug. A role of *exu*, therefore, may be to associate *bcd* mRNA with a microtubule-binding protein. Potential interactions of *exu* protein with membrane and cytoskeletal structures need not be mutually exclusive. Any such interaction must be mediated by a domain of the *exu* protein, and one candidate domain is the segment with potential to form a coiled-coil structure, a motif implicated in protein-protein interactions (Cohen and Parry 1986). This idea may be tested by molecular genetic dissection of the *exu* gene and biochemical analysis of *exu* protein.

Materials and methods

Fly stocks

w¹¹¹⁸ flies were used as wild-type controls and as recipients for P-element transformation. Mutant flies are described in Schüpbach and Wieschaus (1986) or in Tearle and Nüsslein-Volhard (1987). Flies carrying multiple copies of the *bcd* gene have been described (Struhl et al. 1989).

DNA manipulations

The *exu* gene was cloned by P-element transposon tagging, with a P-element allele of *exu* provided by Trudi Schüpbach. DNA of this mutant was used to prepare a genomic phage library that

Figure 5. (See facing page for legend.)

Figure 6. *exu* protein and *bcd* mRNA are both localized to the apical regions of the nurse cells. (A,B) Egg chambers in which *exu* protein and *bcd* mRNA have been detected, respectively. St. Johnston et al. (1989) and Stephenson et al. (1988) previously described the localization of *bcd* mRNA to the apical regions of the nurse cells. In these whole-mount preparations, the optical sections are taken near the center of the nurse cell complexes. The positions of localization between nuclei and outer membranes are seen clearly at the edges of the egg chambers. In nurse cells positioned centrally in these photographs, the apical regions are out of the focal plane, and the localized protein and mRNA can be seen as diffuse patches. Note the presence of *bcd* mRNA at the anterior margin of the oocyte and the absence of any colocalized *exu* protein at that site.

was screened with a P-element probe by standard methods. Candidate clones were tested for chromosomal location, and a phage from the *exu* region was used for further analysis. Northern

blots of ovarian RNA were probed with labeled DNA restriction fragments from the phage, and a region transcribed during oogenesis was identified and later shown to be *exu*. cDNA clones of *exu* were obtained from ovarian and male cDNA libraries, provided by Laura Kalfayan and Steve Elledge, respectively.

DNA sequencing of genomic and cDNA clones of *exu* was performed by standard methods using Sequenase version 2.0 (U.S. Biochemical) according to the manufacturer's directions. Sequencing reactions were performed using both dGTP and dTTP to resolve ambiguities.

To characterize the *exu*^{SC} allele, DNA was prepared from homozygous mutant males and the *exu* gene was amplified by polymerase chain reaction (PCR) (Saiki et al. 1985). Amplification was done in two parts. Oligonucleotide primers to amplify the region from nucleotide 255 in Figure 2 and extending to nucleotide 1588 were 5'-ATGTGGACGAGTTGCAGCTGTCTA and 5'-GCAAGCCGCGTCTTCCTCCTTGG. Primers used to amplify the region from nucleotide 1403–2968 were 5'-CCCTACATGATCCTGGAGTCGTTG and 5'-AAGGAA-GAAAGTTCGTATGTGGTT. PCR reactions followed the suggestions of Innis and Gelfand (1990) and were performed in 100- μ l volumes (overlaid with 75 μ l of mineral oil) containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 50 μ M each dATP, dCTP, dGTP, and dTTP, 20 pmoles of each primer, 100 μ g/ml of gelatin, $\sim$ 1 μ g of *Drosophila* genomic DNA, and 2 units of *Taq* DNA polymerase (Amersham). All components except for the polymerase were assembled in 0.5-ml microcentrifuge tubes and boiled for 5 min. After some cooling at room temperature, the polymerase was added and the amplification was performed in a thermal cycler (MJ Research, Inc.). Twenty-five cycles of 20 sec at 94°C, 30 sec at 55°C, and 90 sec at 72°C were followed by a 5-min incubation at 72°C. PCR products were purified by phenol–chloroform extraction and ethanol precipitation and were isolated after agarose gel electrophoresis. The fragments were cloned into pGEM1 (Promega Biotech) and subjected to double-stranded dideoxy sequencing with Sequenase and a series of internal oligonucleotide primers. DNA sequence corresponding to positions 321–2589 in Figure 2 was determined. Despite the use of conditions designed to minimize replication errors during PCR, there were several changes. Consequently, to distinguish bona fide mutations from replication

Figure 5. Spatial patterns of *exu* protein distribution in ovaries. Distribution of *exu* protein was monitored in whole-mount preparations and in frozen sections. Whole-mount preparations were used for early stages. At late stages, the presence of the vitelline membrane renders the oocyte inaccessible to antibodies, and frozen sections were used to avoid this problem. Ovaries from *exu*^{SC}/*exu*^{SC} females were used as negative controls for these experiments. The *exu*^{SC} mutation truncates the protein early in the ORF (Fig. 2) and makes no detectable protein as determined by Western blot analysis (Fig. 3). (A) Distribution of the *exu* protein in early and middle stages of oogenesis was monitored by immunohistochemical analysis of whole-mount preparations. The stages shown extend from the germarium to stage 9. *exu* protein first appears in regions 2 and 3 of the germarium; however, no distinguishable localization can be observed at this stage. From stages 1 through 6, *exu* protein is localized to the presumptive oocyte. The signal remains intense through stages 7 and 8 despite expansion of the oocyte. A distinct patchy distribution of the *exu* protein begins to appear in the nurse cells at these later stages. At stages 9, 10A, and 10B, the patchy staining becomes more prominent (Fig. 6). During stages 8–10, in the oocyte there is a slight enrichment of *exu* protein at the cortex. (B,C) Distribution of *exu* protein in middle and late stages of oogenesis. Frozen sections were prepared as described in Materials and Methods, and *exu* protein was detected by immunofluorescence using rat primary antisera and fluorescein-coupled secondary antibody. (B) A series of progressively more advanced egg chambers. In the first egg chamber (stage 5 or 6), *exu* protein is present throughout the cytoplasm of all 16 cells but is concentrated in the oocyte. By stages 8–9 (second and third egg chambers), *exu* protein has accumulated to higher levels in the nurse cells, and beginning at this time there is a noticeable concentration of the protein in the apical regions. At stage 10B (last egg chamber), *exu* protein is present at high levels throughout the nurse cells, although higher concentrations are still found at the apical regions. In the oocyte, *exu* protein is present uniformly, with no apparent concentration at the anterior margin. (C) Stage 12 and 14 egg chambers. During stage 11, the contents of the nurse cells are transferred into the oocyte. Most of the *exu* protein is included in this movement, yielding the uniform oocyte distribution seen at stage 12. From stage 11 to 13 the amount of *exu* protein decreases gradually such that none can be detected by stage 14. Uneven illumination in the microscope is responsible for the apparent ventral concentration of *exu* protein seen in the stage 12 oocyte and less noticeably in other egg chambers.

errors, we reamplified the gene and resequenced all regions in which changes were detected.

Several constructs were made to express different parts of the *exu* gene in *Escherichia coli*. All employed the pET expression vectors of Rosenberg et al. (1987). p569 directs expression of the full-length *exu* protein containing no bacterial sequences and was constructed by PCR-mediated introduction of an *Nde*I site at the ATG codon of the cDNA and subsequent cloning of the modified cDNA into pET3a. p574 is a derivative of p569 in which the internal *Hind*III fragment from the *exu* gene has been removed and replaced with an *Nhe*I linker that introduces stop codons in all reading frames. p574, therefore, directs expression of the amino-terminal 191 amino acids of *exu*. In p559, a cDNA fragment including the region corresponding to amino acids 194–531 was cloned into pET3c. In p575, a cDNA fragment including the region corresponding to amino acids 375–502 was cloned into pET3b.

P-element transformation and mutant rescue

To test for rescue of the *exu* mutant phenotype by P-element transformation, a 7.4-kb *Eco*RI *exu* genomic DNA fragment (the region shown in Fig. 2) was inserted into the CaSpeR vector (Pirota 1988) to make pS200. DNA of pS200 was introduced into the germ line of *w*¹¹¹⁸ flies by P-element-mediated transformation (Rubin and Spradling 1982). Transformants were identified by eye color, and balanced stocks were established for independent lines. Ten different lines with insertions on the third chromosome were placed in the *exu*^{SC} background by the appropriate crosses. Virgin females of the genotype *exu*^{SC}/*exu*^{SC}; *P*[*S200-x*]/*TM2* (where *-x* refers to the individual lines) were crossed to wild-type males to test for rescue of maternal-effect lethality. All of these crosses yielded viable and fertile progeny, demonstrating that a single copy of the cloned *exu* gene can rescue the maternal-effect lethality of *exu*^{SC} mutants. To test for the ability to rescue the male sterility defect, males of the genotype *exu*^{SC}/*exu*^{SC}; *P*[*S200-x*]/*TM2* were crossed to *w*¹¹¹⁸ virgin females. For each of five lines examined, the males were only weakly fertile, although the results were not quantitated. Three of these transformant lines were also examined as homozygotes, with no noticeable improvement in male fertility. Thus, this construct does not appear to include all sequences necessary for normal expression of *exu* in males.

We also used the transformant stocks to test the effects of altered *exu* copy number on anterior patterning. Previous work with *bcd* has suggested that the localization machinery is in excess (Driever et al. 1989; Struhl et al. 1989). Given this, it is not surprising that increasing the *exu* gene copy number to six had no effect, as measured by two independent assays. There was no change in the position of the anterior zone of *hunchback* expression, and there was no noticeable change in the pattern of *bcd* mRNA localization at any stage of oogenesis (data not shown).

Antibodies, immunoblotting, immunohistochemistry, and immunofluorescence

exu proteins expressed in *E. coli* (see above) were purified partially and used for antisera production in rats.

For Western blot analysis, crude extracts of *Drosophila* samples were electrophoresed on SDS–polyacrylamide gels. The gels were soaked in transfer buffer (50 mM Tris base, 40 mM glycine, 0.04% SDS, 20% methanol) for 15–30 min, and the proteins were transferred electrophoretically to nitrocellulose in a semidry transfer apparatus (Bio-Rad) according to the manufacturer's instructions. Blots were blocked in binding buffer

[150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 3% BSA, 0.05% thimerosol] for 30 min. After several short washes in washing buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.05% Tween 20], the blots were incubated for 2–4 hr at room temperature with the primary antibody diluted 1 : 1000 in binding buffer. The blots were then rinsed several times in washing buffer and incubated with alkaline phosphatase (AP)-coupled secondary antibodies of the appropriate specificity. After several further washes, the AP reaction was developed in AP buffer containing 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and nitro blue tetrazolium.

Immunohistochemical analysis of whole-mount ovary preparations was performed as follows. Ovaries were dissected from young, well-fed adult females, and the ovarioles were teased apart in PBS. In some cases, the ovaries were incubated in 0.375 M KCl in PBS at 37°C for 30 min. This step significantly enhanced detection of *exu* protein in early stages of oogenesis but did not appear to affect its distribution. The ovaries were washed with PBS twice for 2 min before fixing in 4% formaldehyde in 0.1 M PIPES, 2 mM MgSO₄, for 20 min at room temperature. The fixative was removed by washing extensively with PBS containing 0.1% Tween 20 (PBT). After the final wash, anti-*exu* antisera were added at a dilution of 1 : 150 in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.05% thimerosol (TNBTT) and incubated overnight at 4°C. Ovaries were washed twice with TNBTT and incubated with 2% goat serum in TNBTT for 30 min. The ovaries were washed once again in TNBTT at least twice for 1 hr. Secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-rat IgG] was added at a dilution of 1 : 2000 and incubated for 2 hr at room temperature. After washing the ovaries at least three times with PBT for 15 min, the HRP reaction was developed with diaminobenzidine and hydrogen peroxide. The reaction was stopped by washing the ovaries several times with PBS. Stained ovaries were mounted in GMM.

Immunofluorescent detection of *exu* protein was performed on frozen sections. Ovaries were collected and prefixed as for immunohistochemistry, without the treatment in 0.375 M KCl. After washing in PBS, the prefixed ovaries were embedded in OCT compound (Tissue-Tek) and sectioned with a cryostat. Sections were mounted on polylysine-coated slides, allowed to air-dry for at least 20 min, and postfixed for 20 min in 2% formaldehyde in PBS. After washing once in 3× PBS and twice in PBS, the slides were incubated with *exu* antiserum diluted 1 : 150 in TNBTT for 4–12 hr. The slides were then washed twice in TNBTT, incubated for 30 min in TNBTT containing 2% goat serum, and washed several times in TNBTT. Fluorescein-coupled goat anti-rat secondary antibody (Jackson ImmunoResearch) was added at a dilution of 1 : 1000 in TNBTT and incubated for 1–2 hr. After three washes in PBT, the sections were mounted in a freshly prepared solution of 0.1 M sodium bicarbonate, 50% glycerol, containing a few crystals of *p*-phenylenediamine per 10 ml. Sections were viewed and photographed with a Zeiss Axiophot microscope.

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Protein encoded by the exuperantia gene is concentrated at sites of bicoid mRNA accumulation in Drosophila nurse cells but not in oocytes or embryos.

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