The EMBO Journal vol.10 no.13 pp.4259-4266, 1991

The temporal and spatial distribution pattern of maternal *exuperantia* protein: evidence for a role in establishment but not maintenance of *bicoid* mRNA localization

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Communicated by V.Pirrotta

The *exuperantia* (*exu*) gene of *Drosophila melanogaster* plays a fundamental role in the establishment of polarity of the oocyte and early embryo by ensuring the proper localization of the mRNA of the *bicoid* (*bcd*) gene to anterior regions of the oocyte. We have isolated and sequenced the *exu* gene, sequenced its female-specific transcript and a mutant allele of *exu* that affects primarily *exu*'s female germline function, and determined the temporal and spatial pattern of *exu* protein expression during oogenesis. The *exu* protein is basic, with at least one basic residue being identified as necessary for *exu* function in the female germline, and is present transiently during oogenesis. Our results suggest that *exu* is not required for the maintenance of *bcd* mRNA localization during late stages of oogenesis and early embryogenesis, but rather for the establishment of *bcd* mRNA localization in the developing oocyte. We propose that the *exu* protein may serve to modify a component that binds *bcd* mRNA or to modify the *bcd* message itself, or may perform a role in docking the *bcd* mRNA at its site of localization in the developing oocyte.

*Key words: bicoid/Drosophila/exuperantia/*oogenesis

Introduction

In *Drosophila*, positional cues encoded by maternally active genes and stored in the egg cytoplasm regulate zygotic genes that initiate the regional subdivision of the embryo. Thus, the initial specification of the fly embryonic body plan is a reflection of a spatially differentiated oocyte (reviewed by Ingham, 1988). Although several of the maternally encoded molecules that are responsible for determining embryonic cell fate have been identified, the molecular mechanisms involved in their segregation to distinct regions within the oocyte are largely unknown. This paper concerns the role of the *exuperantia* (*exu*) gene product in localizing *bicoid* (*bcd*) mRNA to the anterior pole of the *Drosophila* oocyte, a key step in the establishment of anterior pattern.

Embryos from females lacking functional copies of the *bcd* gene have no head and thorax and contain duplicated posterior structures (telsons) at their anterior ends. The *bcd* gene codes for a morphogen that is localized to the anterior pole of the egg cytoplasm (Frohnhöfer and Nüsslein-Volhard, 1986). *bcd* mRNA, like many gene products stored in the egg, is manufactured in nurse cells that lie anterior to the developing oocyte. The *bcd* message is shunted into the maturing egg as oogenesis proceeds and is retained at the anterior end, where it remains in mature oocytes and the early developing embryo (Frigerio *et al.*, 1986; Berleth *et al.*, 1988; St Johnston *et al.*, 1989). *bcd* protein first appears at the anterior end of the embryo as translation of the *bcd* mRNA begins shortly after fertilization. It then accumulates in a sharp concentration gradient with a maximum at the anterior tip and a minimum at about two-thirds the length of the embryo. The gradient is probably generated through an appropriate balance of translation of the anteriorly localized message, free diffusion of the protein through the egg cytoplasm, and uniform protein degradation (Driever and Nüsslein-Volhard, 1988a). *bcd* protein becomes localized in embryonic nuclei (in all cellular stages but mitosis) where it is thought to specify position along the anterior–posterior axis by regulating zygotic genes in a concentration dependent manner (Driever and Nüsslein-Volhard, 1988a,b, 1989; Driever *et al.*, 1989; Dalton *et al.*, 1989; Struhl *et al.*, 1989; Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). Thus, the genesis of anterior polarity of the embryo is a consequence of the localization of *bcd* mRNA to the anterior region of the oocyte: the establishment of the protein gradient of positional information, and the subsequent translation of this information into appropriate cell fates by zygotic genes, follow this essential step.

A 3′ untranslated region of ~625 nucleotides in the *bcd* mRNA is required for the proper localization of the message (Macdonald and Struhl, 1988; Macdonald, 1990), but the receptors that directly interact with this localization signal have not yet been determined. Mutant alleles of three maternal genes perturb the anterior localization of the *bcd* mRNA (Berleth *et al.*, 1988; Stephenson *et al.*, 1988; St Johnston *et al.*, 1990) and so identify potential candidates for this role. Of these three genes, *staufen*, *swallow* and *exu*, only the last is required for the initial localization of *bcd* mRNA as it enters the oocyte (Berleth *et al.*, 1988; St Johnston *et al.*, 1989). *bcd* mRNA is also subcellularly localized in nurse cells before it enters the oocyte and *exu* mutations perturb this nurse cell localization (St Johnston *et al.*, 1989; Stephenson and Pokrywka, 1991). Although the early effects of *exu* on *bcd* mRNA localization have been clearly demonstrated, most researchers have also assumed that *exu* is required for the maintained positioning of *bcd* mRNA in the oocyte and early embryo.

The segregation of RNAs to distinct intracellular regions has been observed in cultured vertebrate cells, where the distribution is related to cell polarity (Lawrence and Singer, 1986), and in *Xenopus* oocytes, where the localization of the Vg1 mRNA to the vegetal pole may play an important role in organizing the body plan of the frog embryo (Melton, 1987; Yisraeli *et al.*, 1989). Curiosity about the mechanisms

involved in mRNA localization, and the important role played by the *exu* gene in polarizing the *Drosophila* oocyte and embryo prompted us to begin a molecular analysis of the *exu* gene and extend its phenotypic and genetic analysis (Hazelrigg *et al.*, 1990). *exu* was first genetically identified, and its maternal effect on early embryonic development

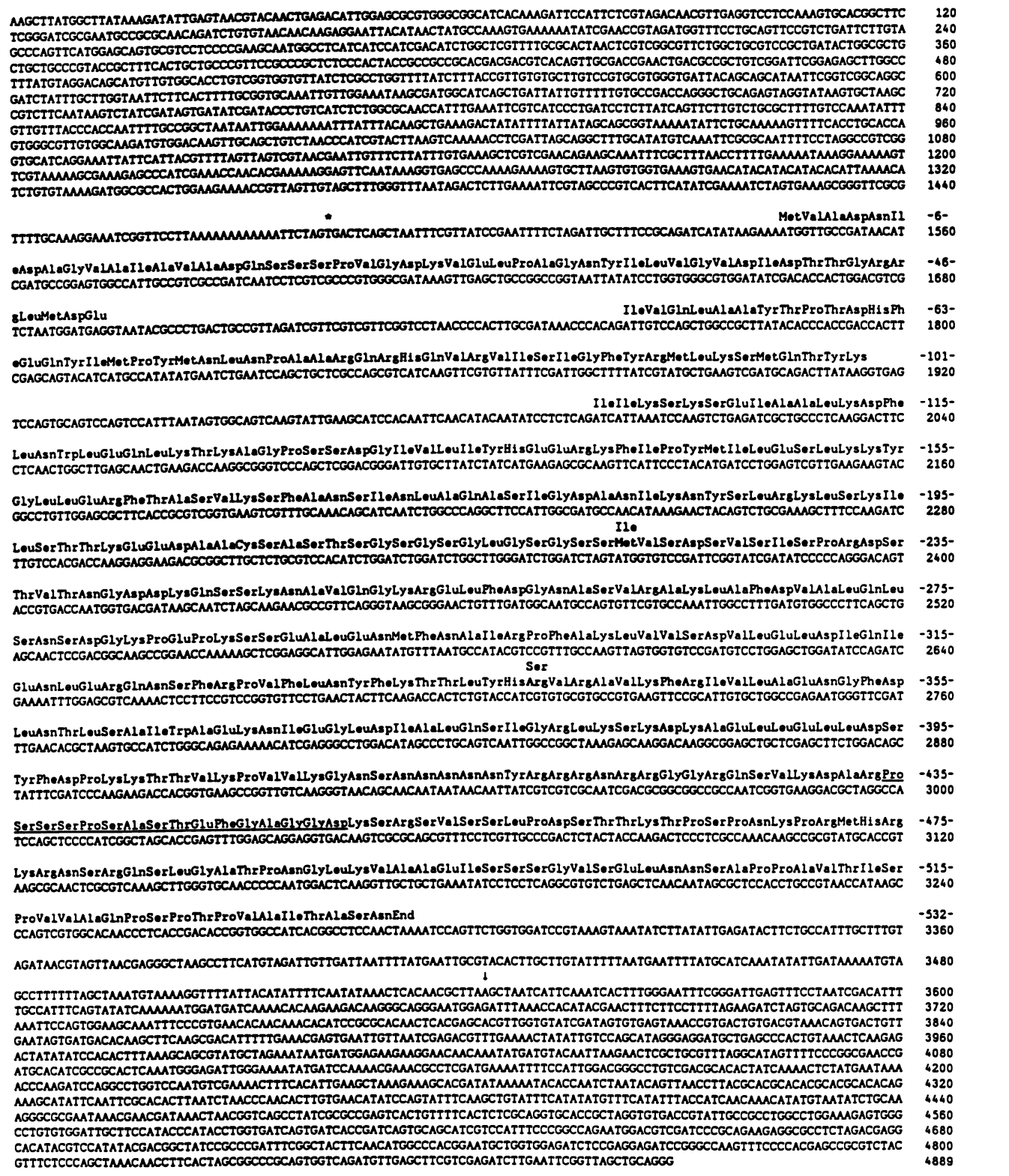

Fig. 1. DNA sequence of *exu* genomic region and wild-type protein sequence predicted from composite sequence of three overlapping female cDNAs, pC7, pC8 and pC10. The genomic sequence extends from nucleotide position 1 to 4889. * indicates the 5′ end (nt 1483) of pc10, the female cDNA with the most 5′ sequence. The small arrow shows the 3′ end of pc8 at nt 3544, the female cDNA that extends the furthest in this direction. The 5′ ends of pC7 and pC8 are at nt 1526 and 1908, respectively. The 3′ end of pC7 is at nt 3110, and the 3′ end of pC10 is at nt 3499. Two amino acids that differ in the mutant *PJ42* allele are in boldface type, with the mutant change shown above the line. The Met to Ile change at residue 223 is shared with the parental *cn bw* (*exu*$^{+}$) strain, whereas the residue 339 Arg to Ser change is specific to the *PJ42* allele. The underlined protein is the predicted PEST domain.

characterized, by Schüpbach and Wieschaus (1986a,b, 1989). Our analysis showed that *exu* also functions in spermatogenesis (Hazelrigg *et al.*, 1990). Six of seven *exu* alleles are male-sterile and produce defects initially in the early stages of spermiogenesis. The remaining mutation, exu^{PJ42}(*PJ42*), affects primarily the maternal function. The *exu* gene encodes overlapping sex-specific transcripts, which are germline-dependent. Here we report the nucleotide sequence and deduced amino acid sequence of the *exu* female transcript, the genomic sequence of wild-type and the *PJ42* mutation, and the distribution of the *exu* protein during oogenesis. Although *exu* is required for the initial anterior sequestering of *bcd* mRNA (Berleth *et al.*, 1988; St Johnston *et al.*, 1990), our results show that it is not involved in the maintenance of this localization in the mature oocyte.

Results

A 9.9 kb genomic fragment introduced into the genome by P-element transformation provides exu function

Previously isolated genomic clones (Hazelrigg *et al.*, 1990) did not contain the *exu* gene on one contiguous fragment. We therefore isolated a phage from the Maniatis wild-type *Drosophila* genomic library that contained an insert that overlapped the two previously identified phage inserts containing *exu*. We mapped the location of *exu* sequences to a 9.9 kb *Eco*RI fragment within this clone by Southern blots probed with an ovary cDNA, pC8 (see below). This *Eco*RI fragment also contains a divergently transcribed, minor 0.95 kb transcript which is not an *exu* message (Hazelrigg *et al.*, 1990, and unpublished data which show that the 0.95 kb transcript is not a germline-dependent transcript and is not affected by *exu* mutations which affect the 2.1 and 2.9 kb transcripts). The *Eco*RI fragment was inserted into the mini-*white* transformation vector, pCaSper (Pirrotta, 1988) to produce P[*white*,*exu*]. Germline transformants carrying P[*white*,*exu*] were selected as pigmented progeny from injected white-eyed parents (see Materials and methods). We obtained three separate transformant lines; one mapped to the second chromosome, and two were X-linked. The appropriate crosses between the X-linked transformant 13−2 with exu^{XL1}(*XL1*)-bearing flies produced flies homozygous for the *XL1* allele, and carrying the X-linked transformed copy of P[*white*,*exu*] (see Materials and methods for a description of the crosses and identification of appropriate genotypes). These flies were rescued for both the male and female sterility associated with the *XL1* allele, indicating that the entire *exu* gene is included in the 9.9 kb *Eco*RI fragment.

exu transcripts in the ovary

A 0.9 kb genomic *Hin*dIII restriction fragment from the *exu* region identified a cDNA, pC8, in an ovarian cDNA library (kindly provided by Dr Laura Kalfayan). pC8 subsequently identified two additional cDNAs, pC7 and pC10. These overlapping cDNAs hybridize to two *exu* transcripts (a major 2.1 kb mRNA and a rare 2.5 kb mRNA) present in wild-type females but not in exu^{VL57}(*VL57*), a partial deletion of the *exu* gene (Hazelrigg *et al.*, 1990, and unpublished data). The major 2.1 kb mRNA is a female germline-specific transcript, whereas the rare 2.5 kb species is expressed in somatic cells (Hazelrigg *et al.*, 1990). (The cDNAs also hybridize to the male-specific, germline 2.9 kb mRNA, which overlaps the female transcript.) The composite size of the isolated cDNAs (1.9 kb), their origin in ovarian tissue, and the germ cell location of the protein recognized by antibodies raised against protein expressed from pC7 and pC8 (see below) argues strongly that they represent the 2.1 kb germline mRNA. The direction of transcription of the cDNAs was determined using strand-specific RNA probes (see Materials and methods). The nucleotide sequence of these cDNAs and of 4.9 kb of genomic DNA spanning the *exu* region is given in Figure 1.

Comparison with the genomic sequence reveals the presence of two introns in the 5′ portion of the *exu* female primary transcript. Both pC7 and pC10 contain an AUG start codon at genomic position 1544, and pC10 has an in-frame UAG stop codon at upstream position 1514 (Figure 1). pC10 has a 146 nt 5′ sequence that does not align with genomic sequences so far determined, and does not hybridize with any other region of the ~120 kb of DNA surrounding *exu* that we have isolated. Since a 10 kb *Eco*RI fragment contains the entire *exu* gene as determined by the transformation results, this indicates that this 146 nt sequence does not come from the *exu* gene. In addition, a fragment of pC10 that includes this 146 nt sequence hybridizes to a 1.3 kb mRNA on northern blots, in addition to the *exu* mRNAs (data not shown), showing that the anomalous 146 nt sequence is an artefact of cDNA construction, due to fusion of two cDNAs. Both pC8 and pC10 contain a UAA stop codon at nt 3292. Although pC8 is incomplete at its 5′ end, it extends further 3′ than both pC7 and pC10, to nt position 3544, and contains a 254 nt 3′ untranslated region; a probable polyadenylation

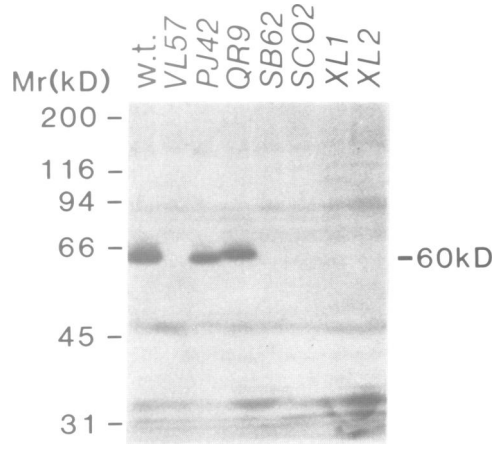

Fig. 2. Western blot of protein extracts from wild-type (w.t.) and *exu* mutant ovaries probed with anti-*exu* antibodies. The *exu* alleles are shown above each lane. Four *exu* alleles (*SB62*, *SCO2*, *XL1* and *XL2*) have been shown to have reduced levels of *exu* mRNA (Hazelrigg *et al.*, 1990, and unpublished). In addition, the chromosomes bearing these alleles contain extraneous recessive lethal mutations or mutations which reduce viability, and were made heterozygous with a deficiency, *Df*(2R)exu^1, to obtain flies for protein extracts; therefore the proteins in these mutant lanes came from genomes containing only one mutant gene copy of *exu*. Thus, it is conceivable that the failure to detect protein in these mutant lanes reflects a very low level of protein, and not a complete absence. However, approximately twice as much protein was intentionally loaded in these mutant lanes (*SB62*, *SCO2*, *XL1* and *XL2*), as confirmed by a duplicate Coomassie-stained gel, in order to compensate for the dosage effect. The *exu* protein migrates with an apparent molecular weight of 60 kDa. The background bands seen in all lanes are also detected with pre-immune serum, whereas the 60 kDa protein band is not.

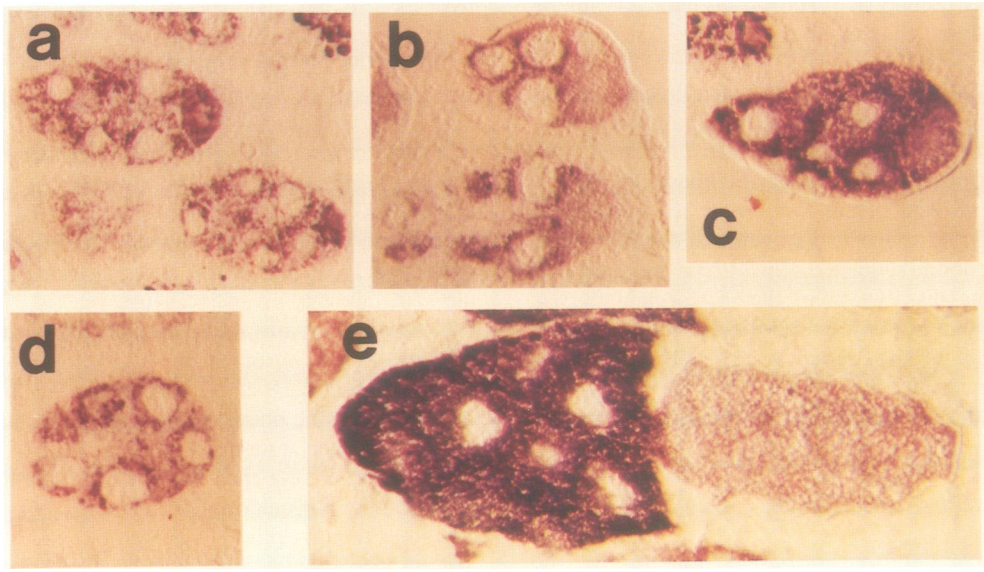

Fig. 3. *exu* protein distribution in wild-type egg chambers. Polyclonal anti-*exu* rat serum was hybridized to sectioned egg chambers of different stages, and bound antibody visualized with goat anti-rat secondary antibody coupled with alkaline phosphatase. In panels a, b, c and e the nurse cells lie to the left of the developing oocyte; panel d is a cross-section through nurse cells. See text for description.

hexamer (AAUAUA) is found 21 nucleotides from the end, at nt position 3523. Assuming a poly(A)$^+$ tail of 100–300 nucleotides, the composite length of the three cDNAs, 1.9 kb, shows that most of the female 2.1 kb transcript is contained within these cDNAs.

The inferred amino acid sequence of the exu protein

The three exons identified above comprise an open reading frame (ORF) of 1596 nt beginning with the AUG at genomic nt position 1544 and terminating at the ocher codon at nt 3292. The presence of an in-frame amber stop codon 30 nt upstream of the start codon in both genomic and pC10 sequences indicates that the entire protein coding portion of *exu* resides in this ORF. Translation of the *exu* ORF yields a protein of 532 amino acids (predicted molecular weight of 57 981 Daltons; Figure 1) The *exu* protein is basic, with a pI of 10.12. There are several basic, arginine-rich stretches (residues 339–346, 419–427 and 472–480).

Database searches for similarity to the entire deduced protein sequence or to fragments of this sequence yielded no significant homology to known proteins. These searches often yielded short stretches of matches to a Ser-Gly-rich region in *exu* (residues 211–222), but the significance of these matches is unclear. Searches of the *exu* protein sequence for common functional motifs (PC Gene prosite program) indicate that residues 435–450 identify a PEST sequence, hypothesized to confer susceptibility to rapid proteolysis (Rogers *et al.*, 1986). No significant similarities to previously identified RNA recognition motifs (Query *et al.*, 1989; Bandziulis *et al.*, 1989) were observed.

The amino acid sequence of exu^{PJ42} indicates that Arg339 is important for exu function in females

All known *exu* mutations except for the *exu*^{PJ42} (*PJ42*) allele cause male sterility in addition to the maternal effect phenotype that originally identified the gene (Hazelrigg *et al.*, 1990). The *PJ42* allele is male fertile at 25°C and shows only a partial reduction in male fertility at 18°C. Since *PJ42* has a much stronger mutant effect in females,

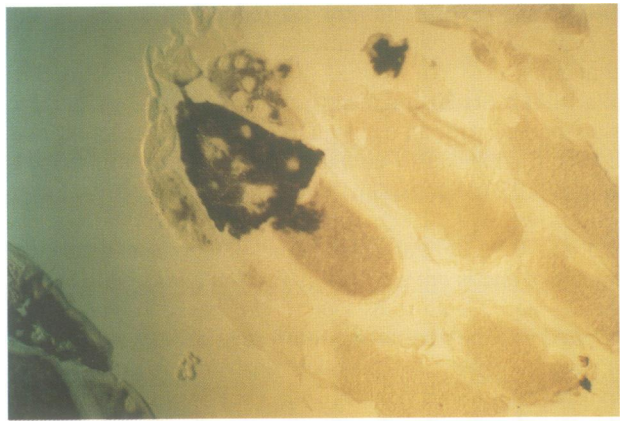

Fig. 4. Mature oocytes lack detectable *exu* protein. Immunohistochemistry was performed as in Figure 5 except that higher concentrations of primary and secondary antibodies were used (see Materials and methods). A section from a wild-type ovary showing early egg chambers, a stage 10 egg chamber and several late-stage oocytes. Although high levels of *exu* protein are seen in the stage 10 nurse cells and oocyte, only background staining (equivalent to control mutant *VL57* mature oocytes) is noted in more mature oocytes.

identification of the *PJ42* defect could reveal a region of the protein required for localization of the *bcd* message in oocytes. We isolated and sequenced *exu* genomic DNA from both *PJ42* homozygous flies and from the *cn bw* (*exu*$^+$) strain in which the *PJ42* allele was induced with ethyl methanesulfonate (Schüpbach and Wieschaus, 1986a). Compared with the *Canton S* (*CS*) wild-type strain, *PJ42* has a conservative methionine to isoleucine change at residue 223, and replaces arginine with serine at residue 339. The *cn bw* parental strain shares the residue 223 methionine to isoleucine change, but contains arginine at residue 339. Thus, this basic residue is important for *exu* function in the female germ line.

The exu protein in wild-type and mutant ovaries

Polyclonal antibodies were raised in rats against *exu* protein made in bacteria (see Materials and methods) and were used

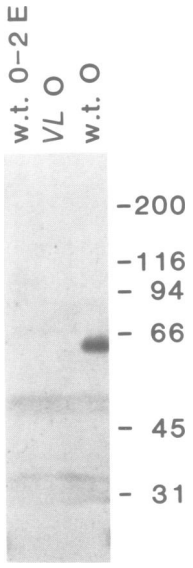

Fig. 5. Western blot of protein extracts from wild-type 0–2 h embryos probed with anti-*exu* antibodies. The right lane is protein from wild-type (w.t.) ovaries (O) to show the position of the 60 kDa *exu* protein. The middle lane is protein from ovaries of flies homozygous for the null allele *VL57*, as a control for the wild-type ovaries lane. The left lane is protein from wild-type 0–2 h embryos (E); no *exu* protein is detected. The faint protein bands are also detected with pre-immune serum and serve as internal controls to show that protein was loaded in the lanes lacking *exu* protein.

to probe Western blots of protein extracts of ovaries from wild-type (*CS*) and various *exu* mutants (Figure 2). The wild-type *exu* protein migrates with an apparent molecular weight of 60 kDa. As predicted from the absence of *exu* mRNA in exu^{VL57} (*VL57*) females (Hazelrigg *et al.*, 1990), ovaries from homozygous *VL57* females lack detectable *exu* protein. Other mutant alleles of *exu* in which no 60 kDa protein is detected are exu^{SB62}, exu^{SCO2}, exu^{XL1} and exu^{XL2}. Ovaries from *PJ42* homozygous females have an *exu* protein of normal mobility, but the protein has a slightly higher apparent molecular weight (~62 kDa) in mutant exu^{QR9} ovaries.

exu protein distribution during oogenesis

Because of the requirement for *exu* in the localization of *bcd* mRNA, we examined the temporal and spatial distribution of *exu* protein in developing wild-type egg chambers using anti-*exu* antibodies. The protein is readily observed in wild-type ovaries incubated with immune serum (Figure 3), but not in *VL57* ovaries treated with immune serum or wild-type ovaries treated with pre-immune serum (data not shown). Although very low levels of *exu* protein can be detected in nurse cells of early egg chambers (stages 5 and 6 of King, 1970), significant amounts are first observed in stages 7 and 8. At this stage, *exu* protein is present in nurse cells and in the oocyte at slightly higher, uniform concentrations (Figure 3a). At stages 7–9, exu protein is observed in a punctate, perinuclear distribution in nurse cell cytoplasm (Figure 3a–d). In stages 8–10, high concentrations of *exu* protein are seen at the anterior pole and cortical regions of the oocyte, and a lower concentration is observed homogeneously distributed throughout the oocyte (Figure 3b, c and e). By stage 10, *exu* protein is more concentrated and more homogeneously distributed within the nurse cells (Figure 3e). The higher apparent levels at the anterior ends of oocytes at these stages could possibly reflect the deposition of the protein by the nurse cells located at the anterior end of the oocyte, followed by diffusion within the oocyte. Analysis of *exu* RNA in developing egg chambers (D.Marcey and T.Hazelrigg, unpublished data) shows that the RNA is detected in the nurse cells but not in the oocyte, supporting the conclusion that *exu* protein is made in the nurse cells and transported to the oocyte. A somewhat surprising result is the virtual absence of *exu* protein in mature oocytes (stage 14) (Figure 4) and early embryos. This absence was confirmed in Western blots of protein extracts of early (0–2 h) wild-type embryos (Figure 5). No 60 kDa protein was detected in these embryos, suggesting that the wild-type oocytes lose all *exu* protein by the time of fertilization.

Discussion

Previous genetic, developmental and molecular studies have shown that the *exu* gene product plays an essential and early role in the establishment of anterior polarity of the *Drosophila* oocyte and embryo (Schüpbach and Wieschaus, 1986a; Frohnhöfer and Nüsslein-Volhard, 1987), and that this role is to localize the *bcd* mRNA anteriorly in the developing oocyte during oogenesis (Berleth et al., 1988; St Johnston *et al.*, 1990). We previously reported that *exu* also functions in spermatogenesis and encodes germline-dependent, overlapping sex-specific transcripts (Hazelrigg *et al*, 1990). In this study we report the sequence of three overlapping ovarian cDNAs encoding a basic protein. Our anti-*exu* antibodies recognize a protein with apparent molecular weight of 60 kDa on Western blots of ovarian protein extracts; five *exu* mutants have no detectable levels of this protein. The *exu* protein is present in nurse cells and in developing oocytes; in oocytes there are higher concentrations at the anterior poles. We found that *exu* protein is not detectable in mature oocytes and early embryos. Analysis of the male transcript and its product will be presented elsewhere.

exu encodes a basic protein that may be short lived

The three overlapping *exu* cDNAs have a composite length of 1.9 kb. Considering the addition of the poly(A)$^+$ tail, this is close to the estimated female mRNA size of 2.1 kb. These cDNAs contain the entire protein coding region. Searches for evolutionarily or functionally related proteins to *exu* yielded no significant homologies. We did not detect previously defined RNA recognition motifs (Bandziulis *et al.*, 1989; Query *et al.*, 1989). If *exu* protein does indeed bind *bcd* mRNA in its role in *bcd* mRNA localization, the recognition may be quite specific for the *bcd* mRNA, and so the lack of significant similarity to previously identified RNA-binding domains may not be relevant to the question of whether *exu* protein binds *bcd* mRNA. The basic nature of the *exu* protein signals potential interaction with negatively charged nucleic acids, and it remains possible that *exu* contains a previously unknown domain that recognizes RNA. Relevant to this speculation is the missense mutation, *PJ42*, in which a serine residue is substituted for the wild-type arginine at position 339 (in a region rich in positively charged amino acids), and which causes loss of *exu* function in females and consequent mislocalization of the *bcd* mRNA. Since *PJ42* mutant male flies are fertile, this mutation does

not simply destroy all *exu* activity. In a 29 amino acid region surrounding and including the Arg339 residue there are three repeats of an eight amino acid motif with conserved residues at positions 1, 5, 6 and 8 (B-X-X-X-F/Y-B-X-V, where B = basic and X = any amino acid; located at amino acids 320–327, 333–340 and 341–348). This sequence is similar to the RNP1 consensus at positions 1 and 5 (Query *et al.*, 1989; Bandziulis *et al.*, 1989). The significance of this repeat is unclear, but this basic region of the protein may be important for *exu*'s function in localizing *bcd* mRNA, since the *PJ42* mutation lies within this region. The existence of a PEST sequence (Rogers *et al.*, 1986) indicates that *exu* may be rapidly degraded, and may account for the disappearance of the *exu* antigen in late stage oocytes. The apparent higher levels of *exu* protein seen at the anterior poles of developing oocytes may reflect the entry into the oocyte at the anterior oocyte pole, followed by degradation of the protein within the oocyte. The higher levels of protein observed in the nurse cells in late stages could be due either to protection from degradation, or to rapid resynthesis of protein from the high levels of *exu* RNA present in nurse cells, but not the oocyte. If this spatial and temporal pattern of loss of *exu* protein is necessary for normal oogenesis, the PEST domain may have an important function in this process. This can be tested by *in vitro* alteration of the PEST sequence and reintroducing the gene by P-element transformation to test the phenotypic effects of the PEST mutations.

The exu protein in oogenesis and embryogenesis

A comparison of the time course of *exu* protein expression and of *bcd* mRNA production during oogenesis is informative with regard to the possible function of the *exu* product in localizing the *bcd* message. The *bcd* and *exu* transcripts must be regulated differently at the translational level: *bcd* mRNA is not translated until early embryogenesis, whereas *exu* mRNA is translated in nurse cells. The appearance of *exu* protein during early to mid-oogenesis coincides with *bcd* RNA expression (Figure 3; Berleth *et al.*, 1988; St Johnston *et al.*, 1989; Stephenson and Pokrywka, 1991). *exu* protein and *bcd* RNA are first seen in follicle stages 5–8. In vitellogenic stages, *exu* protein is concentrated perinuclearly in nurse cell cytoplasm, as is *bcd* RNA (Stephenson and Pokrywka, 1991). During stages 9 and 10, high levels of both molecules are observed in nurse cell cytoplasm, and significant amounts of each are seen entering the oocyte anteriorly (Figure 3; Berleth *et al.*, 1988; St Johnston *et al.*, 1989; Stephenson and Pokrywka, 1991).

In later stages, most of the *bcd* message has been shunted into the oocyte, where it remains as an anterior cap in both mature oocytes and early embryos, disappearing during the formation of the cellular blastoderm (Berleth *et al.*, 1988; St Johnson *et al.*, 1990). In contrast, *exu* protein has disappeared from mature oocytes (Figure 4) and is not detectable in young embryos (Figure 5). These results indicate that although *exu* is required for the proper localization of *bcd* mRNA, it must function early in oogenesis and is not required for the continued sequestration of this message during the minimum of eight hours between stage 11 egg chambers and the cellular blastoderm (King, 1970; Campos-Ortega and Hartenstein, 1985) . Thus, it is unlikely that *exu* protein serves as a molecular bridge between *bcd* mRNA and a structural component of the oocyte. Rather, *exu* protein may function solely in the nurse cells, or may have a transient role in the oocyte in the process of *bcd* mRNA localization.

The time course of *bcd* mRNA entry into the oocyte (beginning at stage 5–6) is not detectably altered in females homozygous or hemizygous for *exu* mutations [Berleth *et al.*, 1988; St Johnston *et al.*, 1990; our own observations (data not shown)], so that it is unlikely that *exu* protein functions solely to transport the *bcd* message into the oocyte. Rather, we propose that *exu* may activate a germline component that binds and sequesters the *bcd* message, may act as a transient docking protein that links the *bcd* message to an appropriate element within the oocyte, or may modify the *bcd* mRNA itself, rendering it 'bindable' by structural components in the egg (e.g. *swallow* or *staufen* protein or other cytoskeletal elements). One interesting hypothetical role for *exu* is suggested by consideration of the *cis*-element required for *bcd* mRNA localization.

Macdonald and Struhl (1988) have identified an ~625 nt sequence in the 3′ untranslated portion of the *bcd* mRNA which is required and sufficient for its proper anterior localization. Secondary structure predictions suggest that this region has the potential to form extensive secondary structure, with many stems and loops (Macdonald and Struhl, 1988). Similar potential secondary structures in this sequence exist in several *Drosophila* species, despite differences in primary sequence (MacDonald, 1990; Seger and Kaufman, 1990).

Although stereotypical secondary structure may be identified by such analysis, many possible folding patterns of the localization signal are possible (Macdonald, 1990). On the assumptions that: (i) the secondary structure of the 3′ localization signal is responsible for the proper binding and localization of the *bcd* message by *trans*-acting factors, and (ii) that only one or perhaps a small number of the potential secondary structures are recognizable by these *trans*-acting factors, it is clear that some mechanism must ensure stabilization of the properly folded 3′ localization signal. The stabilization of intermediate stem–loop structures during the course of folding could promote the production of the final folded form of an RNA. It is thus possible that *exu* protein plays such a folding role and may be a member of a class of proteins whose primary function is to aid in the folding of mRNAs into secondary structures.

Materials and methods

Isolation of cDNAs

cDNAs representing *exu* female transcripts were isolated from a λgt10 ovary cDNA library generously provided by Dr Laura Kalfayan. Phage were plated on bacterial strain Y1088. Library screens were performed according to protocols in Sambrook *et al.* (1989). A 0.9 kb *Hin*dIII fragment from pSW3 (Hazelrigg *et al.*, 1990) which hybridizes to the female-specific 2.1 kb mRNA, was used to isolate cDNAs on the first round of screening. A female cDNA isolated in this initial round, pC8, was used to isolate additional cDNAs in further screens. *exu* cDNAs were isolated from the library at a frequency of 1.3×10^{-5}. Inserts from the λgt10 clones were subcloned into the *Eco*RI site of the polylinker of pBluescript (KS–). pC8 was used on Northern blots of RNA from wild-type and exu^{VL57} mutant females to determine that it did indeed detect the female-specific 2.1 kb *exu* transcript. Five cDNAs (data not shown) were characterized further based on their cross-hybridization with pC8, as determined by Southern blotting.

Isolation of exu clones from cn bw and cn exu^{PJ42} bw

Southern blots of *cn bw* and *cn PJ42 bw* DNA probed with cDNA pC8 revealed the presence of a 6.6 kb *Eco*RI fragment detected by this cDNA.

Both DNAs were digested completely with *Eco*RI and cloned into phage λZAPII (Stratagene). The libraries were screened by standard protocols (Sambrook *et al.*, 1989) using labelled pC8 insert DNA. One positive *PJ42* clone was isolated from $\sim 6 \times 10^4$ plaques screened. Two positive clones from the *cn bw* library were isolated from 1.8×10^4 plaques screened. Phagemids from the genomic phage clones were obtained by *in vivo* excision according to Stratagene's instructions.

DNA sequencing

Double stranded plasmid DNA prepared by mini-preps or CsCl-banding was sequenced by dideoxy sequencing (Sanger *et al.*, 1977) using Sequenase (USB). Reaction products were resolved on 7% urea denaturing polyacrylamide gels. Compressions were resolved using ITP according to USB's specifications. The sequences of pC7, pC8 and pC10 were determined using a succession of synthetic oligonucleotides as primers. Sequence of both DNA strands was determined for all regions encompassing the composite female cDNA sequence. Genomic subclones pHHI and pSX-1 were constructed from plasmid pSW2 (Hazelrigg *et al.*, 1990) by digesting with the appropriate enzymes and ligating into pBluescript (KS−). pHHI, pSX-1 and pSW3 were sequenced with a set of synthetic oligonucleotides and an exonuclease III-generated deletion series was used to determine the partial sequence of pSW4 (Hazelrigg *et al.*, 1990). The region encompassed by the composite female cDNA in the *PJ42* genomic clone was also sequenced with the synthetic primer series. The *cn bw* genomic clone was sequenced in the two regions which were shown to predict amino acid differences between *PJ42* and the wild-type sequence.

The *exu* composite cDNA and genomic sequences were analyzed for alignments and open reading frames with PCGene software (Intelligenetics). PCGene programs were also used to analyze the predicted protein sequence for secondary structure and functional motifs. The predicted protein sequence was divided into 100 amino acid overlapping fragments which were used to search for homologies in the Genbank/EMBL and NBRF databases using the Genetics Computer Group Wisconsin program.

Direction of transcription

To determine the direction of transcription of cDNA pC8, single stranded RNA probes were used to detect the 2.1 kb female *exu* transcript on Northern blots. pC8 was cut with *Pst*I and a single stranded RNA probe was made from the T3 promoter in pBS (KS−). A similar, but full-length RNA probe was made from the T7 promoter after cleaving pC8 with *Sal*I. The RNA probe made from the T3 promoter detected the 2.1 kb *exu* female transcript, whereas the T7 promoter-directed RNA did not, indicating the direction of transcription of the female cDNAs.

Protein preparation and Western blots

Ovaries were dissected in *Drosophila* Ringers and transferred to an Eppendorf tube, the liquid was removed and the weight determined. Embryos were collected at appropriate stages, dechorionated in 50% bleach, rinsed in Ringers and transferred to Eppendorf tubes. To each mg of tissue was added 10 μl of ddH_2O containing a 1:1000 dilution of a protease inhibitor cocktail (100 mM benzamidine−HCl, 1 mg/ml pepstatin A, 100 mM phenyl methyl sulfonyl fluoride, 1 mg/ml phenanthroline). The tissue was homogenized by hand with a glass pestle. An equal volume of 2× Laemmli buffer (4% SDS, 20% glycerol, 200 mM DTT, 120 mM Tris, pH 6.8, 0.2% bromphenol blue) was added and the sample was boiled for 5 min, followed by passage through a 26 gauge needle to shear any DNA present. Proteins were electrophoresed in 10% polyacrylamide gels following standard protocols (Sambrook *et al.*, 1989) and electroblotted to nitrocellulose. The blots were blocked for at least 4 h in 2.5% BSA in TBST (10 mM Tris−HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blot was incubated in rat serum diluted 1:1000 in TBST followed by three 10 min TBST washes. Anti-rat alkaline phospatase-conjugated secondary antibody (Jackson Immuno Research) was used at a concentration of 1:10 000 for an incubation of 30 min. The blots were washed (3×10 min) in TBST and then stained for 10 min in NBT-BCIP (33 μl BCIP and 66 μl NBT/10 ml) in AP buffer (100 mM Tris−HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Construction of P[white,exu] and transformation

To isolate a DNA fragment containing the entire *exu* gene, the Maniatis Charon 4a genomic library was probed with a 2.1 kb *Hin*dIII restriction fragment from phage #9 (Hazelrigg *et al.*, 1990). Positives were rescreened with pSW4, a subclone of phage #10 (Hazelrigg *et al.*, 1990). Clones that were positive on both screens were labelled and used as probes on Southern blots of five plasmid subclones spanning phage #9 and #10. One phage, #9a, hybridized to contiguous restriction fragments from subclones pSW2, pSW3 and pSW4. This phage contained a 9.9 kb *Eco*RI fragment identified by hybridization with ovary cDNA pC8 as containing *exu* gene sequences. This *Eco*RI fragment was gel-isolated and ligated to *Eco*RI-digested pCaSper, and the construct P[CaS,*exu*] was identified in colony screens using pC8 insert as a probe.

Microinjection of DNA was done as described in Spradling and Rubin (1982). The injection host which yielded transformed progeny was $w^{1118};ry^{506}\Delta 2-3$, which contains a stable source of P-element transposase (Robertson *et al.*, 1988). For a description of balancer chromosomes and marker mutations, see Lindsley and Grell (1968). The GO flies were crossed to $w^{1118};CxD/TM3$ flies to replace the $\Delta 2-3$ chromosome by substitution with the *TM3* balancer chromosome. Chromosome linkage was determined by sex linkage and segregation analysis using *TM3* and *SM1* balancer chromosomes. A homozygous stock was established of one of the X-linked transformants, 13−2. Into this stock the *SM1* balancer was introduced; crosses between these flies and a strain with exu^{XL1}(*XL1*) on a chromosome marked with *cn bw* and *sp* ultimately produced progeny homozygous for the exu^{XL1} allele and carrying the X-linked 13−2 insertion. These males and virgin females were crossed to wild-type flies to assess whether the 13−2 insertion rescued the male and female sterility associated with exu^{XL1}.

The generation of antibodies against the exu protein

Two subclones of *exu* cDNAs were inserted into the T7 expression vector, pet 3b (Rosenberg *et al.*, 1987). A *Bam*HI fragment of pC8, extending from the polylinker of pBS(KS-) to the *Bam*HI site near the 3′ untranslated region of this cDNA, was cloned directly into the *Bam*HI site of pet 3b, and the *Eco*RI fragment containing the entire pC7 cDNA was isolated, ligated to *Bam*HI linkers (Pharmacia) and cloned into the pet 3b *Bam*HI site. Correct orientations were identified by the pattern of either *Bgl*II or *Nhe*I restriction fragments. These constructs were transformed into *E.coli* strain BL21(DE3) and induction of *exu* protein from the T7 RNA polymerase promoter in pet 3b was performed as described (Rosenberg *et al.*, 1987). Protein extracts from these induced cells were run on 7.5% polyacrylamide gels and the putative *exu* band removed. Electroelution of these proteins into Amicon microconcentrators was followed by concentration and precipitation with 7 vol of acetone. After drying, the protein pellets (from both constructs) were suspended in distilled H_2O and used to immunize and boost rats (Pocono Rabbit Farms). Polyclonal serum so obtained was used for subsequent analysis.

Immunohistochemistry

Ovaries were dissected in *Drosophila* Ringers and frozen in Tissue-Tek OCT compound prior to sectioning on a SLEE cryostat. Sections were mounted on microscope slides, fixed in fresh 4% paraformaldehyde for 1 h at room temperature and washed (3×20 min) with phosphate buffered saline (PBS). They were then blocked overnight at 4°C with PBS plus 0.1% Triton X-100 (PBST) containing 2% BSA (PBSBT). In most experiments, primary serum was diluted 1:2000 in PBST and incubated with sections overnight at 4°C. Washes with PBST (3×1 h) were followed by incubation with secondary antibody diluted 1:4000 in PBSBT (goat anti-rat IgG conjugated to alkaline phosphatase, Jackson Laboratories) overnight at 4°C. In attempts to detect *exu* protein in late-stage oocytes, higher concentrations of primary (diluted 1:100) and secondary (diluted 1:400) antibodies were used. After three 1 h washes with PBST, sections were incubated for 5 min with alkaline phosphatase buffer (APB, 100 mM Tris−HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and stained for ~15 min with BCIP and NBT in APB. Slides were rinsed for 10 min in distilled H_2O, air dried, mounted in Aquamount (Polysciences) and photographed.

Acknowledgements

Laura Kalfayan's generous gift of the ovary cDNA library is remembered with much admiration for her. We thank Stephen Brown and Esther Fujimoto at Utah and William Hage at Kenyon for excellent technical assistance. Hugh Robertson kindly provided us with the $\Delta 2-3$ fly strain. Ed Stephenson shared unpublished data and along with Mary Beckerle provided helpful discussions and advice. This work was supported by funding from the Howard Hughes Medical Institute.

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Received on June 28, 1991; revised on October 2, 1991