The *exuperantia* Gene Is Required for Drosophila Spermatogenesis as Well as Anteroposterior Polarity of the Developing Oocyte, and Encodes Overlapping Sex-Specific Transcripts

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ABSTRACT

The Drosophila gene exuperantia (*exu*) is a maternal effect gene which is needed for proper localization of the *bcd* RNA during the formation of oocytes. We have extended the characterization of the *exu* phenotype and find that the gene functions in the male as well as the female germline. Six of seven *exu* alleles are male-sterile; mutant defects in spermatogenesis first appear during meiosis. A genetic analysis presented here shows that the *exu* gene does not encode a zygotic vital function. The isolation of two overlapping deficiencies that delete *exu* function localizes the gene cytologically to polytene bands 57A4-B1. We describe the molecular cloning and identification of the gene, and show that it encodes overlapping sex-specific transcripts of 2.9 kb in the male and 2.1 kb in the female. We also show that these two transcripts are limited in expression to the germline. We demonstrate that one allele, exu^{VL57}, is a deletion of about 700 bp which results in a loss of both transcripts.

IN recent years, a number of cases of subcellular localization of RNA have been documented. Actin mRNA is localized in the lamellipodia of chick myoblast and fibroblast cells, where new actin must be synthesized (LAWRENCE and SINGER 1986); transcripts of the Drosophila pair-rule genes become localized in the apical periplasm of the developing embryo (INGHAM, HOWARD and ISH-HOROWITZ 1985; KILCHHERR *et al.* 1986; MACDONALD, INGHAM and STRUHL 1986; EDGAR, ODELL and SCHUBIGER 1987); the Veg-1 RNA of Xenopus was originally isolated in a screen to detect asymmetrically distributed RNAs in the Xenopus egg (REBAGLIATI *et al.* 1985); maternal RNAs in ascidian and annelid eggs are not homogeneously distributed (JEFFREY 1984, 1985).

Is subcellular RNA localization a way for a developing organism to partition or create gradients of morphogens? The most convincing example of such a mechanism comes from the case of localization of the *bcd* mRNA in the Drosophila oocyte. Anterior localization of the *bcd* RNA apparently leads to the formation of an anteroposterior concentration gradient of the *bcd* protein in the developing embryo (DRIEVER and NÜSSLEIN-VOLHARD 1988a,b). This concentration gradient of *bcd* protein is necessary for the initiation of a cascade of zygotic gene expression, the spatial and temporal patterns of which underlie formation of the anteroposterior segmentation pattern of the embryo (AKAM 1987; FROHNHOFER and NÜSSLEIN-VOLHARD 1987; INGHAM 1988; SCHROEDER *et al.* 1988; WINSLOW, CARROLL and SCOTT 1988; TAUTZ 1988; DRIEVER and NÜSSLEIN-VOLHARD 1989; DRIEVER, THOMA and NÜSSLEIN-VOLHARD 1989; HANES and BRENT 1989; STRUHL, STRUHL and MACDONALD 1989). Thus, subcellular RNA localization has, in this case, been implicated to be a way of achieving asymmetric distribution of a morphogen in a developing embryo. The mechanism by which a specific RNA, such as the *bcd* RNA, is subcellularly localized, is not understood.

Two genes, *exuperantia* (*exu*) and *swallow* (*swv*), have been shown to affect the anterior localization of the *bcd* RNA. The *swv* gene (MOHLER and CARROL 1984; STEPHENSON and MAHOWALD 1987; STEPHENSON, CHAO and FACKENTHAL 1988) is required in mothers for normal embryonic development of progeny; in the absence of maternal *swv* product, embryos have defective heads and abdomens. In mutant vitellogenic stage oocytes, the *bcd* RNA is laterally displaced from its normal cap at the anterior end of the oocyte, and mature oocytes have a very shallow anteroposterior gradient of *bcd* RNA (BERLETH *et al.* 1988; STEPHENSON, CHAO and FACKENTHAL 1988). The *exu* gene was first genetically identified and characterized by SCHÜPBACH and WIESCHAUS (1986a,b; 1989). According to the results of BERLETH *et al.* (1988), oocytes produced by mutant *exu* mothers show little or no localization of bcd RNA in early egg chambers and mature oocytes. The resulting embryos are missing head structures and duplicate posterior gastrulation events at their anterior ends.

In this paper we report genetic and molecular experiments which have led to the cloning of the *exu*

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gene. In addition, we describe a new phenotype of *exu* mutations: male sterility. The observed male sterility of mutants correlates with the observation of female-specific and male-specific *exu* transcripts, and our results which show that these transcripts are expressed in the germline. Since *exu* gene function is required for anterior localization of the *bcd* RNA in the developing oocyte, one attractive model is that exu also functions during spermatogenesis to subcellularly localize an RNA for a gene product which must form a gradient for proper development of the polar sperm cell.

MATERIALS AND METHODS

Mutagenesis screens: X-ray mutagenesis screens for new *exu* alleles were done at 25°, and flies were grown on standard cornmeal-molasses medium supplemented with live yeast. Marker mutations and balancer chromosomes are described in LINDSLEY and GRELL (1968). Males from a homozygous *cn bw sp* isoline strain were irradiated with 4000 r with a Philips RT250 orthovoltage X-ray machine (250 kV, 15 mA) using a 1/2 value layer of 3-mm aluminum. The source to target distance was 30 cm, and dose rate was 5 Gy/min. These males were mass mated to *Tft/Cyo* virgin females in bottles, and the parents were discarded after 5 days. Single *cn bw sp/Tft* progeny males were mated in vials to 3–5 *cn* exu^{PJ42} *bw/Cyo* virgin females. The parents were discarded after 1 week. The *cn bw sp/cn* exu^{PJ42} *bw* nonvirgin progeny females were collected and put into plastic egg collection tubes which were inverted over Petri plates containing egg-laying medium. The females were allowed to lay eggs for 24 hr and then discarded. After another 24 hr, the eggs were examined for hatching. In cases in which the eggs did not hatch, the *cn bw sp/Cyo* brothers of these females were collected and mated with *Tft/Cyo* females to establish a stock, and also mated with *cn bw* exu^{PJ42}*/Cyo* females to retest for the female sterile phenotype.

A screen to identify ethyl methanesulfonate (EMS)-induced mutations uncovered by *Df(2R)exu*^{1} was done essentially as the X-ray screen described above, with the following modifications. Treated males came from a *cn bw* isoline strain. Males were fed a 0.025 M EMS-1% sucrose solution for 24 hr, following the protocol of LEWIS and BACHER (1968). The first generation *cn bw/Tft* males were mated singly to *cn bw Df(2R)exu*^{1} *sp/Cyo* females. The *cn bw/cn bw Df(2R)exu*^{1} *sp* test class progeny were examined for visible, lethal and maternal effect mutations. Mutations were rescued by selecting *cn bw/Cyo* brothers of the test class and mating them with *Tft/CyO* virgin females.

To screen for X-ray-induced phenotypic revertants of *F4-1*, males of the genotype w^{1118}*/Y; F4-1/F4-1* were irradiated with 4000 r, using the conditions described above, and mass-mated in bottles to w^{1118}/w^{1118}*; Cyo/Sco* females. (w^{1118} is a partial deletion of the *X*-linked white gene.) After 5 days the parents were discarded. Phenotypic revertants were selected in the first generation progeny as white-eyed flies. Stocks were established by mating these revertant flies with w^{1118}*; Cyo/Sco* flies.

Cytology: All X-ray-induced mutations were examined in aceto-orcein squashes. Flies bearing the mutation were crossed to wild-type Canton S (CS) virgin females, 5 pairs per bottle, and transferred each day to fresh bottles. The larvae were grown at 18° and fed daily with liquid Baker's yeast.

In situ hybridizations to polytene chromosomes were done following the protocols described by ENGELS *et al.* (1986).

Male fertility: Males (3–5 days old) heterozygous for a mutant *exu* allele and the *CyO* balancer chromosome were crossed with *Df(2R)exu*^{1}*/CyO* or *SM1* virgin females. Male progeny heterozygous for the mutant *exu* allele and *Df(2R)exu*^{1} were then mated with Canton-S wild-type virgin females (3–5 days old). All crosses employed roughly equal numbers of males and females. Egg collections were done every 12–24 hr, and hatched eggs were scored after an additional 48 hr. All crosses and egg incubations were done under constant humidity and temperature, at either 18° or 25°.

Spermatogenesis was observed in males of the same genotype used for the fertility tests. These males were raised at room temperature, and were 3–5 days old. Testes squashes were done as described in LIFSCHYTZ and HAREVAN (1977).

Library construction and isolation of genomic clones: Genomic libraries of DNA from *F4-1* and wild-type *Canton S* (*CS*) flies were made in the Stratagene vector lambda-Dash. The *F4-1* library was made by digesting *F4-1* genomic DNA with serial dilutions of *Sau*3A, and ligating the fraction yielding the highest concentration of fragments in the 15–20 kb range with *Bam*HI-digested lambda-Dash DNA. The *CS* genomic library was made using the same protocols, except that the *Sau*3A fragments were size-fractionated by electrophoresis in a low melting point agarose gel before ligation in lambda-Dash.

Libraries were screened according to MANIATIS, FRITSCH and SAMBROOK (1982). The chromosome walk was facilitated by the T3 and T7 promoters present in the polylinkers spanning the insertion site. Positive plaques were picked and rescreened two or three times to purification. All phage DNAs were digested with *Eco*RI, *Sal*I, *Bam*HI or *Hin*dIII, and combinations of double digests to determine their degrees of overlap. The clone that extended the walk the furthest was digested with *Rsa*I, T3 and T7 RNA probes were made from the digested DNA, and these were used to probe a Southern blot which included DNA from the last clone in the walk. The T3 or T7 probe which identified the nonoverlapping region of the new clone was then used to screen the library for the next step in the walk.

Preparation of RNA and DNA and analysis by Northern and Southern transfers: RNA was extracted following the guanidine-HCl procedure described in SAMBROOK, FRITSCH and MANIATIS (1989) with some modifications. Adult flies were separated by sex, and 4 ml of flies were ground to a fine powder in a mortar cooled with liquid N_2. The ground flies were suspended in 8 ml guanidine-HCl buffer (6 M guanidine-HCl,0.2 M Na-acetate), and centrifuged in a Sorval SA600 rotor at 10K for 10 min. The supernatant was layered onto 4 ml of 5 M CsCl, 50 mM EDTA, 0.1% diethyl pyrocarbonate (DEPC) and centrifuged at 20° in a Beckmann SW40 rotor for 16 hr at 30K. After aspiration of the guanidine-CsCl, the pellet was rinsed with cold 70% ethanol, resuspended in 0.5 ml DEPC-treated H_2O, and transferred to an Eppendorf tube. The RNA was Na-acetate-isopropanol precipitated at −20°, followed by centrifugation in a microcentrifuge for 20 min at 4°. Poly(A)$^+$ RNA was separated by passage over an oligo(dT)-cellulose column (Collaborative Research) according to the protocol of AUSUBEL *et al.* (1989) with the modification that the heated RNA was mixed with the pretreated oligo(dT) cellulose and rocked gently for 15 min before the column was prepared. Eluted poly(A)$^+$ RNA was ethanol precipitated and resuspended in 10 mM Tris, pH 7.4.

RNA was electrophoresed in formaldehyde agarose gels following the methods of MANIATIS, FRITSCH and SAM-

BROOK (1982), with the following modifications: 10× gel buffer was 0.2 M MOPS, pH 7.0, 50 mM Na-acetate, 10 mM EDTA. Gels were not alkaline-treated. The BRL 0.24–9.5 kb RNA ladder was used for size standards. RNA was transferred to Schleicher and Schuell Nytran filters following the procedures of MANIATIS, FRITSCH and SAMBROOK (1982). ^{32}P-Labeled probes were made either by nick translation or by using a random-priming labeling kit from Boehringer-Mannheim. Blots were prehybridized and hybridized in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 5% Dextran SO_4. Hybridizations were done for at least 12 hr at 42°, on a rotating platform. Initial washing was in 2 × SSC, 0.1% SDS (3 min at room temperature) followed by washes in 1 × SSC, 0.1% SDS (2 × 15 min, 65°).The final wash was in 0.25 × SSC, 0.1% SDS at 65° (2 × 15 min). Autoradiograms were exposed from 1 to 10 days, at −70°.

Genomic DNA for Southern blot analysis was prepared as described in HAZELRIGG, LEVIS and RUBIN (1984). DNA was electrophoresed in 0.6% agarose gels. Approximately 5 μg of genomic DNA was loaded in each lane. *Hin*dIII-digested lambda DNA (BRL) was used for size standards. Southern blots were prepared and hybridized following the methods of MANIATIS, FRITSCH and SAMBROOK (1982), with some modifications. Blots were first prehybridized for 4 hr at 65° in 4 × SSCP, 5 × Denhardt's solution. A second prehybridization was for 2 hr, at 65°, in hybridization buffer: 4 × SSCP, 5 × Denhardt's solution, 0.5% SDS, and 0.2 mg/ml sonicated salmon sperm DNA. Hybridizations were done for at least 12 hr at 65°, on a rotating shaker. Filters were first washed in 2 × SSC, 0.1% SDS (2 × 15 min, 65°). Final washes were done in 1 × SSC, 0.1% SDS (2 × 15 min, 65°). Autoradiograms were exposed for 3–5 days.

RESULTS

Isolation of deficiencies for *exu* and cytological mapping: To localize *exu* cytologically and to obtain useful breakpoints for subsequent molecular analysis of the gene, we screened for X-ray induced *exu* alleles as outlined in Figure 1a (for a detailed description of the screening protocol, see MATERIALS AND METHODS). Two chromosomes lacking *exu* function were isolated from 9804 mutagenized 2nd chromosomes. Analysis of polytene chromosome squashes revealed that one, exu^{XL1}, was cytologically normal, whereas the second, $Df(2R)exu^{1}$ (hereafter referred to as exu^{1}), was deleted for polytene bands 57A2-B1 (Figure 2).

The exu^{VL57} allele was isolated in a dysgenic screen by T. SCHÜPBACH (unpublished results). We have analyzed two different exu^{VL57} isolates ($exu^{VL57-12}$ and $exu^{VL57-14}$) and since these appeared identical in all our analyses, we will refer to both these isolates as exu^{VL57} in this paper. *In situ* hybridization of a biotinylated *P*-element probe to the polytene chromosomes of exu^{VL57}/Canton S larvae did not show a detectable *P* element in the 57A2-B1 region. Furthermore, attempts to dysgenically revert the exu^{VL57} mutation were unsuccessful (our unpublished data). These observations suggest that the two exu^{VL57} lines described here do not contain a *P* element capable of excision, or detectable by *in situ* hybridization, inserted in the *exu* gene. However, a very small, defective *P*-element

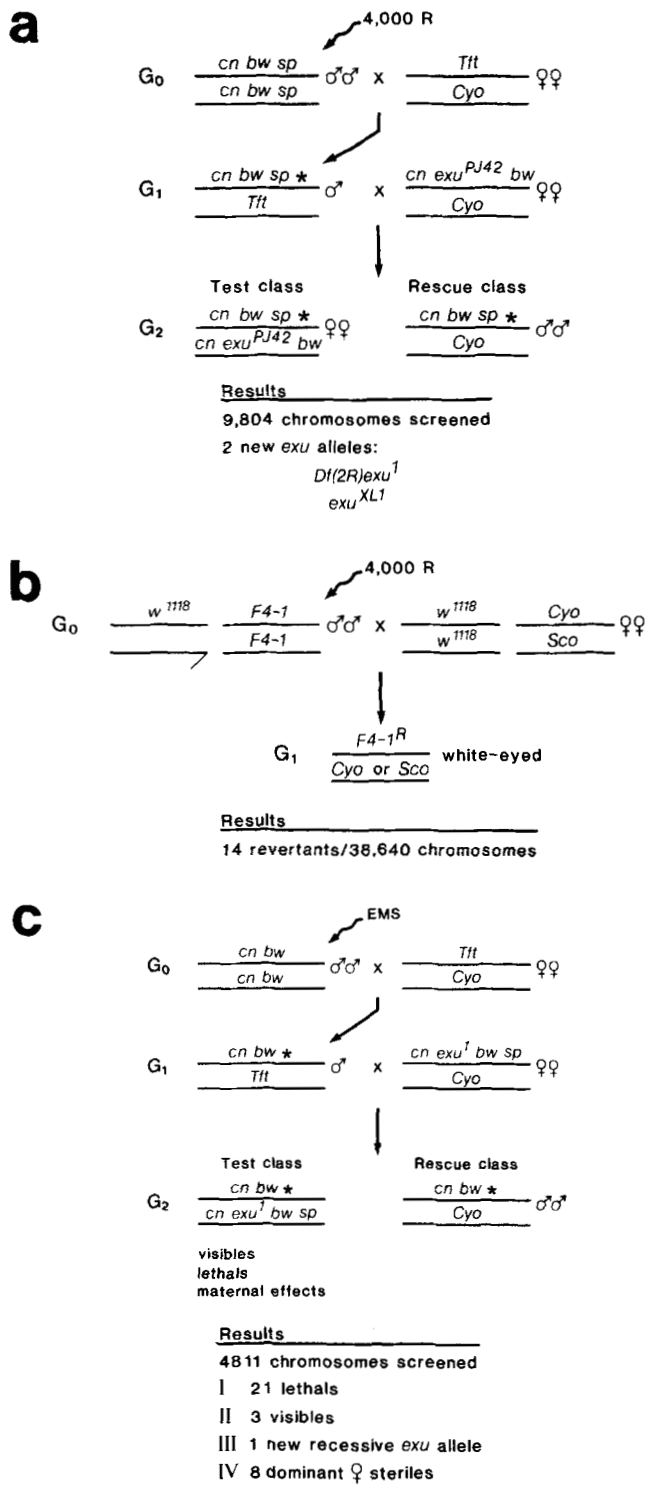


FIGURE 1.—Mutagenesis screens. (a) Screen to isolate X-ray-induced *exu* alleles. The G_2 test class females were scored for the *exu* maternal-effect phenotype. (b) Screen to isolate phenotypic revertants of *F4-1*. X rays were used to generate white-eyed derivatives of *F4-1*, a *P*-element insertion at 57B1,2 that expresses the *white* gene. *F4-1* flies have pigmented eyes; the phenotypic revertants have lost the function of the *white* gene. (c) Screen to isolate EMS-induced mutations in the region deleted by *Df(2R)MK1*. The G_2 test class progeny were assayed for lethal, visible and female sterile phenotypes. In the cases of females that laid eggs that failed to hatch, the progeny were observed for mutant phenotypes. See MATERIALS AND METHODS for more detailed descriptions of these screens.

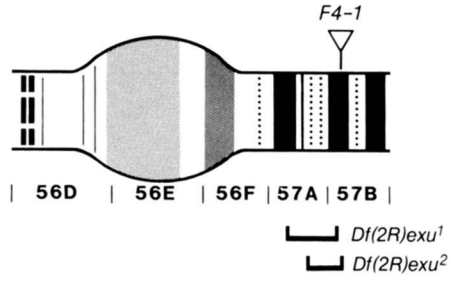


FIGURE 2.—Cytological map of the *exu* region. The region depicted is part of polytene sections 56 and 57 of the right arm of chromosome 2. The *exu* mutations shown were isolated in this laboratory. The site of insertion of *P(white,rosy)F4-1* is indicated by an open triangle. The cytological extents of the two deficiencies are shown by dark bracketed bars.

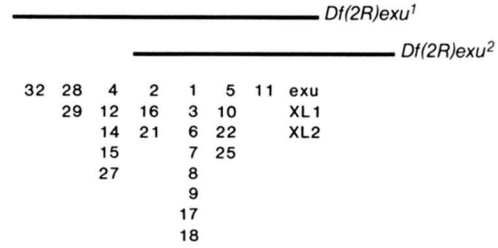


FIGURE 3.—Complementation map of EMS- and X-ray-induced mutations. The solid lines at the top represent the overlapping deficiencies *Df(2R)exu*^{1} and *Df(2R)exu*^{2}. The mutations uncovered by both *exu*^{1} and *exu*^{2} are distal on the chromosome relative to the proximal mutations uncovered only by *exu*^{1}. The order of complementation groups within each region, proximal and distal, is arbitrary and does not necessarily represent the true order of the genes.

insertion could have been undetected by these techniques.

The distal breakpoint of *exu*^{1}, 57B1,2, is also the site of insertion of a *P* element expressing the *white* gene, *P(white,rosy)F4-1* (LEVIS, HAZELRIGG and RUBIN 1985). Phenotypic revertants of *F4-1* (white-eyed flies) were induced with X rays (see Figure 1b, and MATERIALS AND METHODS for details), with the intention of creating deletions in the DNA flanking *F4-1*. One of 14 phenotypic revertants, *Df(2R)exu*^{2} (hereafter referred to as *exu*^{2}), failed to complement *exu*^{PJ42}. Cytologically, *exu*^{2} is deleted for polytene bands 57A4-B1. This deficiency further refined the cytological mapping of *exu*, localizing the gene to a region containing two faint dotted bands, and part of the 57B1,2 doublet (Figure 2). This suggested that *exu* could be cloned by a chromosomal walk initiated from the *F4-1* insertion, since at most we would have to isolate all of the DNA contained within the small region of overlap of the *exu*^{1} and *exu*^{2} deficiencies.

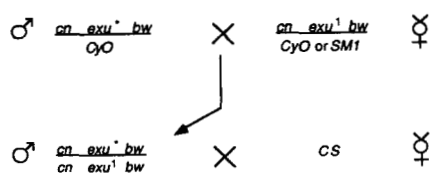
Identification of other genes deleted by *Df(2R)exu*^{1}: All *exu* alleles previously isolated were identified by their strict maternal-effect phenotype. It was possible that these alleles did not represent the true null state of the gene, and that true null alleles would be zygotic lethals. To determine if *exu* could be mutated to produce zygotic lethal alleles, and also to determine if there were other strict maternal-effect genes closely linked to *exu*, an EMS mutagenesis screen was done to identify other genes in the region deleted by *exu*^{1} (see Figure 1c and MATERIALS AND METHODS for details). Lethal, visible and maternal-effect mutations were sought. From a screen of 4811 mutagenized second chromosomes, 21 lethal mutations, one mutation causing a held-out wing phenotype, two mutations producing aberrant wing and thorax phenotypes, and one new *exu* allele (*exu*^{XL1}) were isolated. In addition, eight dominant maternal-effect mutations mapping to the second chromosome (but not necessarily within the region deleted in *exu*^{1}) were recovered. Each of these eight mutations, when heterozygous in a mother, causes embryonic lethality in the progeny; the exact natures of the embryonic lethal phenotypes will be described in another report.

The *exu*^{1} and *exu*^{2} deficiencies are overlapping. Crosses of each recessive mutation with these two deletions defined two groups of mutations: a distal group uncovered by both *exu*^{1} and *exu*^{2}, and a proximal group uncovered only by *exu*^{1}. Complementation crosses were done to produce all pairwise combinations of the mutations within the proximal and distal groups. Figure 3 shows the results of this complementation analysis. The lethal and visible mutations fall into seven complementation groups. Since mutations causing male sterility were not sought in this screen, we cannot discount the presence of other genes in this region necessary for male fertility; current screens for such mutations are underway.

Three mutations have recessive visible phenotypes. Mutation 32 has reduced viability when heterozygous with *exu*^{1} (survival = 20% of the expected value); the viable flies have wings that are held out slightly from the bodies. Mutations 28 and 29 are allelic and cause flies to have short wings with an indentation at the border of the wing where the fourth wing vein meets the edge. In addition, the thoraces of these flies have abnormal ridges surrounded by whorls of bristles, reminiscent of the dumpy-vortex phenotype. Mutation 28 is semilethal when heterozygous with the *exu*^{1} deficiency (survival = 1.5% of the expected value), whereas it survives when heterozygous with mutation 29 (survival = 91% of the expected value). Mutation 29 is viable with *exu*^{1} (survival = 75% of the expected value).

To determine if any of the lethal or visible mutations represented more extreme alleles of *exu*, each allele of all of the complementation groups that are uncovered by both *exu*^{1} and *exu*^{2} was assayed for its ability to complement *exu*^{PJ42}. All mutations thus tested complemented with *exu*^{PJ42} for the lethal, visible and maternal-effect phenotypes, indicating that the

Male Sterility of *exu* Alleles



Allele	Induced by	25° C Eggs Hatched / Eggs Total	25° C Percent Hatching	Motile Sperm	18° C Eggs Hatched / Eggs Total	18° C Percent Hatching
QR9	EMS	0/2355	0%	absent	0/458	0%
SB62	EMS	0/1483	0%	absent	0/624	0%
Sco2	EMS	0/1588	0%	absent	0/311	0%
VL5712	P-element	0/727	0%	absent	0/518	0%
VL5714	P-element	0/833	0%	absent	-	-
XL1	X-rays	0/140	0%	absent	0/404	0%
XL2	EMS	0/1189	0%	absent	0/365	0%
PJ42	EMS	564/625	90%	present	309/723	43%
CS	-	1119/1236	91%	present	574/614	93%

FIGURE 4.—Male sterility of *exu* mutants. Fertility was assessed for flies grown at either 18° or 25°. See MATERIALS AND METHODS for crossing schemes. All *exu*-bearing chromosomes are marked with the *cinnabar* (*cn*) and *brown* (*bw*) mutations (in some cases other marker mutations are present, but these are not relevant for this experiment), identifying the appropriate heterozygous males in the F_1 generation. Fertility was measured in two ways. First, mutant males were mated with wild-type *CS* females and the percentage of hatched eggs from these matings was determined (at both 18° and 25°). Second, phase contrast microscopy of squashes of mutant testes determined whether motile sperm were present (25°). * indicates an *exu* allele.

maternal-effect *exu* alleles are not a special class of alleles at any of these loci. Thus, the region deleted in $Df(2R)exu^1$ genetically consists of five lethal complementation groups, one locus defined by mutations which produce visible wing and thoracic defects and cause semi-lethality, one locus defined by a single mutation which causes a visible wing defect, and the *exu* locus.

Male sterility of *exu* alleles: To determine the effects of exu alleles on male fertility, we generated males that were mutant for each *exu* allele but free of effects of other recessive mutations which might be present on the second chromosome bearing the *exu* allele in question. The appropriate crosses produced males heterozygous for each exu allele and the exu^1 deficiency. Figure 4 shows the results of assays of fertility of males hemizygous for each *exu* allele. Six of the seven alleles were completely male-sterile at both 18° and 25°. An EMS-induced allele, exu^{PJ42} (SCHÜPBACH and WIESCHAUS 1986a), was fertile at 25°, but fertility was reduced by about 50% at 18°. Sibling females (exu/exu^1) of all males grown at 25° were collected as virgins and mated with Canton-S wild-type males. Female sterility was confirmed for every allele, and all females produced embryos with the *exu* phenotype.

Phase contrast microscopy of testes of exu^{VL57}/exu^1 males, as well as males homozygous for different exu alleles including exu^{VL57}, reveals that primary spermatocytes appear morphologically normal (compare Figure 5, A and B). Defects in spermatogenesis are first seen during meiosis, since early postmeiotic cysts contain nuclei that are variable in size, many being smaller than normal. In the early stages of spermiogenesis, these micronuclei do not associate regularly with mitochondrial derivatives. Instead of a one-to-one association of nuclei and mitochondrial derivatives, as is seen in wild-type (Figure 5C), in mutant testes variable numbers of micronuclei associate with each mitochondrial derivative, and the mitochondrial derivatives appear irregular in size (Figure 5D). Subsequent spermatid differentiation is also abnormal in mutants. Aceto-orcein stained mutant testes reveal that spermatid nuclei do not fully elongate or completely condense. The mutant spermatid bundles are irregularly arranged, and the heads are distributed along the length of each bundle instead of being clustered at the apical ends. Although coiled bundles of spermatids are observed at the testis base, the seminal vesicles do not contain functional motile sperm.

Molecular isolation of the *exu* gene and identification of its transcripts: The fact that exu^2 is a small deficiency that removes *exu* function suggested that the *exu* gene could be isolated by initiating a chromosomal walk at the insertion site of the *P(white,rosy)* insertion *F4-1*. The *P(white,rosy)* insertion at 57B1,2 is the only *P* element present in *F4-1* flies. Genomic libraries of *F4-1* and wild-type Canton S (CS) DNA were made in lambda vectors. Initially the *F4-1* library was screened using the complete *P*-element pπ25.7 as a probe. Approximately 3×10^4 phage were screened, and 5 plaques rescreened twice as positives. One of these was chosen to initiate the walk, after *in situ* hybridizations to polytene chromosomes confirmed that the clone did indeed come from 57B1,2. An overlapping phage was isolated from the CS library, and the chromosomal walk was extended in both directions from the starting point. Our final set of overlapping phage are numbered 1–14, distal to proximal, and span a distance of about 150 kb.

The direction of the walk was determined by in situ hybridizations of biotinylated genomic DNA probes to polytene chromosomes of $Df(2R)exu^1/CS$ larvae. The initial clones isolated were located outside of exu^1, but phage insert 10 mapped entirely within the exu^1 deficiency. This suggested that the distal breakpoint of exu^1 lay in phage 9. This breakpoint was confirmed since phage 9 insert DNA hybridizes only weakly to exu^1 chromosomes. Since the two overlapping deficiencies exu^1 and exu^2 both remove the function of *exu* (see Figure 3), the DNA contained between the distal breakpoint of exu^1 and the proximal breakpoint of exu^2 should contain at least part of the *exu* gene. *In situ* hybridizations of labeled phage probes to polytene chromosomes of exu^2/CS larvae showed that exu^2 deletes the DNA contained within phage 12 but retains DNA present in phage 13. These data indicated that

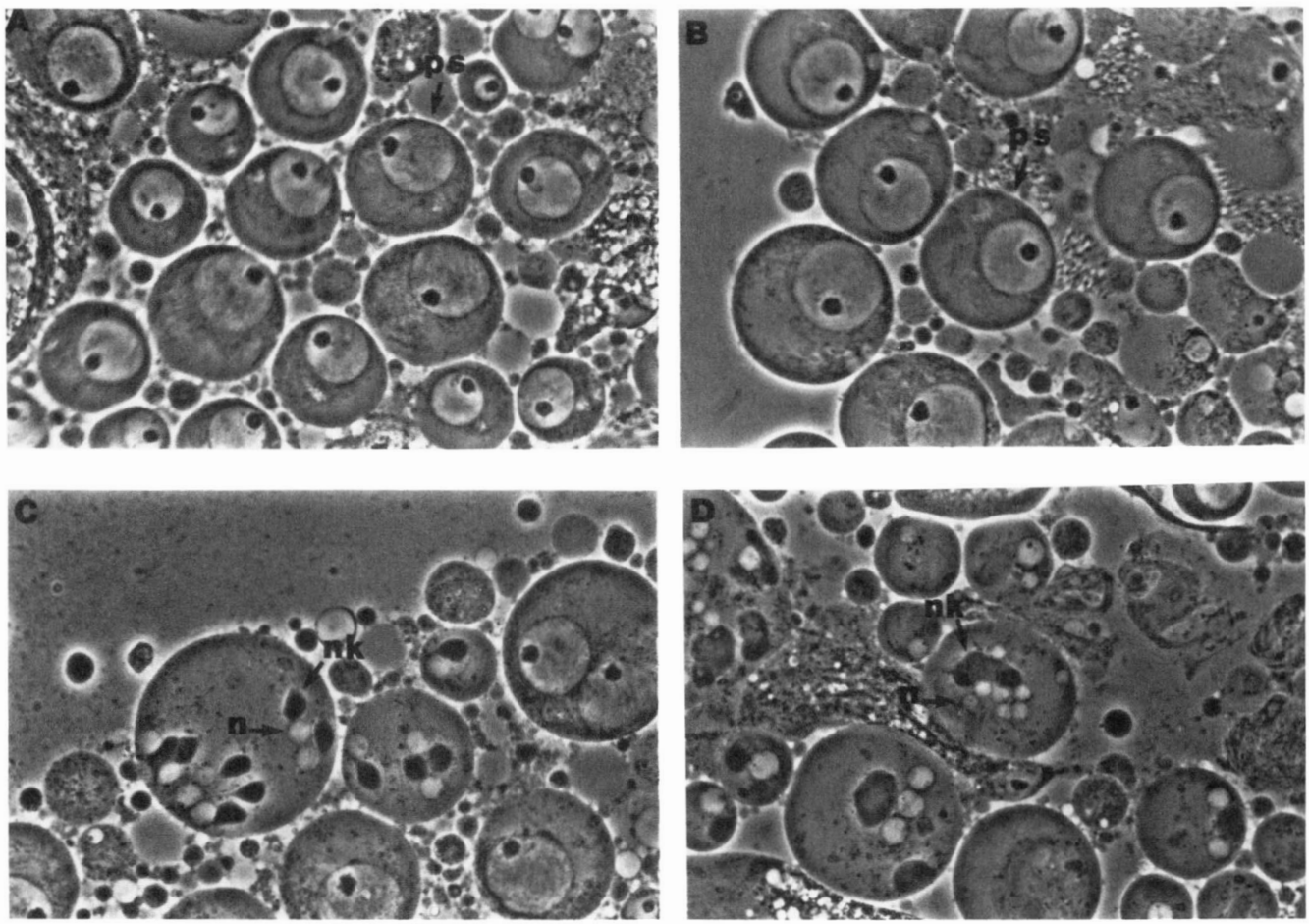


FIGURE 5.—Phase contrast microscopy of spermatogenesis in wild-type and mutant testes. (A) Wild-type testis squash showing primary spermatocytes (ps). (B). exu^{VL57} testis squash; the primary spermatocytes (ps) appear morphologically normal. (C) Wild-type spermatid nuclei (n) with associated mitochondrial derivatives, the nebenkerns (nk). (D) exu^{VL57}/exu^{VL57} testis squash showing abnormal sized nebenkerns (nk) and small nuclei (n).

the *exu* gene should lie between the distal end of phage 9 and the proximal end of phage 13 of our walk, a distance of about 65 kb.

Mutant strains were examined by Southern blot analyses to determine if any molecular alterations existed in the region of the walk encompassed by phages 9–13. These strains included all homozygous-viable *exu* alleles: four EMS-induced *exu* alleles (exu^{PJ42}, exu^{QR9}, exu^{SB62}, exu^{SCO2}), the dysgenically induced exu^{VL57} mutation (isolines 12 and 14 were identical in our analyses), and one X-ray-induced mutation (exu^{XL1}). Comparisons were made between DNA from each exu mutation and the parental chromosomes on which each mutation was induced. Blots were probed with phages 9, 10, 11, 12 and 13. This analysis revealed a change in the restriction fragment pattern of mutant exu^{VL57} DNA, when probed with phages 9 and 10. No other mutants showed alterations by these Southern blot analyses. Figure 6 shows a Southern blot in which DNA from parental and homozygous-viable mutant lines has been digested with *Bgl*II. This Southern blot and others done with other enzymes consistently indicate that exu^{VL57} contains a deletion of about 700 bp. The position of this deletion is shown in Figure 7, a molecular map of phage inserts 9 and 10. The deletion lies within a 2.0 kb *Bgl*II fragment and deletes an intervening *Xba*I site. The exu^{VL57} mutation was isolated in a dysgenic screen (T. SCHÜPBACH, personal communication). It is possible that it arose by insertion and subsequent imprecise excision of a *P* element. These results are consistent with the absence of a detectable *P* element by *in situ* hybridizations to chromosomes of exu^{VL57} larvae.

The molecular alteration present in the exu^{VL57} mutation indicated that the region of DNA contained within phages 9 and 10 was likely to contain at least part of the exu gene. To examine the transcription pattern of this region, we probed Northern blots of poly(A)$^+$ RNA from adult males and females with labelled genomic DNA from phages 9 and 10. Phages 9 and 10 both detect the same major male and female specific RNAs: a 2.9-kb male RNA and a 2.1-Kb female RNA. In addition, a minor 2.5-kb RNA is sometimes observed. Figure 8 is an autoradiogram of

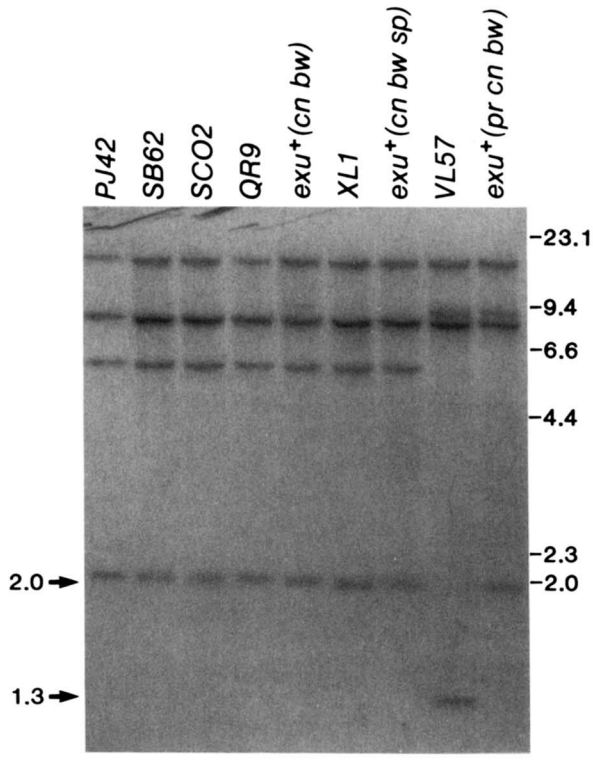


FIGURE 6.—Southern blot of mutant and wild-type DNAs. DNA was digested with BglII and probed with phage 9 (see MATERIALS AND METHODS for details). Mutant DNA lanes are followed by a lane of DNA from the parental strain in which each mutation was induced. Thus, lane 5 is DNA from *cn bw* flies, the parental strain of mutants exu^{PJ42} (lane 1), exu^{SB62} (lane 2), exu^{SCO2} (lane 3), and exu^{QR9} (lane 4). Lane 7 is DNA from *cn bw sp* flies, the parental strain of mutant exu^{XL1} (lane 6). Lane 9 is DNA from the *pr cn bw* parental strain of exu^{VL57}, and lane 8 is exu^{VL57} DNA. (The faint band present in the exu^{+}(*cn bw*) lane is due to a partial digest, and does not indicate a difference between the parental and mutant strains.)

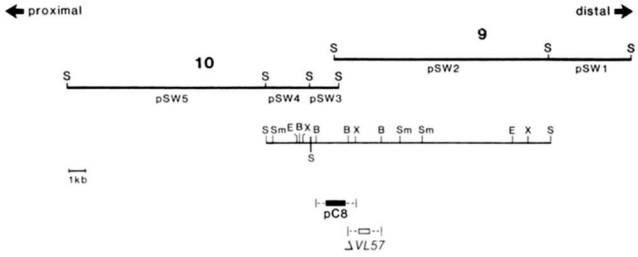


FIGURE 7.—Molecular map of cloned DNA contained within phages 9 and 10. The two top lines show the *Sal*I restriction sites of the phage 9 and 10 genomic DNA inserts. The outer *Sal*I sites of each insert are artificial sites created during cloning and are not present in the genomic DNA. pSW1, pSW2, pSW3, pSW4 and pSW5 are *Sal*I subclones of this region. The third line is a restriction map of the region contained in pSW2, pSW3 and pSW4. The open bar designated exu^{VL57} represents the 700-bp deletion present in the exu^{VL57} allele; this deletion maps within the 2.0-kb *Bgl*II fragment of pSW2. The solid bar designated pC8 represents a cDNA which maps between the *Sal*I and *Xba*I sites indicated. Restriction enzymes are: B = *Bgl*II, E = *Eco*RI, S = *Sal*I, Sm = *Sma*I, X = *Xba*I.

a northern blot with wild-type male and female RNA probed with pSW3, a subclone which includes the region of overlap of phages 9 and 10 (see Figure 7).

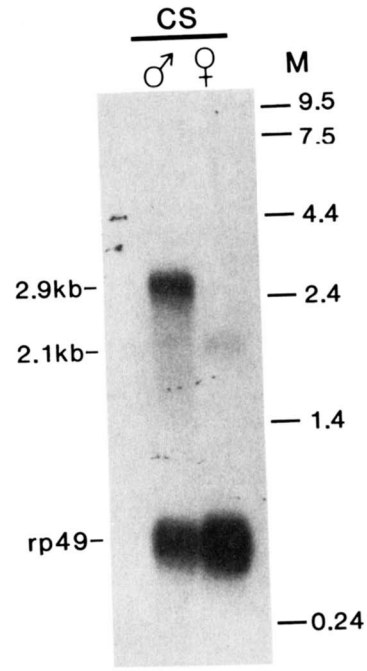


FIGURE 8.—Northern blot of wild-type *CS* male and female poly(A)$^{+}$ RNA. Approximately 5 μg of poly(A)$^{+}$ RNA was loaded in each lane. The blot was probed with the *Sal*I insert of pSW3 and also with a probe to detect the rp49 transcript. Exposure of the autoradiogram was for 1 day. The pSW3 probe detects a 2.9-kb transcript in males, and a 2.1-kb transcript in females. A minor 2.5-kb transcript, can be detected with this probe with longer exposures of the autoradiogram.

This blot has also been probed with a DNA clone which recognizes the ribosomal protein 49 (rp49) gene transcript (O'CONNELL and ROSBASH 1984), to independently measure the relative amounts of RNA loaded in each lane. The relatively low signal present for the female specific transcript on this blot may be due either to differences in abundance of the male and female transcripts, or to the extent of sex-specific exons present in pSW3.

To determine if the exu^{VL57} mutation affects the levels or sizes of these transcripts, we probed a Northern blot of wild-type and exu^{VL57} RNA (Figure 9) with a cDNA, pc8, isolated from an ovary cDNA library provided us by LAURA KALFAYAN (S. WATKINS and T. HAZELRIGG, unpublished data). This cDNA maps in the region of overlap of phages 9 and 10 (Figure 7). The transcripts detected by pc8 in wild-type flies (the major 2.9 and 2.1-kb sex-specific RNAs and the minor 2.5-kb RNA) are missing in exu^{VL57} flies. The fact that pC8 hybridizes to both the female and male specific RNAs reveals that these transcripts are at least partially overlapping. The basis of this overlap could be due to differential RNA processing in males and females, or utilization of different promoters and/or polyadenylation sites; current experiments are examining these possibilities. Since the 2.5-kb transcript is also detected by pc8, and since this transcript is missing in exu^{VL57} flies, we believe it is also transcribed

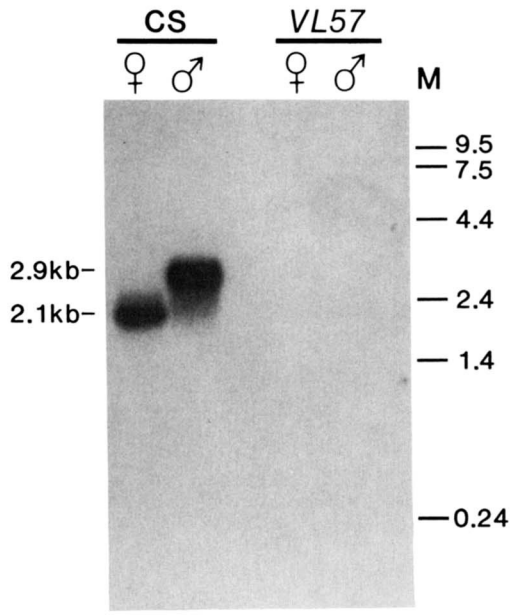


FIGURE 9.—Northern blot of wild-type *CS* and mutant exu^{VL57} male and female poly(A)$^+$ RNA. An aliquot of 5 μg of poly(A)$^+$ RNA was loaded in each lane. The blot was probed with pC8, a cDNA isolated from an ovary cDNA library (see RESULTS and Figure 6), and the autoradiogram was exposed for 1 day. In addition to the major transcripts indicated, this probe detects a minor 2.5-kb transcript. This blot was reprobed for actin RNA to show that RNA was present in the exu^{VL57} lanes, in amounts equal to those present in the *CS* lanes (data not shown).

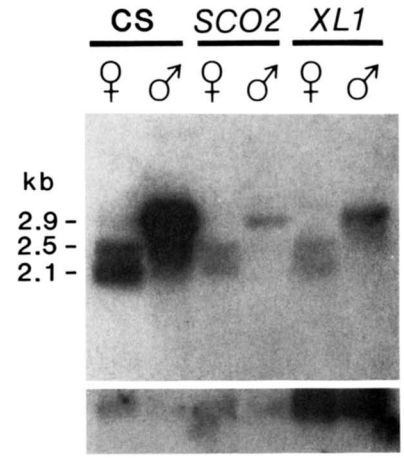


FIGURE 10.—Northern blot of RNA from mutant flies. Approximately 5 μg of poly(A)$^+$ were loaded in the CS (wild-type) and exu^{SCO2} lanes, and 30 μg were loaded in the exu^{XL1} lanes. The blot was probed with ^{32}P-labeled pc8 DNA, and the autoradiogram was exposed for 10 days. It was subsequently reprobed for rp49 RNA, which is shown in the bottom panel.

from DNA contained in the exu gene, but arguments presented below suggest it is not a transcript required for the germline functions of *exu*.

Adult poly(A)$^+$ RNA was isolated from the following exu mutants: exu^{PJ42}, exu^{SCO2}, and exu^{XL1}. Northern blots of these RNAs were probed with pc8; these results show that whereas exu^{PJ42} mutant flies have normal levels of the male and female transcripts, exu^{SCO2} and exu^{XL1} mutants have reduced levels of both transcripts (Figure 10). Using levels of rp49 RNA as internal standards, we estimate that in exu^{SCO2} mutants the levels of these transcripts are reduced to about 30–40% of wild-type, and in exu^{XL1} mutants the reduction is to about 5% wild-type.

The *tudor* gene is one of the grandchildless-knirps class of genes (BOSWELL and MAHOWALD 1985). Progeny from mutant *tudor* mothers are missing germ cells. Work by SCHÜPBACH and WIESCHAUS (1986b) has shown that wild-type expression of *exu* is required in the female germline. Mosaic females that have *exu* mutant germ cells produce progeny with the *exu* phenotype. Our observations of male-sterility in exu mutants leads us to expect that the gene is expressed in the germline of males as well as females. To determine if the transcripts we have observed in the region of phages 9 and 10 of our walk are germline transcripts, we isolated RNA from progeny of homozygous tud^1 females, and probed Northern blots of this RNA with pc8 (Figure 11). These flies are missing the 2.9- and 2.1-kb male and female-specific transcripts, showing that these transcripts are germline-specific. Long exposure of the same blot (data not shown) reveals that the minor 2.5-kb RNA is present in flies of both sexes, indicating that although this transcript is detected by the cDNA probe, it is not germline-specific. Thus, the minor 2.5-kb transcript is not likely to be the transcript required for the maternal and spermatogenic functions of *exu*.

The fact that the exu^{VL57} allele contains a 700 bp deletion, that exu^{VL57} mutants are missing the male and female specific transcripts, that exu^{XL1} and exu^{SCO2} mutants have reduced levels of these RNAs, and that these transcripts are germline-specific, strongly indicate that the 2.9-kb male RNA and the 2.1-kb female RNA transcribed in the region of overlap of phage 9 and 10 are the *exu* transcripts required for spermatogenesis in the male, and formation of oocytes with normal anterior-posterior polarity in the female.

DISCUSSION

Two genes in Drosophila have been identified which affect localization of the *bcd* RNA. Mutations in both *exu* and *swv* have maternal effects on the early development of embryos, and eggs from mutant mothers have been shown to have abnormal *bcd* RNA localization (FRIGERIO *et al.* 1986; BERLETH *et al.* 1988; STEPHENSON, CHAO and FACKENTHAL 1988). There are several notable differences in the phenotypic effects of *exu* and *swv* mutations (FROHNHOFER and NÜSSLEIN-VOLHARD 1987). Alleles of both genes have more extreme effects at low temperatures, and the different phenotypic effects of the two genes are more pronounced at lower temperatures. In the case of *swv*, embryos produced by mutant mothers have abnormalities in syncytial nuclear migrations, head

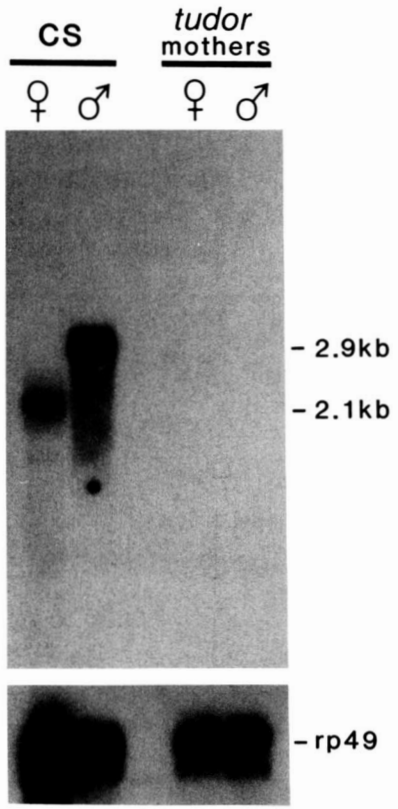


FIGURE 11.—Northern blot of RNA from flies lacking germ cells. Approximately 5 μg of poly(A)$^+$ RNA was loaded in each lane. The lanes labeled tudor mothers are RNA from flies produced by homozygous *tud*^{1} mothers. These flies are missing germ cells. The blot was probed first with a probe made from pc8 DNA, the autoradiogram was exposed for 2 days, and subsequently the blot was reprobed for rp49 RNA (bottom panel).

defects, and a high frequency of abdominal defects (STEPHENSON and MAHOWALD 1987). In contrast, *exu* mutant mothers produce embryos without the nuclear migration defects, and also with a lower frequency of abdominal defects. In addition, embryos from *exu* mothers duplicate posterior gastrulation events anteriorly, which leads to ectopically appearing posterior structures at their anterior ends (SCHÜPBACH and WIESCHAUS 1986a). We have shown in this paper that *exu* has an additional role in spermatogenesis. It is not known if *swv* plays a role in spermatogenesis. The extant *X*-linked mutations are all male fertile, but were isolated in screens that demanded male fertility, and therefore could represent special alleles of *swv*. Thus, although the products of both genes are needed for correct localization of the *bcd* RNA, the phenotypic differences suggest that the products of these genes serve different functions in that process.

The *exu* gene was originally identified in genetic screens for maternal-effect mutations. X-ray induction of germline clones of *exu*$^-$ cells has shown that the maternal phenotype is germline autonomous (SCHÜPBACH and WIESCHAUS 1986b). The observations reported here show that the exu gene also apparently functions in the male germline, since six of the seven existing alleles are male-sterile. Mutant males do not produce functional sperm. The first defects we have observed in spermatogenesis occur at meiosis, with the production of many micronuclei. However, the defects are not simply meiotic, since subsequent stages of spermatogenesis are also abnormal. Molecular data supports the phenotypic data; a male-specific as well as a female-specific transcript are produced by the gene. We have shown that these transcripts are limited in expression to the germline, since flies without germ cells are lacking these sex-specific transcripts. The data presented here shows that these transcripts are overlapping. Another maternal-effect gene, *vasa*, is expressed in the male germline (HAY, JAN and JAN 1988) but has no known function in spermatogenesis (LASKO and ASHBURNER 1988). Thus, among genes identified for maternal effects, *exu* mutations are unusual, and may be unique, in that they produce a maternal-effect phenotype in the developing embryos from mutant mothers, and also alter spermatogenesis so that normal sperm are not produced.

Of particular interest to us is the *exu*^{PJ42} mutation, since it is like other *exu* alleles in terms of its maternal phenotype, but is fully male-fertile at 25°. We do not yet know whether the male and female specific *exu* transcripts encode different proteins in the two sexes. However, we do know that the transcripts are overlapping, and therefore are likely to share some (if not all) of their protein-coding regions. Analysis of the molecular nature of the *exu*^{PJ42} mutation may help to determine a region of the *exu* product which is more important for its maternal function.

Two models appear likely to account for the functions of *exu* in both male and female gametogenesis. The first is that the *exu* product is a cytoskeletal element which is utilized in the architecture of both sperm and eggs. By this model, the effect of *exu* mutations on *bcd* RNA localization in oocytes would be a secondary consequence of destabilizing a cytoskeleton which acts as an anchor for a protein (or RNA) which binds the *bcd* RNA. A second model is that the *exu* product binds directly to the *bcd* RNA and subcellularly localizes it in the oocyte, and that it also functions to subcellularly localize an RNA during spermatogenesis. In this latter model, the *exu* product could be associated with the cytoskeleton, or it could be a membrane component. Since the male and female *exu* transcripts are different, they may encode domains which recognize different RNAs. During Drosophila spermatogenesis there is a long period of growth of the primary spermatocyte, during which RNAs are transcribed which will be utilized later during the long and complex process of spermiogenesis (for review see LINDSLEY and TOKUYASU 1980). It is possible that

subcellular RNA localization may be a mechanism employed in spermatogenesis to yield a state of localized or graded concentrations of proteins. Thus, the *exu* product could be serving similar morphogenetic functions during both oogenesis and spermatogenesis.

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