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Components Acting in Localization of *bicoid* mRNA Are Conserved Among *Drosophila* Species

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ABSTRACT

Substantial insights into basic strategies for embryonic body patterning have been obtained from genetic analyses of *Drosophila melanogaster*. This knowledge has been used in evolutionary comparisons to ask if genes and functions are conserved. To begin to ask how highly conserved are the mechanisms of mRNA localization, a process crucial to Drosophila body patterning, we have focused on the localization of *bcd* mRNA to the anterior pole of the embryo. Here we consider two components involved in that process: the *exuperantia* (*exu*) gene, required for an early step in localization; and the *cis*-acting signal that directs *bcd* mRNA localization. First, we use the cloned *D. melanogaster exu* gene to identify the *exu* genes from *Drosophila virilis* and *Drosophila pseudoobscura* and to isolate them for comparisons at the structural and functional levels. Surprisingly, *D. pseudoobscura* has two closely related *exu* genes, while *D. melanogaster* and *D. virilis* have only one each. When expressed in *D. melanogaster* ovaries, the *D. virilis exu* gene and one of the *D. pseudoobscura exu* genes can substitute for the endogenous *exu* gene in supporting localization of *bcd* mRNA, demonstrating that function is conserved. Second, we reevaluate the ability of the *D. pseudoobscura bcd* mRNA localization signal to function in *D. melanogaster*. In contrast to a previous report, we find that function is retained. Thus, among these Drosophila species there is substantial conservation of components acting in mRNA localization, and presumably the mechanisms underlying this process.

ANTERIOR body patterning of the Drosophila embryo relies in large part on a small set of maternally expressed genes, often called the anterior group genes (NÜSSLEIN-VOLHARD *et al.* 1987). One of these genes, *bicoid* (*bcd*), encodes the anterior body patterning morphogen (FROHNHÖFER and NÜSSLEIN-VOLHARD 1986; DRIEVER and NÜSSLEIN-VOLHARD 1988a,b). The bcd protein is deployed in an anterior concentration gradient in the embryo (DRIEVER and NÜSSLEIN-VOLHARD 1988a). Formation of the bcd protein gradient occurs as a consequence of the prelocalization of *bcd* mRNA to the anterior pole of the developing oocyte (FRIGERIO *et al.* 1986; BERLETH *et al.* 1988). The other anterior group genes, *exuperantia* (*exu*), *swallow* (*sww*) and *staufen* (*stau*) (GANS *et al.* 1975; SCHÜPBACH and WIESCHAUS 1986), are involved in this process (FROHNHÖFER and NÜSSLEIN-VOLHARD 1987; STEPHENSON *et al.* 1988; ST. JOHNSTON *et al.* 1989), and their action requires RNA regulatory elements within the 3′-untranslated region (3′-UTR) of the *bcd* mRNA that direct localization (MACDONALD and STRUHL 1988; MACDONALD *et al.* 1993). Once the bcd protein is deployed as a gradient in the embryo, it serves as a concentration-dependent transcriptional activator, regulating the expression of zygotic segmentation genes (DRIEVER and NÜSSLEIN-VOLHARD 1988b, 1989; DRIEVER *et al.* 1989; STRUHL *et al.* 1989).

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Previous studies have shown that the basic anterior body patterning strategies revealed by analysis of *Drosophila melanogaster* are substantially conserved among higher dipterans. The *bcd* gene is conserved, as is the use of mRNA prelocalization to deploy the bcd protein gradient; anteriorly localized *bcd* mRNA has been observed in species as distant as *Musca* (MACDONALD 1990; SEEGER and KAUFMAN 1990; SOMMER and TAUTZ 1991). Evolutionary comparisons of embryonic events that occur subsequent to deployment of the bcd gradient further demonstrate a high level of conservation; the situation is less clear for the mRNA localization processes that occur during oogenesis.

Several studies have addressed the embryonic events. A number of zygotic segmentation genes that act downstream from *bcd* are conserved, as are their patterns of expression in the early embryo, indicating that both proteins and transcriptional regulatory elements have been maintained during evolution (KASSIS *et al.* 1986; TREIER *et al.* 1989; SOMMER and TAUTZ 1991). In addition, conservation of regulatory elements among segmentation genes of higher dipterans probably extends to RNA elements that act post-transcriptionally, as suggested by the behavior of maternal *hunchback* mRNA. In *D. melanogaster*, sequences (called NREs) in the 3′-UTR of this mRNA mediate its degradation in a spatially restricted pattern in early embryos (WHARTON and STRUHL 1991; HÜLSKAMP *et al.* 1989); the maternal *hunchback* mRNA of *Musca* is also degraded in a similar pattern (SOMMER

and TAUTZ 1991), and so the NRE sequences may be conserved.

Uncertainty about the degree to which the *bcd* mRNA localization processes have been conserved owes to two reasons. First, evolutionary comparisons of the genes acting during oogenesis to localize *bcd* mRNA have not been described. Second, the *bcd* mRNA localization signal has been found to be inconsistently conserved when tested for function interspecifically in transgenic *D. melanogaster* flies; MACDONALD (1990) found that the localization signal from several species ranging as far as *Drosophila virilis* retained activity, while SEEGER and KAUFMANN (1990) found that the *Drosophila pseudoobscura* signal did not, despite substantial conservation of sequence and potential secondary structure of the localization signal.

The work reported here begins to address the uncertainties about the evolutionary conservation of the mechanisms underlying *bcd* mRNA localization. First, we consider *exu*, a gene required for this process in *D. melanogaster* ovaries (SCHÜPBACH and WIESCHAUS 1986; FROHNHÖFER and NÜSSLEIN-VOLHARD 1987; ST. JOHNSTON *et al.* 1989). We describe a phylogenetic comparison of both structure and ovarian function of the *exu* genes from *D. melanogaster*, *D. pseudoobscura* and *D. virilis*. Curiously, the *exu* gene is duplicated in *D. pseudoobscura*. Nevertheless, one *exu* gene from *D. pseudoobscura* and the single *exu* genes from the other species are functionally interchangable for *bcd* mRNA localization, arguing for conservation of basic mechanisms. Second, we describe a further analysis of the *D. pseudoobscura bcd* mRNA localization signal, and find that in our experiments it does retain function when tested in *D. melanogaster*. Thus, all available results argue that mechanisms of *bcd* mRNA localization are at least partially conserved, much as is the function of the bcd protein itself.

MATERIALS AND METHODS

DNA manipulations: Libraries of *D. pseudoobscura* (in λEMBL4, from S. SCHAEFFER, Pennsylvania State University) and *D. virilis* (in λEMBL3, from R. BLACKMAN, University of Illinois) genomic DNA were screened at low stringency (MCGINNIS *et al.* 1984) with a cDNA probe from the *D. melanogaster exu* gene [spanning nucleotides (nt) 360–2114 of MACDONALD *et al.* (1991)]. Several hybridizing phage from each library were isolated, and part or all of the DNA inserts from each were subcloned into plasmid vectors. To establish the position and orientation of the *exu* gene within the cloned DNAs, we identified restriction fragments that hybridized in Southern blot analyses to various parts of the *D. melanogaster exu* gene.

One subclone of the *D. virilis exu* gene and two different subclones of *D. pseudoobscura* DNA, each containing a different *exu* gene, were used for sequencing. A set of oligonucleotides, used previously for sequencing the *D. melanogaster exu* gene, were used as primers. Only two gave readable sequences. To obtain most of the remaining sequences, each subclone was fragmented with restriction enzymes for further subcloning prior to sequencing with primers corresponding to flanking vector DNA. Some regions could not be readily sequenced in this manner, and a few additional oligonucleotide primers were obtained. All sequencing was performed with Sequenase 2.0 (U.S. Biochemical Corp.) on denatured double-stranded templates, generally using both dGTP and dITP reactions. The sequences have been submitted to GenBank with the following accession numbers: *D. pseudoobscura exu1*, L22553; *D. pseudoobscura exu2*, L22554; and *D. virilis exu*, L22555.

Southern blots of genomic DNA were hybridized at reduced stringency (42% formamide, 6 × SSPE, 37°) and washed in 2 × SSPE at 45°. The probe was a *D. melanogaster exu* cDNA fragment extending from nt 360 to 1528 [coordinates from (MACDONALD *et al.* 1991)].

Constructs for *P* element transformation: The *D. virilis exu* gene was cloned as an approximately 7-kb *Sal*I fragment (with ends converted into *Asp*718 sites) into a slightly modified version of the CaSpeR transformation vector (PIROTTA 1988) to make pVE. Neither of the two *D. pseudoobscura exu* genes was isolated with extensive 5′-flanking sequences. Consequently, each was fused to a 5′-region of the *D. melanogaster exu* gene previously shown to be sufficient to direct expression of an *exu* transgene (MACDONALD *et al.* 1991). Fusion was accomplished by joining 5′ *D. melanogaster exu* gene sequences to *D. pseudoobscura exu* gene sequences at a common *Cla*I site located at codons 6–7 of all genes. This site appears naturally in the *D. melanogaster* gene, but had to be introduced into the *D. pseudoobscura* genes. This was accomplished in short subclones by PCR using 5′-primers AGCATCGATGATTGTGTTACCATTGCCGCG (for *D. pseudoobscura exu1*; the *Cla*I site is underlined) and GTTATCGATGATAGTGCTTCCGCTACCGCCT (for *D. pseudoobscura exu2*) and 3′-primers corresponding to vector sequences. The hybrid genes were assembled in a slightly modified version of CaSpeR to make p594 (*D. pseudoobscura exu1*) and p595 (*D. pseudoobscura exu2*).

Two *bcd* hybrid genes were constructed. Both are derived from p2001, which consists of the wild-type *D. melanogaster bcd* gene in CaSpeR and contains all sequences necessary for normal expression in flies. In one hybrid gene, p2163, the 3′-UTR of the *D. melanogaster bcd* gene was replaced with the 3′-UTR of the *D. pseudoobscura bcd* gene (SEEGER and KAUFMAN 1990). To make p2163, an *Eco*RV-*Xba*I fragment [nt 4098–4883 of BERLETH *et al.* (1988)] was removed from p2001 and replaced with a similar region from the *D. pseudoobscura bcd* gene, an *Eco*RV-*Nru*I fragment [nt 2527–3422 of SEEGER and KAUFMAN (1990)] in which the *Nru*I site had been converted into an *Xba*I site by fusion with plasmid polylinker sequences. The *D. pseudoobscura* fragment includes the polyadenylation site, which in the *D. melanogaster* gene lies downstream from the region removed in p2163. Thus, the hybrid *bcd* gene of p2163 has tandem polyadenylation sites. In the second hybrid *bcd* gene, the *D. melanogaster* 5′ flanking region was fused to the entire *D. pseudoobscura bcd* transcription unit, including the coding region and both 5′ and 3′-untranslated regions. Fusion of the two genes was accomplished by first inserting a *Pst*I linker at a *Cla*I site positioned 13 nucleotides 5′ to the 5′-most predicted transcription start site of the *D. pseudoobscura bcd* gene. DNA 3′ to this *Pst*I site was then joined to a 5′ *D. melanogaster bcd* gene fragment extending to a *Pst*I site at the 3′-most transcription start site (SEEGER 1989).

A *nanos*/*D. pseudoobscura bcd* hybrid gene, in which the promoter and coding region are from the *nanos* gene (WANG and LEHMANN 1991), and the 3′-untranslated and flanking regions are from the *D. pseudoobscura bcd* gene, was assembled in CaSpeR to make p2173. The *D. pseudoobscura bcd* gene fragment was the same as that used in p2163, except that the

5′- and 3′-ends were converted into *Bgl*II and *Eco*RI sites, respectively. The *nanos* gene fragment extends from a *Sac*I site, located about 1.3 kb 5′ to the transcription start site, to a *Hpa*I site (converted to a *Bam*HI site by addition of a linker), located 93 bp downstream from the stop codon.

Drosophila manipulations: *D. melanogaster* fly strains used, including w^{1118} and exu^4, are described in LINDSLEY and ZIMM (1992). *P* element transformation was by standard procedures (RUBIN and SPRADLING 1982; SPRADLING and RUBIN 1982) using w^{1118} embryos as recipients for injection. Chromosomes bearing transgenes were identified by segregation tests. Lines with *exu* transgenes on the third chromosome were crossed into the exu^4 background (*exu* is on the second chromosome). Females homozygous for the exu^4 mutation and carrying one copy of a transgene were mated to exu^+ males (*exu* mutant males are sterile; HAZELRIGG *et al.* 1990) and allowed to lay eggs in a vial over several days. Failure of all of a large number (>100) of embryos to develop into adults was scored as no rescue of the exu^4 phenotype. When rescue did occur, most embryos developed into viable adults. Rescue of the male sterility of *exu* mutants was also tested by crossing males homozygous for exu^4 and carrying one copy of a transgene with w^{1118} virgin females. Rescue in this case was scored as the appearance of any progeny. Of all transgenic lines tested, only one of the two carrying the *D. pseudoobscura exu1* gene rescued the male defect. Previously we found that transgenes of the *D. melanogaster exu* gene provided only partial male *exu* function (MACDONALD *et al.* 1991), and it may be that extensive flanking sequences are required for normal expression in males.

RNA analysis: RNA preparations and RNase protection assays were performed as described (MACDONALD *et al.* 1986). RNA probes for the different *exu* genes were as follows.

D. melanogaster: pRO3, an *Eco*RV [nt 910–1634 of MACDONALD *et al.* (1991)] fragment of *D. melanogaster* genomic *exu* DNA in the vector pGem1, was cut with *Ava*II. Transcription of this DNA with T7 RNA polymerase produces a probe complementary to nt 1326–1634 of the *D. melanogaster exu* mRNA.

D. virilis: pV28, a *Hpa*I-*Stu*I (nt 454–973) subclone of *D. virilis* genomic *exu* DNA in the vector pGEM2, was cut with *Sty*I. Transcription of this DNA with T7 RNA polymerase produces a probe complementary to nt 778–973 of the *D. virilis exu* mRNA (nucleotide coordinates for the *D. virilis* and *D. pseudoobscura exu* genes are as in the sequences submitted to GenBank).

D. pseudoobscura exu1: pM79, an *Apo*I-*Apo*I (nt ~−50 to 324) subclone of *D. pseudoobscura* genomic *exu1* DNA in the vector pGEM2, was cut with *Ava*II. Transcription of this DNA with T7 RNA polymerase produces a probe complementary to nt 229–324 of the *D. pseudoobscura exu*1 mRNA.

D. pseudoobscura exu2: pM914, an *Apo*I-*Apo*I (nt 320–546) subclone of *D. pseudoobscura* genomic *exu2* DNA in the vector pSP73, was cut with *Eco*RV. Transcription of this DNA with T7 RNA polymerase produces a probe complementary to nt 320–377 and to nt 428–546 of the *D. pseudoobscura exu2* mRNA.

In situ hybridization to whole mount ovaries and embryos (TAUTZ and PFEIFLE 1989) was performed as described (KIM-HA *et al.* 1993). Probes were labeled with digoxigenin. The probe for *D. melanogaster bcd* mRNA was a cDNA clone lacking the 3′-UTR. The probe for *D. pseudoobscura bcd* mRNA was an *Eco*RV-*Nru*I fragment [nt 2527–3422 of SEEGER and KAUFMAN (1990)] from the 3′-UTR. The probe for *nanos* mRNA was a 0.8-kb *Sty*I fragment of a *nanos* cDNA, including most of the coding region.

Computer analysis: Sequence comparisons were performed using the University of Wisconsin GCG programs, Version 7.1 (Genetics Computer Group 1991). Compare and Dotplot programs were run using the default parameters to generate the comparisons shown in Figure 2.

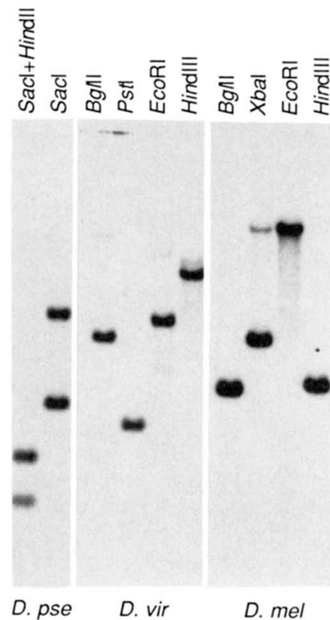

FIGURE 1.—Genomic Southern blots probed for *exu* genes. Genomic DNAs were digested with restriction enzymes as indicated and used to prepare a Southern blot. The blot was probed at reduced stringency with a fragment of the *D. melanogaster exu* cDNA corresponding to the most conserved part of the gene. Under the conditions used, hybridization of the probe to the different genes occurs at similar efficiencies (all blots represent equivalent exposures when adjusted for the amount of DNA loaded; also, compare the different bands in the *D. pseudoobscura* DNA), and so all closely related *exu* genes should be readily detectable. Notably, both genes recovered from the *D. pseudoobscura* library are found in the *D. pseudoobscura* DNA. For example, the DNA sequences of the two genes predict that digestion with *Sac*I and *Hind*III will produce fragments of 1.2 kb (for *D. pseudoobscura exu1*) and 0.7 kb (for *D. pseudoobscura exu2*)–both appear. Similarly, digestion with *Sac*I is expected to produce single fragments of different sizes for the two genes, as is found. The *D. virilis* and *D. melanogaster* genomic DNAs were digested with several different enzymes to increase the probability of detecting multiple genes. In both *D. virilis* and *D. melanogaster* DNAs a single set of strongly hybridizing bands are detected, indicating the presence of single *exu* genes in each species (the weak upper band in the *Xba*I digestion of *D. melanogaster* DNA appears to result from incomplete digestion). In the *D. virilis* DNA a series of faint bands are also detected; they may correspond to sequences substantially less well conserved than even the divergent *D. pseudoobscura exu2* gene, which hybridizes strongly under these conditions.

RESULTS

The *exu* gene was initially identified in *D. melanogaster* (SCHÜPBACH and WIESCHAUS 1986), and was subsequently cloned from that species (HAZELRIGG *et al.* 1990; MACDONALD *et al.* 1991). For a phylogenetic comparison of *exu* genes, we chose *D. virilis* and *D. pseudoobscura*. These species have diverged, both from *D. melanogaster* and from each other, enough to allow

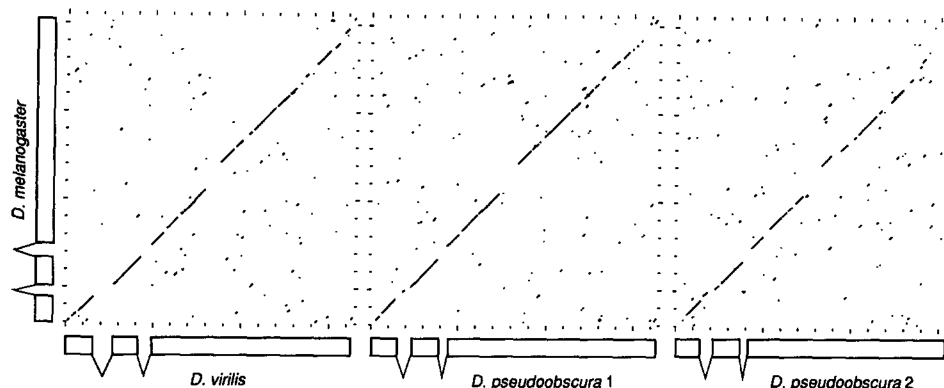

FIGURE 2.—Dot matrix comparisons of *exu* DNA sequences. Genomic DNA sequences of the *D. virilis* and *D. pseudoobscura* *exu* genes (submitted to GenBank) were compared to the equivalent region of the *D. melanogaster exu* gene [nt 796–2546 of MACDONALD *et al.* (1991)] and the results displayed in dot matrices. For each panel the scale increments are 100 nt. The positions of introns within each gene (identified from cDNAs for the *D. melanogaster* gene, and predicted for the other genes) are indicated. The boxes represent predicted protein coding regions; for each gene the first box begins with the ATG translational start codon, and the last box ends with the termination codon. Note that additional flanking sequences of the *D. melanogaster* gene are transcribed, and include at least one 5′ intron, and a 3′ male-specific intron (MACDONALD *et al.* 1991; MARCEY *et al.* 1991). Sequences of the flanking regions of the other *exu* genes have not been determined.

considerable change in gene sequences (BEVERLEY and WILSON 1984).

One *D. virilis* and two *D. pseudoobscura exuperantia* genes: Bacteriophage libraries of *D. virilis* and *D. pseudoobscura* genomic DNAs were screened by low stringency hybridization with a *D. melanogaster exu* gene probe. We recovered several copies of the same *D. virilis exu* gene and, surprisingly, two different *exu* genes from the *D. pseudoobscura* library. To confirm that both genes were from *D. pseudoobscura,* and to ask if multiple *exu* genes are also present in the other species, Southern blots of *D. melanogaster, D. virilis* and *D. pseudoobscura* genomic DNA restriction fragments were probed at reduced stringency with a DNA fragment corresponding to the most highly conserved region of the *exu* gene (Figure 1). Although it is difficult to rule out the existence of *exu* genes with highly diverged sequences, it appears that *D. melanogaster* and *D. virilis* have a single *exu* gene, while *D. pseudoobscura* has two *exu* genes. Thus, we have cloned all readily detectable *exu* genes of the three species.

A segment of each gene, including all of the predicted protein coding region, was sequenced. In Figure 2 each sequence is compared by dot matrix to the *D. melanogaster exu* gene sequence (no DNA sequences are presented here, but all have been submitted to GenBank). Clearly, there are regions of high conservation, and regions of little similarity. The latter include the introns (predicted by comparison of the open reading frames with the *D. melanogaster* gene) as well as part of the protein coding region. Each suggested exon is flanked, where appropriate, by typical splice donor and acceptor sites (data not shown). All of the *exu* genes display a stereotypical intron/exon organization within their coding regions, with only modest variation in intron size.

Comparison of predicted exu protein sequences indicates that all proteins are 55–70% identical (Table 1). Notably, the *D. virilis* gene is more closely related to the *D. melanogaster* gene than are either of the *D. pseudoobscura* genes. Typically, the situation is reversed [*e.g.*, COLOT *et al.* (1988) and JONES *et al.* (1991)], and *D. virilis* is thought to have diverged prior to the split between *D. melanogaster* and *D. pseudoobscura* (BEVERLEY and WILSON 1984). For all genes, amino acid sequences are not uniformly conserved along the lengths of the proteins. The sequences are presented in Figure 3A, with the patterns of conservation summarized schematically in Figure 3B. There are two highly conserved domains in which greater than 90% of the residues are identical for all but the *D. pseudoobscura* exu2 protein (80–85% identity). The larger (about 96 amino acids) of the two domains contains no known protein motifs, and is not strikingly similar to any sequence in the GenBank, PIR and Swissprot databases. The other highly conserved region includes a series of three repeats with partial homology to an RNA binding motif, as noted by (MARCEY *et al.* 1991). Residues which comprise this homology are fully conserved, except in the *D. pseudoobscura* exu2 protein, where two of the repeats are altered.

Three regions have intermediate sequence conservation (about 55–75% identity; again, *D. pseudoobscura* exu2 is substantially less conserved), with the two *D. pseudoobscura* proteins exhibiting higher conservation relative to one another. One region of intermediate conservation contains a PEST domain (MARCEY *et al.* 1991), thought to confer protein instability (ROGERS *et al.* 1986) and rich in proline, glutamate, serine and threonine residues and flanked by basic residues. Notably, this region has been largely deleted from the *D. pseudoobscura* exu2 protein, but a region rich in proline, glutamate and serine is created by an insertion within an upstream domain of intermediate conserva-

TABLE 1

Comparisons of exu protein sequences

exu gene	Amino acid identities (%)[a]		
	D. virilis	*D. pseudoobscura exu1*	*D. pseudoobscura exu2*
D. melanogaster	69	63	55
D. virilis		65	56
D. pseudoobscura exu1			70

[a] Values represent percent amino acid identities in the pairwise comparisons.

tion (this region lacks basic residues at both ends and thus lacks strong PEST character). Upon further scrutiny of these regions, it appears that both of the regions rich in proline, glutamate, or remnants thereof, can be found in individual proteins. In the *D. virilis* and *D. pseudoobscura* exu1 proteins the appearance of a basic amino acid interrupts the original downstream domain (pro 439 of the *D. melanogaster* protein is changed to arg), while the deletion of basic residues (Lys-281 and/or Lys-285 of the *D. melanogaster* protein) lengthens the upstream domain.

Among the several regions with low sequence conservation, the central one displays a bias in amino acid content; in the *D. melanogaster* protein, amino acids 207–246 are serine-rich, while the equivalent regions of the other proteins have a high proportion of acidic residues.

Differential expression of the *D. pseudoobscura exuperantia* genes: Expression of the single *D. melanogaster exu* gene is largely restricted to the gonads, where differential RNA processing produces female- and male-specific mRNAs (HAZELRIGG *et al.* 1990; MACDONALD *et al.* 1991; MARCEY *et al.* 1991). By analogy, the two *D. pseudoobscura exu* genes could represent sex-specific forms. If one *D. pseudoobscura exu* gene is active only in males, the encoded protein would not normally act in the ovary-specific localization of *bcd* mRNA, and might not be expected to provide *exu* function if expressed in *D. melanogaster* ovaries. RNA was purified from adult ovaries and testes and probed for both *exu* transcripts by RNase protection assays (Figure 4). The *exu1* gene is expressed in both testes and ovary (lanes 1 and 2). Expression of the *exu2* gene is strong in testes (lane 3), and weak or absent in ovaries (lane 4).

Conservation of *exuperantia* gene function in the ovary: To determine if the structural conservation within various *exu* genes is accompanied by conservation of ovarian function, we asked which genes could provide *exu* activity in *D. melanogaster* females. The *D. virilis exu* gene and each of the *D. pseudoobscura exu* genes were introduced by *P* element transformation into *D. melanogaster* flies. For the *D. virilis* transgene, a large restriction fragment that contains the gene and is likely to direct normal patterns of ovarian expression was used. Unfortunately, neither *D. pseudoobscura exu* gene was isolated with substantial 5′-flanking sequences and so the available DNA fragments are unlikely to provide normal expression. Consequently, we constructed hybrid transgenes that are expected to be expressed in ovaries (see MATERIALS AND METHODS). Briefly, *D. melanogaster exu* 5′-flanking sequences, including the coding region for the first 7 amino acids, were joined to each *D. pseudoobscura* gene. Fusion was at a *Cla*I restriction site at codons 6–7 of each gene (the *Cla*I site occurs naturally in the *D. melanogaster* gene, and was introduced by PCR into the *D. pseudoobscura* genes); hence the hybrid genes consist almost exclusively of *D. pseudoobscura* coding regions.

To confirm that the transgenes are expressed in *D. melanogaster* ovaries, RNase protection assays were performed. Ovarian RNA was prepared from the transgenic stocks and analyzed using probes specific for each of the genes. All three transgenes are expressed at readily detectable levels in ovaries (Figure 4; lanes 6, 10 and 11).

Females lacking *exu* activity are maternal-effect lethal; their progeny all die as embryos. To determine if an interspecific *exu* transgene can rescue this lethality, *D. melanogaster* females lacking the endogenous *exu* gene and carrying one of the transgenes were tested for the viability of their progeny. *D. virilis exu* and *D. pseudoobscura exu1* will substitute for *D. melanogaster exu* in this assay, while *D. pseudoobscura exu2* will not (Table 2). The sequence comparisons of the various *exu* genes suggested that the *D. pseudoobscura* genes might be specialized in their functions; if so, the *D. pseudoobscura exu1* gene that provides ovarian function might be unable to perform in testes. We therefore tested the ability of the transgenes to rescue the male sterility defect of *exu* mutants (HAZELRIGG *et al.* 1990). Of all lines tested, only one carrying the *D. pseudoobscura exu1* gene had rescuing activity. The significance of negative rescue results with the other transgenes is uncertain, since even our *D. melanogaster exu* transgenes provided only poor rescue (Table 2) (MACDONALD *et al.* 1991). Nevertheless, it is clear that the *D. pseudoobscura exu1* gene by itself can at least partially provide both of the known *exu* functions, in female and male gonads. Thus, a specialization of male and female functions would appear not to be sufficient to account for the observed patterns of sequence divergence.

***bcd* mRNA localization signals are also conserved among Drosophila species:** When these experiments were initiated, we anticipated that cloning the *D.*

A

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         •         •         •         •         •         •
MVADNIDAGVAIAVADQS..SSPVGDKVELPAGNYILVGVDIDTTGRRLMDEIVQLAAYTPT   mel
-V-VS--SVID-SA----TAA-G-AI-D---N-D----A----------I------------   vir
---EISKDC-T--A---C.....-DM-E-------------V-------I-----------K   pse1
--SAISEDSASATASG-C.....EVV-E----------A-E--------I-------G--SK   pse2

         •         •         •   100•    •         •
DHFEQYIMPYMNLNPAARQRHQVRVISIGFYRMLKSMQTYKIIKSKSEIAALKDFLNWLEQL   mel
-----------------------------F-----------------V-------D--D--   vir
-N-Q-----------------I-------------------------V---M-------M-   pse1
GN-Q----------Q-------I-------------------------V---M-------T-   pse2

       •         •         •         •         •         •
KTKAGPSSDGIVLIYHEERKFIPYMILESLKKYGLLERFTASVKSFANSINLAKASIGDANI   mel
-S-NPNT.-G-----------------Q--V---M-D---KT----V--F----S----T..   vir
VA-Q.--T----ML-----------V--A------I---NRT----V-TF-M----L----L   pse1
LA-Q.-NKE---ML--DD----------A----S-ID--NR---A---TCPM--TFL-KHG-   pse2

     •   200•      •         •         •         •
KNYSLRKLSKILSTTKEEDAACSASTSGSGSGLGSGSSMVSDSVSISPRDSTVTNGDDKQSS   mel
-H----N------KA--DNARVDNDNEADSNSSSADKHV.......................   vir
--CG-----LL-AKSNDESANKENEPENVNRNGSSNDKCH......................   pse1
--CG-----ML-AKSKDGNSTKEDEHENPEGNSSITDNSGH.....................   pse2

   •         •         •         •                  •      300
KNAVQGKRELFDGNASVRAKLAFDVALQLSNSDGKPEPK.........SSEALENMFNAIRP   mel
--GL-KE-DE---S-------T-N-------L-S.T--.........E-----S-L---LK-   vir
--GMNQEHNF-E-S-N----MVYE-----T---RTS--.........E---E-V-L---VK-   pse1
--QK--A...-E-S-N----MVYEM----IE-ESTES-ESFESPESSE----EVKLL--V--   pse2

•         •                   •                    •         •
FAKLVVSDVLELDIQIENLERQNSFRPVFLNYFKTTLYHRVRAVKFRIVLAENGFDLNTLSA   mel
-TEAISG--K---T-NVH---------------------------------------TTLND   vir
-----S--IM--QT-N--MG-------------R----------------------T-DS-K-   pse1
-SQ-LS-TI---KD-NHS-G-------------R---NY------Y--G---H--T-KS-K-   pse2

         •         •         •   400•              •         •
IWAEKNIEGLDIALQSIGRLKSKDKAELLELLDSYFDPKKTTVKPVVKGNSNNNNNYRRRNR   mel
--T--R-----A---G-AS--AE--N--V-----F---A-V-I--II-PST.....I----K   vir
--T--RK---EL--TN-DT--TEE-T--V-----F---S-A-I--SF-P-G-GP...---I-   pse1
--SD-RKP--ELV-TA-DS--TEET----D-----Y--S---I--RC-RSG-GT...-----   pse2

      •                                  •         •         •
RGGRQSVKDARPSSSPSASTEFGAGGDKSRSVS...SLPDSTTKTPSPNK..PRMHRKRNSR   mel
-NT.PAH-PGVG-G-R---D---------Q---S...V----------I-NGG-PQ------   vir
AN-AA-S-NGAM.--R-T-T---------Q-EGLSAPV------S---G-TGG-P-----NT   pse1
AK-AA-S-NGAI..........-----N-........V---A--P......GG-PR----NI   pse2

         •   500•              •         •         •
QSLGATPNGLKVAAEISSSGVSELNNSAPPAVTISPVVAQPSPTPVAITASN            mel
P--.........HTKEVK-AE-A---T--.A.S--L......-NY-P-A-T-            vir
RN....SF-SAKGPKKAETLNIAAPE-L--Q...---AVA...-----A-T-            pse1
RNNILG-QNTEKGSPKAEMKT-TPKSMSIKPPSEFADI                          pse2
```

B

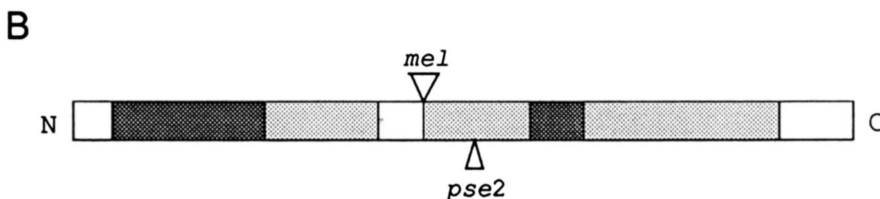

FIGURE 3.—(A) Predicted exu protein sequences. The sequence of the *D. melanogaster* protein is shown in full using the one-letter amino acid code. For the other proteins, amino acids identical to those of the *D. melanogaster* protein are shown as dashes, and nonconserved amino acids are identified. Gaps introduced into the sequence to allow alignment are indicated by dots. The *D. melanogaster* protein is the largest, at 532 amino acids. The *D. virilis* exu protein and *D. pseudoobscura* exu1 and exu2 proteins contain 486, 497 and 477 amino acids, respectively. Two possible PEST domains are indicated, one by bold overlining above the *D. melanogaster* sequence (MARCEY *et al.* 1991), and one by bold underlining beneath the *D. pseudoobscura* exu2 sequence. Three copies of a sequence with partial homology to an RNA binding motif (MARCEY *et al.* 1991) are indicated with arrows. The repeat unit is B*XXX*F/YB*X*V, where B is a basic amino acid, *X* is variable, and F, Y and V are phenylalanine, tyrosine and valine, respectively. Additional abbreviations: *mel*, *D. melanogaster*; *vir*, *D. virilis*; *pse*, *D. pseudoobscura*. (B) Conservation of exu protein structure. The exu protein is shown in schematic form, drawn to scale along the horizontal axis. Regions with high, intermediate and low sequence conservation are indicated by dark, light and no stippling, respectively. Regions present in only one of the proteins are indicated by triangles. The numerous small deletions, found primarily in the poorly conserved regions and especially in the carboxy-terminal region, are not indicated.

pseudoobscura exu gene would allow us to explore a possible role of *exu*, namely, specific recognition of *bcd* mRNA. SEEGER and KAUFMAN (1990) found that the *D. pseudoobscura bcd* mRNA was not localized when present in *D. melanogaster* embryos, presumably because of a failure to be recognized by a specific localization factor. If *exu* encoded that factor, then expressing the *D. pseudoobscura exu* gene in *D. melanogaster* might restore localization of the *D. pseudoobscura bcd* mRNA. However, in the course of experiments to test this notion we find that in our hands the *D. pseudoobscura bcd* mRNA does in fact become localized to the

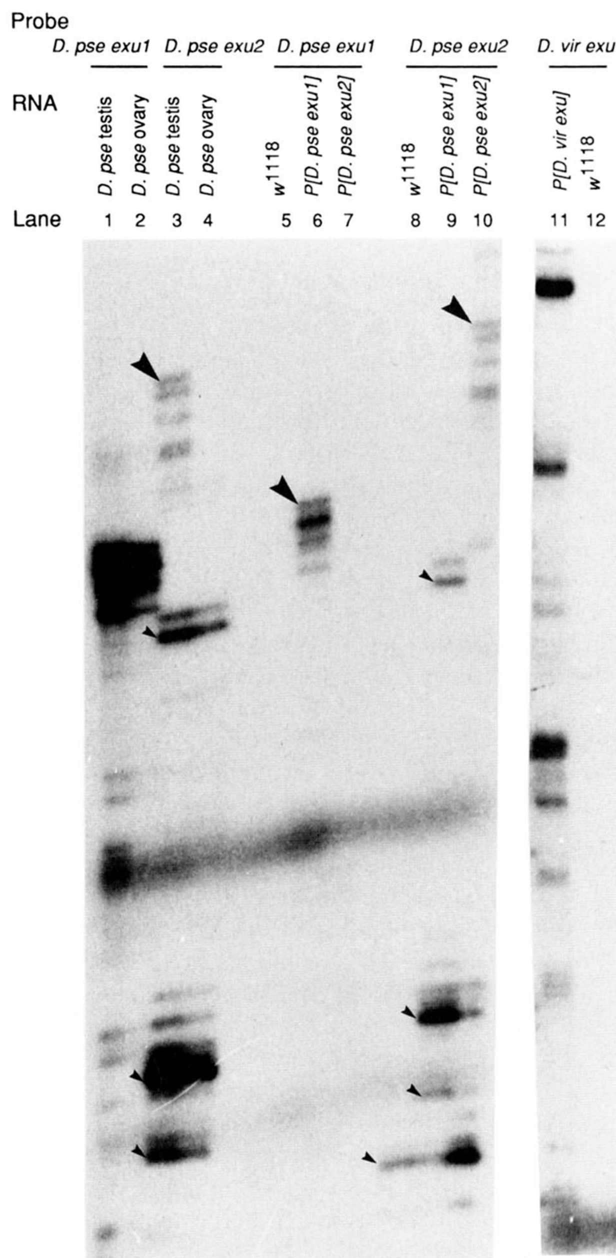

FIGURE 4.—Expression of endogenous and transgenic *exu* genes. RNAs were detected by RNase protection assays using probes corresponding to the different genes. The *D. pseudoobscura exu2* probe protects fragments from both the *D. pseudoobscura exu2* mRNA and the *D. pseudoobscura exu1* mRNA. However, it is straightforward to identify which bands correspond to which mRNA by probing RNA samples containing only one of the two *D. pseudoobscura exu* gene mRNAs; such RNA samples have been obtained from *D. melanogaster* flies carrying a single *D. pseudoobscura exu* transgene (see lanes 6–7 and 9–10). In lanes 1–4, RNA samples (1 fly equivalent per lane) from *D. pseudoobscura* testis (*D. pse* testis; lanes 1 and 3) or ovary (*D. pse* ovary; lanes 2 and 4) were probed for the *exu1* gene (lanes 1 and 2) or the *exu2* gene (lanes 3 and 4). The *exu1* transcript is abundant in both testes and ovaries (lanes 1 and 2; the cluster of bands is indicated by a large arrowhead). In contrast, *exu2* transcripts are detected only in testes (lane 3; the largest in a cluster of bands is marked with a large arrowhead). Clusters of background bands owing to cross-reactivity of the probe with the *exu1* transcripts are each indicated with a single small arrowhead. Expression of the various transgenes in *D. melanogaster* ovaries is monitored in lanes 5–12. RNA samples are from w^{1118}, the recipient for *P* element transformation, and transgenic flies carrying *D. pseudoobscura exu1* (P[*D. pse exu1*]), *exu2* (P[*D. pse exu2*]), or *D. virilis exu* (P[*D. vir exu*]). Each group of lanes (5–7, 8–10, 11–12) corresponds to a single probe, as indicated (abbreviations as above). The different RNA samples were tested with each probe (i) to detect expression of the transgene and (ii) to determine if the probe protected portions of other *exu* mRNAs. Samples in lanes 5–7 were all tested with the *D. pseudoobscura exu1* probe. No protected fragments are observed in RNA from w^{1118} flies (lane 5) or in transgenic flies carrying the *D. pseudoobscura exu2* gene (lane 7), indicating that the probe does not protect those mRNAs. However, the probe does protect a cluster of prominent bands in the RNA from transgenic flies carrying the *D. pseudoobscura exu1* gene (lane 6; large arrowhead), demonstrating that the transgene is expressed. Using the *D. pseudoobscura exu2* probe (lanes 8–10), protected fragments are observed in RNA from flies carrying the *D. pseudoobscura exu2* transgene (lane 10; as in lane 3, the largest in a cluster of bands is marked with a large arrowhead–an additional protected fragment migrates much slower and is not shown here), demonstrating that it is expressed. The probe cross-reacts with the *D. melanogaster exu* mRNA (lane 8; the band marked with a small arrowhead), as well as the *D. pseudoobscura exu1* mRNA (lane 9; clusters of bands are marked with single small arrowheads). Because the fragments of the *D. pseudoobscura exu2* mRNA protected by the *exu2* probe differ in size from the bands resulting from cross-reactivity with the *exu1* mRNA, it is possible to identify which bands in lanes 3 and 4 arise from which mRNA. Samples in lanes 11 and 12 were tested with the *D. virilis exu* probe. No protected fragments appear when RNA from w^{1118} flies is used (lane 11). In contrast, protected fragments do appear when RNA from transgenic flies carrying the *D. virilis exu* gene (P[*D. vir exu*]) is used (lane 12), indicating that the *D. virilis* gene is expressed.

TABLE 2

Transgene rescue of *D. melanogaster* mutations

Transgene	Independent lines displaying rescue of	
	exu^4 females	exu^4 males[a]
D. melanogaster exu[b]	10/10	5/5
D. pseudoobscura exu1	5/5	1/2
D. pseudoobscura exu2	0/2	0/2
D. virilis exu	5/5	0/2

[a] In all cases in which male rescue was detected, male fertility remained substantially less than for wild type.
[b] Data from MACDONALD, *et al.* (1991).

anterior pole when expressed in transgenic *D. melanogaster* embryos.

Previous experiments involving expression of the *D. pseudoobscura bcd* gene in *D. melanogaster* used a genomic DNA fragment containing the *bcd* transcription unit and flanking regions. Anterior localization of the transgene mRNA did not occur. Because expression of the transgene was initially variable and ceased in later generations (SEEGER and KAUFMAN 1990), we made new transgenic flies, using a hybrid *bcd* gene expected to be expressed more consistently than the *D. pseudoobscura*

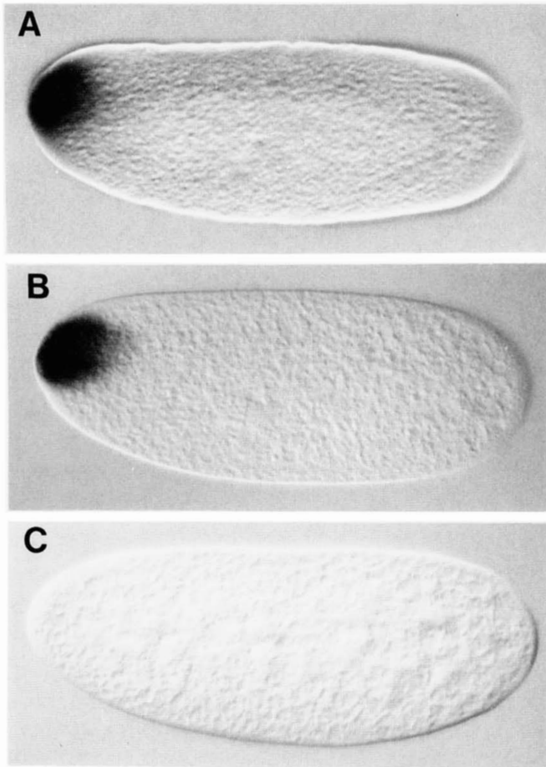

FIGURE 5.—Activity of the *D. pseudoobscura bcd* mRNA localization signal in *D. melanogaster* embryos. In all panels mRNA distributions within embryos are revealed by whole mount *in situ* hybridization. All embryos are oriented with anterior to the left. (A) Distribution of the endogenous *bcd* mRNA in a wild-type embryo. (B) Distribution of the mRNA from a hybrid *bcd* in which the *D. melanogaster bcd* promoter regions is fused to the*D. pseudoobscura bcd* transcription unit. The hybridization probe is complementary to the *D. pseudoobscura bcd* 3′-UTR; no cross hybridization to the *D. melanogaster bcd* mRNA can be detected in control embryos lacking the transgene (panel C).

bcd gene. The entire *D. pseudoobscura bcd* transcription unit (5′- and 3′-flanking regions and coding region) was fused to the promoter of the *D melanogaster bcd* gene (see MATERIALS AND METHODS). Surprisingly, transcripts of this transgene were found to be localized to the anterior pole of embryos from transgenic mothers (Figure 5B). Two additional hybrid genes were also created and tested for localization of their transcripts in transgenic *D. melanogaster* flies, and the results support the notion that the *D. pseudoobscura* localization signal is functional in *D. melanogaster*. One was a hybrid *bcd* gene in which the 3′-UTR [which contains the localization signal in each of several *Drosophila* species tested (MACDONALD and STRUHL 1988; MACDONALD 1990)] is from *D. pseudoobscura bcd*, but the rest of the gene is from *D. melanogaster bcd*. In the other hybrid gene, the *D. pseudoobscura bcd* 3′-UTR is joined to a heterologous transcription unit [the *nanos* gene lacking most of its 3′-UTR; the *nanos* mRNA 3′ UTR also contains a localization signal, but that signal directs the mRNA to the posterior embryonic pole (WANG and LEHMANN 1991)]. Transcripts of both hybrid genes were also localized to the anterior pole of early embryos (data not shown). It appears that the *D. pseudoobscura bcd* mRNA localization signal can function in *D. melanogaster*, and we are unable to use combinations of *bcd* mRNAs and exu proteins from these species to address questions of specific interactions.

DISCUSSION

An unexpected result of our phylogenetic comparison was the discovery that *D. pseudoobscura* has two *exu* genes, while *D. melanogaster* and *D. virilis* each have only a single *exu* gene. Patterns of evolution among these species suggest that functions performed by the exu protein may be divided among the duplicated *exu* genes of *D. pseudoobscura*. Typically, *Drosophila* genes are more closely related between *melanogaster* and *pseudoobscura* than between *melanogaster* and *virilis* [*e.g.*, COLOT *et al.* (1988) and JONES *et al.* (1991)]. In contrast, we find the opposite for the *exu* gene. If the exu protein performs two or more different (but perhaps closely related) functions, each function would be expected to place somewhat different constraints on evolutionary divergence of the *exu* gene sequence. Upon duplication of the gene and division of function in *D. pseudoobscura*, each individual gene would be subject to fewer constraints. Hence, the appearance of tolerable sequence changes would be accelerated. What might the different functions be? One possibility is that *exu* has different roles in the male and female germ lines, its two major sites of expression. Indeed, one *exu* mutation specifically disrupts the female function, without affecting male fertility (HAZELRIGG *et al.* 1990). Nevertheless, at least one of the two *D. pseudoobscura exu* genes does not appear to be specifically tailored for the male or the female functions; when expressed in exu^- *D. melanogaster*, the *exu1* gene fully rescues the female maternal-effect lethality and at least partially restores male fertility. Thus, the degree of divergence displayed by the *exu1* gene cannot be attributed to a specialization for either of the recognized *exu* roles in the female and male germ lines; perhaps there is an additional *exu* function not yet recognized, which is not essential for viability or fertility.

Three of the *exu* genes can act interchangeably in *D. melanogaster* ovaries, and must share protein motifs essential for the role of *exu* in localization of *bcd* mRNA. Consequently, we wish to know what domains are conserved among the proteins and likely to be important for function, and what domains are not conserved and perhaps of less importance. Obvious examples of functionally conserved regions exhibit extensive sequence identity. Among the three genes, there are two regions with particularly high sequence conservation; unfortunately, neither region contains protein motifs of known function. Other regions are also conserved at the sequence level, but to a lesser degree. There are, in addition, two

possible examples in which protein domains are conserved, but with minimal sequence identity. One example involves PEST-like domains. PEST domains, implicated in protein degradation, are regions with an abundance of certain amino acids, and a paucity or outright absence of others (ROGERS *et al.* 1986). Given these constraints, PEST domains need not require the conservation of a particular sequence to conserve their function. Two regions that are not highly conserved at the sequence level are nevertheless rich in the amino acids common to PEST domains. During evolution of the *exu* genes, contraction of one PEST-like domain appears to be accompanied by expansion of the other.

Another example of potential domain conservation involves a region of the exu protein that has the lowest degree of sequence identity between *D. melanogaster exu* and any of the other genes (the largest gap in the dot matrix comparisons of Figure 2). In the *D. melanogaster* protein, this region is rich in serine, while in all other exu proteins acidic amino acids predominate. If some of these serine residues in the *D. melanogaster* protein are phosphorylated *in vivo*, this region would represent an acidic domain conserved among all three species. Notably, the *D. melanogaster* protein is multiply phosphorylated, although the sites of phosphorylation have not been identified (MACDONALD *et al.* 1991; S. K-S. LUK and P. M. MACDONALD, unpublished). The possibilities raised here, although unquestionably speculative, suggest that regions without striking sequence conservation may still be important for *exu* function.

Our finding that the *D. pseudoobscura bcd* mRNA localization signal can act in *D. melanogaster* flies differs from a previous report (SEEGER and KAUFMAN 1990). Although we do not know the explanation for the difference, it may be noteworthy that in the earlier experiments the expression of the *D. pseudoobscura bcd* gene was driven by its own promoter, while in our experiments the *D. melanogaster bcd* promoter was used. In the former case, expression of the transgene was clearly not normal, although the evidence indicating at least some level of maternal expression was strong. In our experiments, essentially all embryos expressed similar, readily detectable levels of anteriorly localized mRNAs. Perhaps defects in the level or consistency of *bcd* mRNA expression also impair localization. Another explanation would invoke the presence of sequences in the *D. pseudoobscura bcd* mRNA that impair localization in *D. melanogaster*. However, one of our hybrid *bcd* genes encodes an mRNA that should contain all of the normal *D. pseudoobscura bcd* transcript, and little or none of the *D. melanogaster bcd* transcript; this mRNA is localized normally (Figure 5).

Demonstration that the mRNA localization signal of the *D. pseudoobscura bcd* gene does act in *D. melanogaster* provides a more straightforward conclusion to comparisons of the 3′-UTRs of *bcd* genes from a variety of *Drosophila* species (MACDONALD 1990; SEEGER and KAUFMAN 1990). Previously, it was necessary to explain why, of the examples tested, the *D. pseudoobscura bcd* 3′-UTR alone did not direct localization in *D. melanogaster*, yet it was not the most divergent. Our new results alleviate the need to speculate about why functional divergence does not parallel sequence divergence during evolution. Overall, then, it seems that components involved in the localization of *bcd* mRNA are substantially conserved in widely different Drosophila species, and the mechanisms underlying mRNA localization events are themselves likely to be conserved.

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