courtless, the Drosophila UBC7 Homolog, Is Involved in Male Courtship Behavior and Spermatogenesis

Sara Orgad,* Galit Rosenfeld,* Ralph J. Greenspan† and Daniel Segal*

**Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel-Aviv 69978, Israel and †The Neurosciences Institute, San Diego, California 92121*

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ABSTRACT

The *courtless* (*col*) mutation disrupts early steps of courtship behavior in Drosophila males, as well as the development of their sperm. Most of the homozygous *col*/*col* males (78%) do not court at all. Only 5% perform the entire ritual and copulate, yet these matings produce no progeny. The *col* gene maps to polytene chromosome band 47D. It encodes two proteins that differ in their carboxy termini and are the Drosophila homologs of the yeast ubiquitin-conjugating enzyme UBC7. The *col* mutation is caused by an insertion of a *P* element into the 3′ UTR of the gene, which probably disrupts translational regulatory elements. As a consequence, the homozygous mutants exhibit a six- to sevenfold increase in the level of the COL protein. The *col* product is essential, and deletions that remove the *col* gene are lethal. During embryonic development *col* is expressed primarily in the CNS. Our results implicate the ubiquitin-mediated system in the development and function of the nervous system and in meiosis during spermatogenesis.

THE genetic foundation of complex, programmed behaviors has been assumed by biologists and ethologists ever since Darwin. Most studies of their interrelationship, however, have been carried out in organisms in which single gene analysis and molecular genetics are not readily available. An exception to this trend is the male courtship ritual of the fruit fly, *Drosophila melanogaster*, which is known to be affected by a variety of mutations (reviewed by Hall 1994; Yamamoto and Nakano 1998).

Sexual behavior in Drosophila is under the control of the sex determination pathway. Chromosomal females that are genetically transformed into males by mutations in *Sex-lethal*, *transformer*, or *transformer-2* display courtship behavior characteristic of males (McRobert and Tompkins 1985; Bernstein *et al.* 1992). These are genes that govern sex determination in a global way, affecting a wide array of sexual dimorphisms. It has generally been assumed that there are other genes governing somatic sex determination—less global but still multifunctional and pleiotropic—that act downstream from these to affect courtship behavior (Taylor *et al.* 1994). The first example of such a downstream gene is *fruitless* (*fru*; reviewed by Yamamoto *et al.* 1998), mutants of which cause males to court each other and to fail to attempt copulation with females (Gailey and Hall 1989) and also to lack a male-specific muscle in the abdomen (Gailey *et al.* 1991). The phenotypes of *fru* suggest that such downstream genes may indeed be pleiotropic.

Corresponding author: Daniel Segal, Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel-Aviv 69978, Israel. E-mail: dsegal@ccsg.tau.ac.il

Pleiotropic genes affecting both behavior and the execution of sex determination have been found previously in the mouse. The well-known *weaver* mutant affects not only ataxia but also spermatogenesis (Vogelweid *et al.* 1993). Likewise, the mutants *Purkinje cell degeneration* (Mullen *et al.* 1976), *hotfoot* (Gordon *et al.* 1990), and *quaking* (Bennett *et al.* 1971) all affect motor coordination and male fertility. Beyond the connection shown in these mutants, a variety of cloned genes in different organisms have been found to show expression common (and in some cases restricted) to brain and testes. These include the *Wilms' tumor* gene (Sharma *et al.* 1992), the *Xwnt-4* gene (McGrew *et al.* 1992), the *c-kit* and *Sl* gene products (Motro *et al.* 1991), and the *Oct-2a* and *Oct-2b* genes (Hatzopoulos *et al.* 1990).

Such genes have been described in Drosophila as well. An example of a gene affecting both behavior and sexual differentiation is *dunce*, of which all of the available alleles disrupt associative learning and cause female sterility (although males are fertile; Bellen and Gregory 1987). Several male sterile mutations affecting mitochondrial aggregation during spermatogenesis display in addition a common behavioral defect in that they shake their appendages abnormally (Lifschytz and Hareven 1977).

We report here a new mutation, *courtless* (*col*), in which homozygous mutant males have a very low probability of courting females and are also defective in spermatogenesis. The *col* locus encodes the Drosophila homolog of the yeast ubiquitin-conjugating enzyme UBC7 (Jungmann *et al.* 1993). The *courtless* mutant, together with a previously described neurological mutant in the fly, *bendless* (*ben*), affecting a different ubiquitin-conjugating

enzyme (Muralidhar and Thomas 1993), suggests a surprisingly sophisticated and selective role for ubiquitin-mediated protein degradation or modification in the development and function of the nervous system.

MATERIALS AND METHODS

Fly stocks and genetic crosses: All stocks were reared, flies were aged, and crosses were maintained at 25° on standard cornmeal-agar-molasses medium. Canton-S served as a wild-type control. Except for *col*, genetic markers and balancer chromosomes used are described in Lindsley and Zimm (1992). The *col* strain (original name *ms(2)P[ry$^+$]4 cn/CyO; ry/ry*) is one of the *P*-element-induced mutants generated by the Spradling laboratory, by mobilization of the Carnegie-20 vector (Rubin and Spradling 1983). It was obtained from the Drosophila Stock Center at Bloomington, Indiana. *P[ry$^+$] col cn/P[ry$^+$]col cn; ry/ry* flies are referred to hereafter as *col* homozygotes.

A strain carrying a multiply marked second chromosome, *al nub lt stw sca sp/CyO; ry/ry*, was used for recombination analysis. *P[ry$^+$]col cn/al nub lt stw sca sp; ry/ry* females were crossed to *al nub lt stw sca sp/CyO; ry/ry* males. Resultant males that were recombinant for the second chromosome markers or for the *P*-element-associated *ry*$^+$ marker were individually backcrossed to *al nub lt stw sca sp/CyO; ry/ry* females and a line, balanced over *CyO*, was generated from each recombinant male. Males from each line were examined for fertility and courtship behavior.

To generate revertants, as well as new alleles of *col*, we mobilized the *col*-associated *P* element by crossing *col* homozygous females to males from a strain bearing a source of transposase, *Sp/CyO; Sb ry Δ2-3(99B)/TM6, Ubx e*, according to Robertson *et al.* (1988). Resultant *col/CyO; Sb ry Δ2-3(99B)/ry* males were crossed to *col/CyO; ry/ry* females and progeny from this cross were scored for presumptive excision of the *P* element by screening for loss of the *ry*$^+$ eye color marker. Thirty such independent putative *col^{ex}/CyO; ry/ry* lines (where *ex* represents presumed excision of the original *P* insert in the *col* gene) were recovered and a stock was established from each of them by balancing over *CyO*. They were backcrossed several times to *col/CyO; ry/ry* to obtain a genetic background similar to the original *col* mutant. To facilitate identification of homozygous mutant embryos and larvae, the original *col* mutant and the excision lines were balanced over either *CyO, wg$^{en11\text{-}lacZ}$* (FlyBase 1999) or over *CyO, pAct-GFP* (Reichhart and Ferrandon 1998).

Transgenic flies were generated by subcloning a 4.3-kb genomic fragment containing the *col* gene plus 1 kb of 5′ sequences and 1 kb of 3′ sequences into the CaSper vector and transforming it into embryos following standard procedures.

Behavioral tests: 1. Courtship: Males were collected upon eclosion and were maintained individually in separate vials for 3 days till sexual maturation. On the 4th day each was transferred by aspiration, avoiding anesthesia, into a cylindrical Plexiglas mating chamber and was presented with a wild-type (Canton-S) virgin female. The courtship behavior of the pair of flies was monitored until they copulated, or for 30 min, whichever occurred first. The behaviors that the male performed were recorded and the courtship index (C.I., the fraction of the observation period, expressed in percentages, during which the male performed any courtship activity) was calculated (Gailey *et al.* 1986). At the end of the observation period, the males were returned to separate vials for 24 hr and were retested with fresh virgins. After each trial the mating chamber was washed, rinsed with ethanol, and air dried.

2. Olfaction: A trap assay that tests the ability of flies to detect and migrate toward a source of olfactory attractant was used (Woodard *et al.* 1989). Briefly, 10 flies of a given sex and genotype were placed in a petri dish containing a microfuge tube that holds the attractant. Flies can reach the attractant only by passing through a narrow orifice. This trap was constructed by cutting off the narrow end of a microfuge tube and placing in it the narrow end of a truncated micropipette tip. Another truncated micropipette tip is placed over the first with its narrow end pointing out. The tip whose narrow end points outward makes entry into the trap difficult, and the other tip interferes with flies exiting the trap, once they have entered. Response to the attractant is measured by counting the number of trapped flies 72 hr later. The assay was performed four times on wild-type and four times on mutant flies. Ten flies were assayed each time.
3. Vision: (a) For each genotype, flies (five groups of 20 each) were placed in a dark vial. The flies were allowed to go into a Y-maze composed of one dark vial, and another vial, whose bottom 90% was covered with aluminum foil. Light was shined onto the uncovered part of that vial for 1 min, and the number of flies in each vial was determined. (b) Electroretinograms were performed according to Minke *et al.* (1975).
4. Locomotion: Locomotor activity of *col/col* homozygous flies was compared to that of wild-type males according to Griffith *et al.* (1993).

Testis analysis: Testes were dissected from 10 males of each genotype, prepared, stained, and visualized by phase contrast and light microscopy according to Lifschytz and Hareven (1977).

Library construction and screening: A genomic library was constructed from *col* homozygous flies in the λZapII vector (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. The library was screened with a probe comprising a 580-bp *Hin*dIII fragment that contains *P*-element sequences derived from the Carnegie 20 vector (Rubin and Spradling 1983). Genomic DNA flanking the *P*-element insert was identified by Southern analysis and was used as a probe for screening a genomic library of wild-type Drosophila, as well as two adult head cDNA libraries (gift of Dr. Steve Russell, Cambridge University). Library screening was performed by standard methods (Sambrook *et al.* 1989) under stringent conditions.

Northern analysis: Poly(A)$^+$ RNA was isolated from males and females of the following genotypes: *col/col, col/CyO*, and wild type (Canton-S) using an RNA purification kit (Promega, Madison, WI), according to the instructions of the manufacturer. A total of 10 μg of poly(A)$^+$ RNA from each preparation was resolved by electrophoresis on a 1.4% formaldehyde-agarose gel, blotted to Hybond (Amersham, Arlington Heights, IL), and probed with class 1a *col* cDNA or with the retained fourth intron. A probe of the ribosomal protein gene rp49 served as an internal control for normalizing the amounts of RNA loaded.

***In situ* hybridization:** Hybridization to larval polytene chromosomes was carried out according to Engels *et al.* (1986). Hybridization to whole-mount embryos was essentially as described by Tautz and Pfeifle (1989). Probes for all *in situ* hybridizations were labeled by alkaline phosphatase-conjugated digoxygenin (Boehringer, Indianapolis). Labeling conditions were according to the instructions of the manufacturer.

PCR analysis: Primers corresponding to the *P* element and to sequences along the *col* cDNA were used in PCRs (Saiki *et*

al. 1988) to determine the site of the *P*-element insertion in *col* homozygous flies. Primers (5′-GTGCGTCACCGTCAATAC 3′), corresponding to position 706–724 (Figure 5), and 5′-CTAGATAAACTTGAGTTAG-3′, position 848–867, were used to amplify the region containing the fourth intron. Several primers corresponding to different positions along the *col* transcript were used to define the extent of the deletion in the *col* excision lines.

DNA sequencing: Inserts of cDNA clones corresponding to the *col* gene were subcloned into Bluescript (Stratagene) and were subjected to exonuclease III digestion to generate a series of nested deletions. Sequencing was carried out by the dideoxy chain termination method (Sanger *et al.* 1977) using Sequenase version 2.0 (United States Biochemical, Cleveland). Specific primers were designed to fill in the gaps. The sequence was determined on both strands. Protein databases were searched using the FASTA program (Pearson and Lipman 1988).

Primer extension: One picomole of 5′-γ-^{32}P-end labeled antisense primer (5′-GGCGGCAATCGCGAACTTTC-3′) corresponding to the 5′ end of the *col* cDNA was annealed to 10 μg poly(A)$^+$ RNA from adult wild-type (Canton-S) flies and was reverse transcribed using AMV reverse transcriptase (Boehringer) according to the instructions of the manufacturer. The same primer was used for sequencing the genomic 4.3-kb *Sac*I fragment containing the *col* gene. Reaction mixtures were loaded on a 6% polyacrylamide gel.

Antibodies: 1. The coding region of class 1a *col* cDNA was cloned into the pET-28a expression vector (Novagen) and transformed into BL21 bacteria. The resulting His-tagged COL protein was purified on Ni-NTA agarose and was used to immunize rabbits.

2. Two peptides, corresponding to the carboxy termini of the two putative COL proteins (Figure 4B), were synthesized, coupled to KLH, and injected into rats.

Immunoblot: Fifty males from each of the genotypes *col*/*col*, *col*/*CyO*, and wild type (Canton-S) were homogenized in 250 μl of 2× protein loading buffer, boiled for 10 min, and centrifuged. The same procedure was used for preparing embryonic or larval extracts. Protein concentration was determined using the Bio-Rad (Richmond, CA) protein assay reagent. Equal amounts of protein were resolved on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and incubated with a 1:100 dilution of the antibodies generated with the class 1a *col* cDNA. Peroxidase-conjugated anti-rabbit IgG and the ECL system (Amersham) were used for visualization.

Immunohistochemistry: Immunohistochemistry was performed as described in Zhou *et al.* (1995), using a rat polyclonal antibody directed against the C-terminal part of COL1 at a 1:500 dilution. Visualization of antibody binding was performed using rabbit anti-rat IgG coupled to horseradish peroxidase.

The sequence data presented in this article have been submitted to the EMBL Data Library under accession no. AJ277746.

RESULTS

Courtship behavior is abnormal in *col* homozygous males: Direct screening of a large population of mutagenized flies for males displaying aberrant courtship behavior is very laborious. Therefore, our strategy was to examine courtship behavior among available male sterile mutants. We tested 64 *P*-insertional male sterile lines obtained from the Drosophila Stock Center, Bloomington, Indiana and have identified three that courted abnormally (Orgad *et al.* 1997). One of these lines (*ms(2)P[ry$^+$]4*), which we call *courtless* (*col*), is the subject of this article.

When wild-type (Canton-S) mature males are presented with virgin females, they initiate the courtship ritual almost instantaneously, spending most of their time performing the different courtship steps, culminating in copulation of >90% of them (Figure 1A). In homozygous *col* males, on the other hand, 78% of the males tested did not court at all, and 17% performed only some of the courtship steps. Most of these courting mutant males (13%) performed only early steps of courtship (orienting, following, and wing extension; Figure 1A). Only 5% of the *col*/*col* males eventually copulated, yet these matings gave no progeny. The C.I. representing the fraction of the observation time that each male actually spent courting was 72% for wild-type (Canton-S) males and only 12% for *col* homozygous males (breaking down this value for the courting and noncourting mutant males gives a C.I. value of 0 for the noncourting and of 23% for the courting). In contrast, *col*/*col* females are fertile when mated to wild-type (Canton-S) males, behave normally, and are as receptive to males as are wild-type females.

Blind or olfaction-defective mutants in Drosophila are able to court and mate with a C.I. of ~50% of that of the wild type. Double mutants that are blind and olfaction defective have a C.I. of only 7% of the wild type (Hall 1981). We were interested in excluding the possibility that the low C.I. obtained for *col* homozygous males is due to a combined effect of defective vision and olfaction. We tested the visual response of the *col* mutants. Homozygous *col* flies were attracted to light to the same extent as wild-type (Canton-S) flies. The number of flies attracted to light was 17 ± 2 for wild-type (Canton-S) flies, 16 ± 2 for *col*/*CyO*, and 15 ± 2 for *col*/*col* (see materials and methods). A similar conclusion was drawn from examination of electroretinograms of *col* homozygous mutant flies and *col*/*CyO* flies as compared to wild-type (Canton-S) flies (Figure 1B). Likewise, no gross defect in the olfactory response of *col* homozygous males was recorded, using the trap assay (Woodard *et al.* 1989; see materials and methods). Two olfactory attractants were used, Drosophila culture medium and wild-type (Canton-S) virgin females. In both cases no difference was observed in the olfactory response between *col* homozygous, *col*/*CyO*, and wild-type (Canton-S) flies, suggesting that olfaction defects are not a major cause of the altered courtship behavior in *col* (Figure 1C).

To rule out the possibility that the defective courtship behavior is due to general sluggishness of the mutant flies, we compared locomotor activity of *col* homozygous flies to that of *col*/*CyO* and wild-type (Canton-S) flies. No difference was observed between the locomotor activities of the three genotypes (Figure 1C).

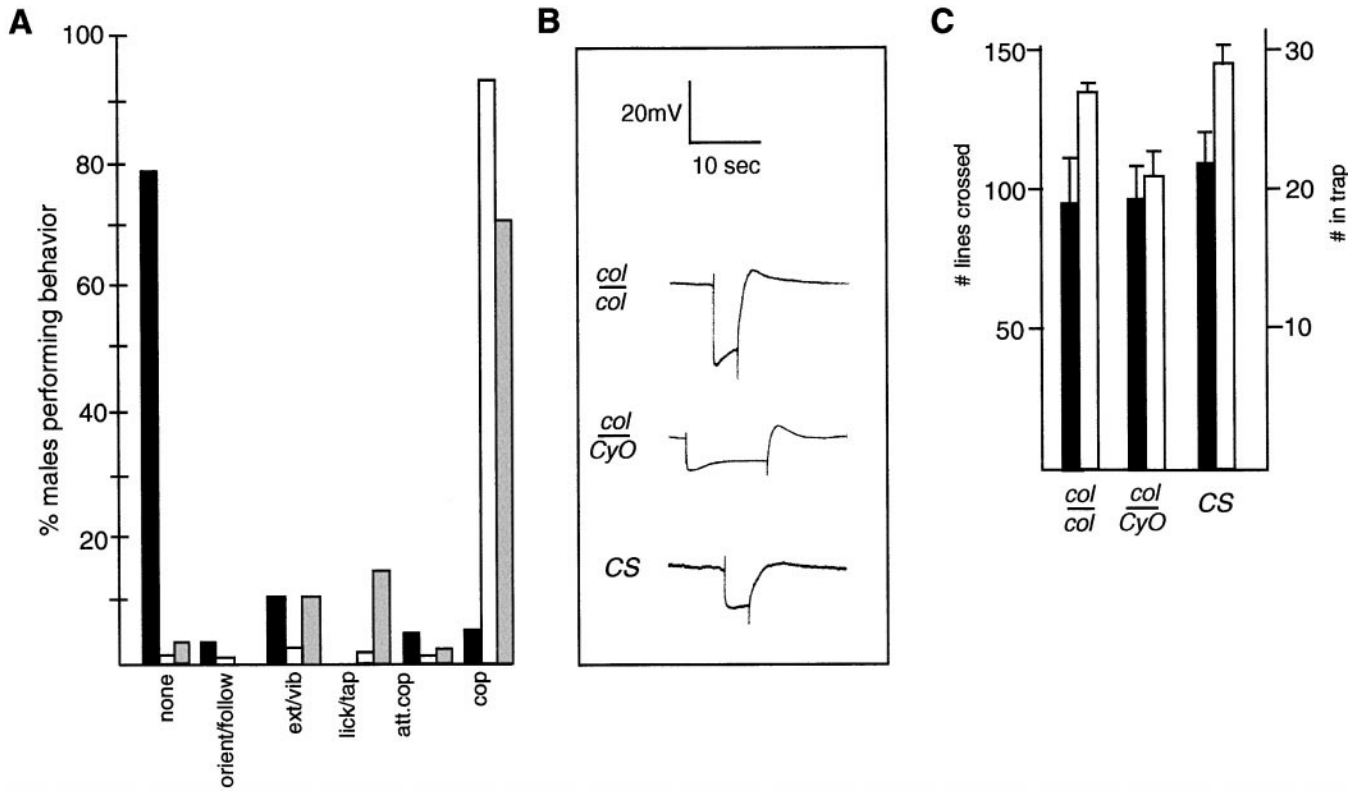


Figure 1.—(A) Profiles of courtship behavior. Three days after eclosion, *col* homozygous males (solid bars, $n = 86$), wild-type (Canton-S) males (open bars, $n = 104$), or *col*/*CyO* heterozygous males (shaded bars, $n = 73$) were confronted with wild-type (Canton-S) virgins and courtship behavior was monitored. Horizontal axes represent the most advanced step of courtship displayed by each individual male: (none) no male courtship behavior observed; (orient/follow) orienting toward and following the female; (ext/vib) wing extension and vibration; (lick/tap) licking the female's genitalia and/or tapping her abdomen; (att.cop) attempted copulation; (cop) copulation. A fly carrying out any indicated step of courtship must have carried out all prior steps in the courtship sequence. (B) Electroretinogram. Electroretinograms of *col*/*col* homozygous males, *col*/*CyO* heterozygous males, and wild-type (Canton-S, CS) males are shown. (C) Olfaction (open bars) and locomotor activity (solid bars) of *col*/*col*, *col*/*CyO*, and Canton-S. Five groups of 10 flies each were tested for each value given, and standard errors are shown.

Spermatogenesis is defective in *col* homozygous males: In Drosophila spermatogenesis, four gonial mitotic divisions of the primary spermatogonial cell produce a cohort of 16 cells, which remain connected by cytoplasmic bridges throughout spermatocyte development and spermatid differentiation (Fuller 1993). Two consecutive meiotic divisions result in a cyst containing 64 haploid spermatids. Many intracellular morphogenetic events take place, leading to a dramatic change in the shape of the spermatids, whereby both the cells and the nuclei elongate. Nuclear elongation is accompanied by chromatin condensation, and in the mature sperm the nucleus is shaped as a slightly curved needle. The last stage of spermatogenesis is individualization and coiling (Fuller 1993).

Microscopic examination indicates that in homozygous *col* males the four mitotic divisions of the primary spermatogonial cells occur normally, and cysts containing 16 primary spermatocytes are evident. However, the two consecutive meiotic divisions that should follow do not take place, and no cysts with 32 or 64 haploid spermatids are found. The primary spermatocytes undergo an immediate transition to elongated spermatids that have rather long tails, and heads that are larger in size and different in shape than those of normal spermatids (Figure 2, C and D). The bundles of spermatids are not as well organized in the mutant cysts as in wild-type (Canton-S) males (Figure 2, A and B).

Generation of new *col* alleles: *In situ* hybridization to polytene chromosomes of mutant *col* larvae using the 0.58-kb *Hin*dIII fragment of the *P*-element sequence originating from Carnegie-20 as a probe (see materials and methods) revealed a single *P*-element insertion at band 47D on the right arm of the second chromosome (Figure 3).

Two approaches were taken to verify that the behavioral defect in the *col* mutant is caused by this *P*-element insert. First, we tested whether the ry^+ marker on the *P* element cosegregates with the behavioral defect. To that end, *P*[ry^+]*col cn*/*al nub lt stw sca sp; ry*/*ry* females were crossed to *al nub lt stw sca sp*/*CyO; ry*/*ry* males. Twenty-two lines were generated from individual resultant males that were recombinant for the second chromosome markers or for the *P*-element-associated ry^+ eye

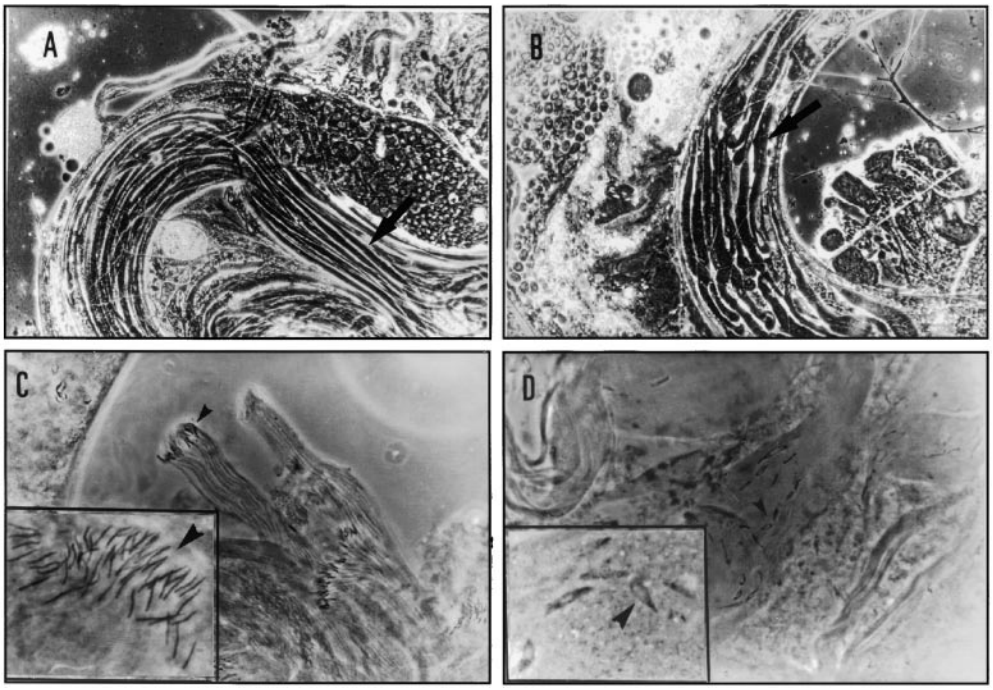


Figure 2.—*col* causes defects in sperm development. (A and B) Phase contrast micrographs of squashed testes from (A) wild-type (Canton-S) males, showing well-organized flagella in long parallel arrays within cysts (arrow), as opposed to (B) disorganization of the flagella within the cysts (arrow) in *col*/*col* males. (C and D) Higher magnification of orcein-stained testes from (C) wild-type (Canton-S) males showing several cysts in which needle-shaped and aligned nuclei in a spermatid bundle are apparent (arrowhead, and magnified in the inset) and from (D) *col*/*col* males focusing on one such abnormal cyst where defective sperm heads are apparent (arrowhead, and magnified in the inset).

color marker (see materials and methods). Fertility and courtship behavior were examined for homozygous recombinant males from these lines. The *P*-element-associated ry^+ marker cosegregated with both the behavioral defect and male sterility in all of the lines (data not shown). Second, we tested whether excision of this *P* element leads to restoration of fertility and normal courtship behavior. To that end, we mobilized the *P* element in the *col* strain by hybrid dysgenesis (see materials and methods), and screened for loss of the *P*-element-associated ry^+ marker. Males homozygous for the putative excision events were tested for their fertility

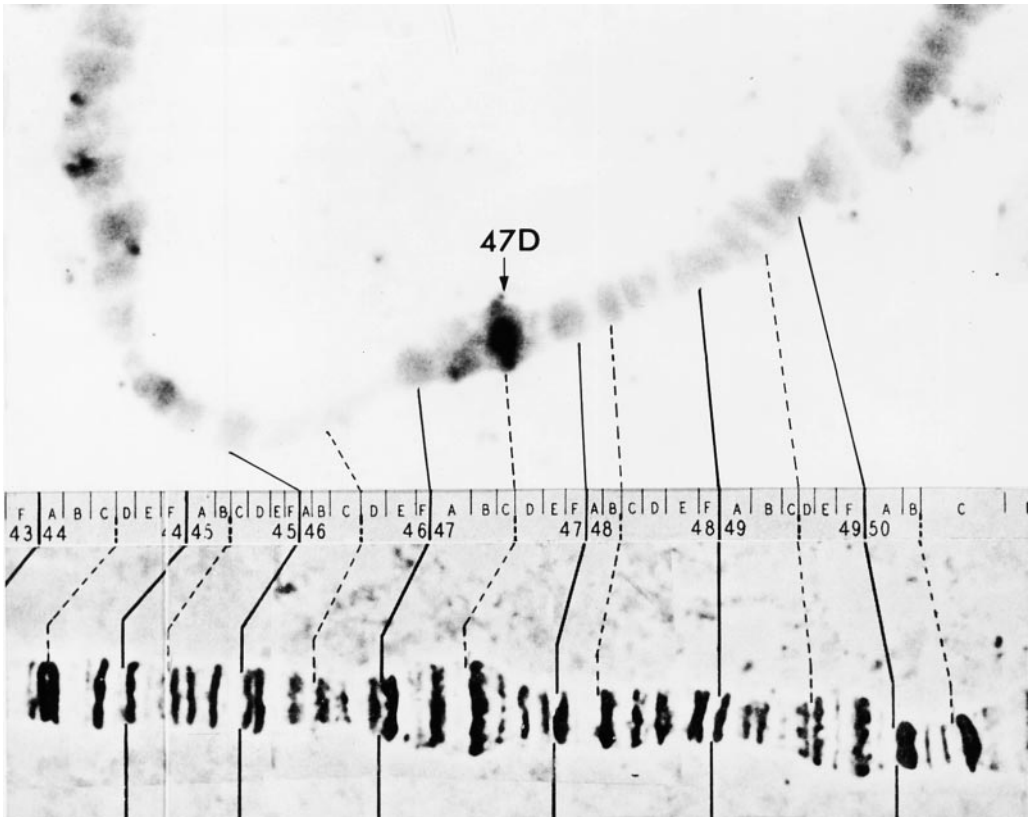


Figure 3.—Cytological localization of *col*. *In situ* hybridization to mutant larval polytene chromosomes using a digoxigenin-labeled *P*-element probe. *col* is located at band 47D on the right arm of the second chromosome.

TABLE 1

Nature of deletions in homozygous-lethal *col* mutants

Line no.	Size of deletion (kb)	*col* sequences deleted (kb)	Sequences deleted downstream of *col* (kb)
5-4	1.8	Entire *col* transcript	0.2
6-8	2	0.7 (3′ UTR + 64 codons)	1.3
52-13	1	1 (3′ UTR + 162 codons)	—
59-7	0.9	0.9 (3′ UTR + 158 codons)	—
60-7	1.1	1.1 (3′ UTR + 186 codons)	—

and courtship behavior. All of the 21 independent lines whose homozygous males were fertile and courted normally were found to be precise excisions of the *P* element, within the limits of Southern and PCR analyses (data not shown).

The mobilization of the *P* element in the *col* mutant resulted, in addition, in 28 independent lines that had lost the *P*-element-associated ry^+ marker but remained recessive male sterile and displayed the defective male courtship behavior typical of the original *col* mutant. They were found to have deletions internal to the *P* element, leaving most of it, as well as the flanking genomic regions, unaffected. Five additional excision lines obtained from this hybrid dysgenesis experiment were found to be homozygous lethal. Embryogenesis progresses normally in mutants of these five lines, and the lethal phase in all of them is between the first and the second larval instar.

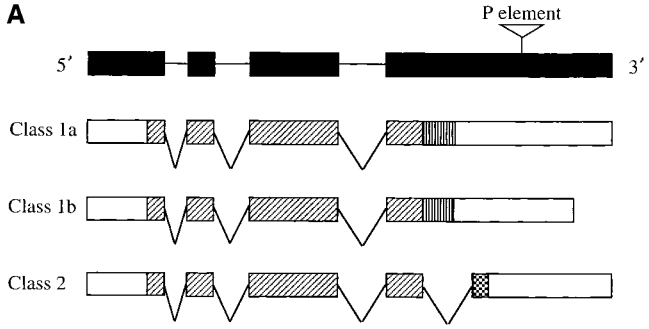
Heteroallelic males generated by crossing each of these homozygous lethals to the original *col* mutant exhibited normal courtship behavior (10 males from each heteroallelic combination were tested). The proportion of males copulating varied between the different heteroallelic combinations and ranged from 60 to 80% of the males tested; however, none produced offspring. Combined Southern and PCR analyses revealed that in all the homozygous lethal lines the excision of the *P* element was accompanied by deletion of genomic sequences flanking the insertion site, which ranged in size from 0.9 to 2 kb. In all of them, at least part of the *col* transcriptional unit was deleted (see Table 1 for the sequence details of each homozygous lethal line).

Cloning of the *col* gene: A genomic library was constructed from *col* homozygous flies and was probed with a 0.58-kb *Hin*dIII fragment of the *P* element. A positive clone containing a 5-kb genomic insert was isolated. Southern analysis of this genomic clone, using DNA of Carnegie 20 as a probe (see materials and methods), identified genomic sequences flanking the insertion site of the *P* element. These flanking sequences were used, in turn, as a probe to screen a genomic library of wild-type Drosophila, as well as adult head cDNA libraries (see materials and methods). Several genomic and cDNA clones were isolated. The intron-exon structure of the *col* gene and the insertion site of the *P* element were determined by a combination of Southern blots, PCR analysis, and DNA sequencing (see materials and methods). These revealed that the *col* gene spans only 1.65 kb and is composed of five exons separated by small (50–150 bp) introns. The *P* element has inserted 200 bp upstream from the end of the transcription unit, in the 3′ untranslated region (UTR) of the gene.

***col* is the Drosophila homolog of the yeast UBC7:** Five cDNA clones corresponding to the *col* transcripts were isolated from adult head cDNA libraries (see materials and methods). Sequencing of all of them revealed the existence of three classes of cDNAs. Classes 1a and 1b (1.3 and 1.1 kb long, respectively) both retain the fourth intron, and have the same open reading frame, which is capable of encoding a protein of 200 amino acids with a calculated molecular weight of 22,344 D (Figure 4A). The two cDNA classes differ in their 3′ untranslated region, with class 1b having a shorter 3′ UTR. Class 2 (1.1 kb long) is an alternatively spliced species, which is the result of splicing out of the fourth intron that is retained in the other two classes. Thus, the deduced class 2-derived protein is shorter, 185 amino acids long with a molecular weight of 20,390 (Figure 4A). The 3′ UTR of class 2 is identical to that of class 1a. The two predicted protein products of the *col* gene differ in their carboxy termini (Figure 4B).

Primer extension experiment assigned the transcription start site to the adenine at position 0 (Figure 5). The predicted TATA box is located 58 nucleotides upstream of the transcription start site (Figure 5). Several AU-rich sequences, one K-like box, and two Brd-like boxes are present in the 3′ UTR. These elements are known to confer a short half-life to transcripts (Chen and Shyu 1995; Lai and Posakony 1997; Lai *et al.* 1998). The Brd-box was shown to reduce translation as well (Lai and Posakony 1997).

Analysis of the predicted protein product of *col* revealed that it is highly homologous to the yeast ubiquitin-conjugating enzyme UBC7. This enzyme is a member of a large family of proteins that are found in all eukaryotes (Jentsch *et al.* 1990) and function in the covalent transfer of ubiquitin to specific protein substrates. A comparison of the sequence of the COL protein and



B

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COL1 MAGSALRRLMAEYKQLTLDPPEGIVAGPISEDNFFEWEALIAGPEGT
COL2 --------------------------------------------------

COL1 CFEGGVFPARLIFPTDYPLSPPKMKFTCDMFHPNIFADGRVCISILH
COL2 --------------------------------------------------

COL1 APGDDPMGYELSAERWSPVQSVEKILLSVVSMLAEPNDESGANVDAA
COL2 --------------------------------------------------

COL1 IMWREQRDEFNAIADGWCAKLLVYRRKWIMCVTVNTFYPEQHDSNNS
COL2 ----------------------------ITLTASPDLTGGPIT...

COL1 LTHSFLKNPSLS
COL2 ............
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Figure 4.—Structure of *col* transcripts and predicted COL proteins. (A) Structure of the three different classes of transcripts identified for the *col* gene. Solid line and boxes represent introns and exons, respectively. Coding sequences shared by all three classes are indicated by hatched boxes. Open boxes represent the untranslated regions of the transcripts. Striped boxes indicate the alternatively spliced intron (intron 4). The checkered box represents the different carboxy terminus of the transcript in which the fourth intron has been spliced out. (B) The two predicted COL1 and COL2 proteins differing in their carboxy termini are shown. Dashes represent identical residues. Dots represent the 15 C-terminal residues where COL1 is longer than COL2.

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-71  tcgattcattcgatataaataaacacggaacaccgtttgtgtttattatattacgacgtg
-11  tctgtattttgACCCGTGCCCGAGGATCTGGAAAGTTCGCGATTGCCGCCAAAACAAGCA
 50  AGGACCCCATTCGGGTGTAGCAGGTGCAGGAGCAGGTGGACAGGAAACGGAGAAGCAGCT
110  GAAGGAAACTCAAAGGAAGTGGTCACAGTGGGGAGAAGGAGCCCGTGAACTGAACCATCA
170  TCATTACCATCGAGCGCATTTAGGATGGCTGGGTCCGCACTGCGCCGCCTGATGGCGGAA
                          M  A  G  S  A  L  R  R  L  M  A  E
230  TACAAACAGTTAACACTTGACCCGCCCGAGGGCATTGTGGCCGGCCCCATCAGCGAGGAC
     Y  K  Q  L  T  L  D  P  P  E  G  I  V  A  G  P  I  S  E  D
290  AACTTCTTCGAGTGGGAGGCACTGATTGCCGGACCTGAGGGCACTTGTTTCGAGGGCGGA
     N  F  F  E  W  E  A  L  I  A  G  P  E  G  T  C  F  E  G  G
350  GTGTTTCCTGCCCGGCTCATCTTTCCGACCGACTATCCTCTGAGTCCGCCTAAAATGAAA
     V  F  P  A  R  L  I  F  P  T  D  Y  P  L  S  P  P  K  M  K
410  TTCACTTGTGACATGTTCCATCCCAACATATTCGCCGACGGGCGGGTCTGCATATCAATA
     F  T  C  D  M  F  H  P  N  I  F  A  D  G  R  V  C  I  S  I
470  CTACACGCACCCGGCGACGATCCGATGGGCTACGAGCTATCCGCGGAGCGCTGGAGTCCT
     L  H  A  P  G  D  D  P  M  G  Y  E  L  S  A  E  R  W  S  P
530  GTCCAGAGCGTGGAGAAGATTTTGCTTAGTGTGGTCAGCATGCTGGCGGAACCCAACGAT
     V  Q  S  V  E  K  I  L  L  S  V  V  S  M  L  A  E  P  N  D
590  GAGAGCGGCGCTAATGTGGATGCAGCAATTATGTGGCGCGAACAGAGGGATGAGTTCAAC
     E  S  G  A  N  V  D  A  A  I  M  W  R  E  Q  R  D  E  F  N
650  GCCATCGCCGACGGCTGGTGCGCAAAACTCTTGGTTTACCGGCGTAAATGGATCATGTGC
     A  I  A  D  G  W  C  A  K  L  L  V  Y  R  R  K  W  I  M  C
710  GTCACCGTCAATACTTTCTACCCTGAACAACACGATTCCAACAACTCGCTAACCCATAGT
     V  T  V  N  T  F  Y  P  E  Q  H  D  S  N  N  S  L  T  H  S
770  TTTCTCAAAAATCCCTCGTTGAGCTAACCGACACAAGTTTCAAATTAGCAAAAATGAAGA
     F  L  K  N  P  S  L  S  *
830  ACCTAACGTCAAAATCGACTAACTCAAGTTTATCTAGCACACTGACTGCCTCACCGGACT
890  TAACTGGAGGGCCTATTACCTGAAGTACATCACTGGTAGTTGGACACGAGGCCAGGTGAA
950  GGCGGAGTGAGAAGTTGGCAAGAAAAGTGAAAATTCTAATAATTTCTATTATTAAAGGTT
1010 TATTTTTTTTATATATTTTCACTAGATATAATTTGTATTGATGCTTTATAAAGATGCTGT
1070 TTTGAAAAGTTAAATAGAAATTACGATAATTCAAGTGCAAGGGCAAAGTATTCGAAGTGA
1130 TTGAGGCGTTTGATTTGCATTATGCTTTATGTTTGGTTTATATAGTCATTACACACTTAA
1190 TTCGACTGATCATTAACTGAAATCCAACATGAAATTGTCCTATGTACTCATAAAATACTA
1250 CACAATTGTAAACGTTTCATTCGATTAAGTAAATTGATTAATGTTCACTTTCGATAAAAA
1310 AAAAAAAAAAAAAAAAAAAAAAA
```

Figure 5.—Nucleotide and predicted amino acid sequences of the *col* cDNA. The longest *col* cDNA isolated has 1332 nucleotides. The open reading frame extends from nucleotide 195 to 794 and is capable of encoding a protein of 200 amino acids. The predicted TATA sequence is boxed. The transcription start site, identified by primer extension, is indicated by an arrowhead. The alternatively spliced fourth intron is underlined and the corresponding donor and acceptor splice signals are doubly underlined. The end of the shorter 3′ noncoding sequence is indicated by an arrow. The two Brd-like boxes in the 3′ UTR are boxed and in boldface letters. The K-box is in boldface letters and the AU-rich motifs are underlined with dashed lines. The cysteine corresponding to the putative active site, which is required for the formation of thiolester bond with ubiquitin, is circled.

UBC7 proteins from different organisms is shown in Figure 6. The COL sequence is 60% identical to the yeast UBC7, and taking into account conservative substitutions raises the similarity to 72%. It is 52 and 46% identical to the UBC7 of Arabidopsis and wheat, respectively, indicating high evolutionary conservation. The conserved cysteine residue at position 89 (Figure 5) is the putative active site for formation of a thiolester bond with ubiquitin, which is essential for the transfer of ubiquitin to the substrate (Pickart and Rose 1985).

Expression of the *col* gene: The *col* gene is expressed throughout Drosophila development, and its expression is developmentally regulated. In embryos two transcripts, 1.1 and 1.3 kb, are present in equimolar amounts. The smaller (1.1 kb) transcript is probably composed of a mixture of the two classes of *col* transcripts, which are similar in size (classes 1b and 2, Figure 4A). Expression of these transcripts declines in larvae but increases at the pupal stage, and even more at the adult stage (data not shown). We compared the level of expression of the *col* transcripts in males and females of *col*/*col*, *col*/*CyO*, and wild-type (Canton-S) flies by Northern blot. When the entire class 1a cDNA was used as a probe (potentially capable of detecting all three transcripts), the same two bands were visible in both sexes, although higher levels were found in males than in females in all three genotypes (Figure 7A; densitometry shows the difference to be 5-fold in *col*/*col*, 9-fold in *col*/*CyO*, and 17-fold in Canton-S). Densitometry further indicates that in homozygous *col* males the level of expression is four times lower than in wild-type (Canton-S) males. When the membrane was stripped and reprobed with the fourth intron, it became evident that classes

```
COL1     ....MAGSALRRLMAEYKQLTLDPPEGIVAGPISEDNFFEWEALIAGPEGTCFEGGVFPARLI
Scubc7   ....-SKT-QK--LK-LQ--IK-S-P------K--N-I-I-DC--Q--PD-PYAD---N-K-E
Taubc7   MATAP-RR-S..SSRSSSESRTT-SM-FQL-FVDDS-V---QVT-I--PE-LYD--Y-N-IMS
Atubc7   ....--SQ-SLL-QKQL-D-CKH-VD-FS--LVD-K-I---SVT-I--PD-LY---F-N-IMT

COL1     FPTDYPLSPPKMKFTCDMFHPNIFADGRVCISILHAPGDDPMGYELSAERWSPVQSVEKILLS
Scubc7   --K--------LT--PSIL----YPN-E-------S-----NM---AE---------------
Taubc7   --QN--N---TVR--SE-W---VYP--------.-P-----N----AS---T--HT--S-V--
Atubc7   --QN--N---TVR--S--W---VYS----------P-----S----AS---T--HT--S-M--

COL1     VVSMLAEPNDESGANVDAAIMWREQRDEFNAI....ADGWCAKLLVYRRKWIMCVTVNTFYPE
Scubc7   -M---S---I-----I--C-L--DN-P--ERQ....VKLSIL-S-GF.................
Taubc7   II---SS-----P--IE--KD---KQ---KKKVRRAVR-KSQEM-.................
Atubc7   II---SG-----P---E--KE--DK----KKKVSRCVR.KSQEMF.................

COL1     QHDSNNSLTHSFLKNPSLS
Scubc7   ...................
Taubc7   ...................
Atubc7   ...................
```

Figure 6.—Comparison of the amino acid sequence of COL and other UBC7s. Alignment of the predicted sequence of COL, UBC7 from yeast (Scubc7), wheat (Taubc7), and Arabidopsis (Atubc7) proteins. Identical residues are indicated by dashes. COL is 60, 46, and 52% identical to the yeast, wheat, and Arabidopsis proteins, respectively.

1a and 1b transcripts that retain this intron are male specific (Figure 7B). The only 1.3-kb cDNA we have isolated corresponds to the transcript that retains the fourth intron. Figure 7B indicates that such a transcript is not present in females, yet Figure 7A shows that females do have a 1.3-kb transcript. This indicates that at least one additional 1.3-kb *col* transcript has yet to be identified.

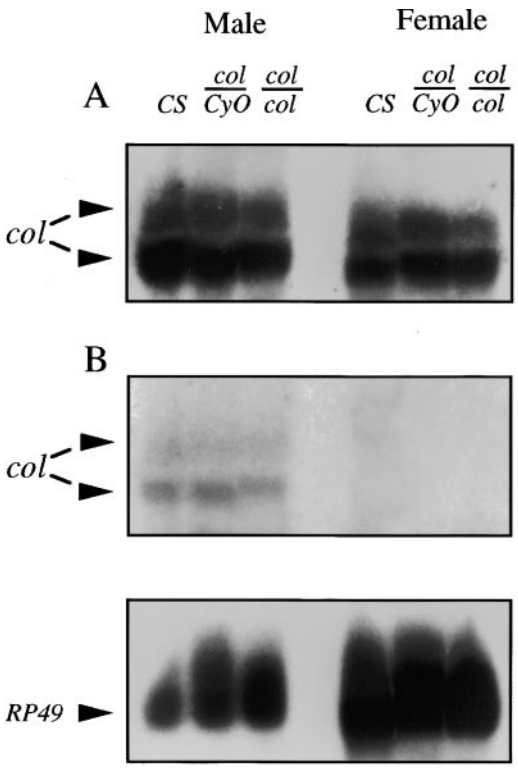

Figure 7.—Northern blot analysis of *col*. (A) Poly(A)$^+$ RNA (10 μg/lane) from wild-type (Canton-S), *col*/*col*, and *col*/*CyO* males and females was resolved on a 1.4% formaldehyde denaturing gel and was probed with Class 1a *col* cDNA. (B) The same RNA blot was stripped and reprobed with the alternatively spliced fourth intron. The amount of RNA loaded was standardized by comparison to the expression level of the Drosophila *RP49* gene.

The spatial distribution of *col* transcripts was determined by *in situ* hybridization of digoxigenin-labeled RNA probes corresponding to class 1a cDNA. Early in the blastoderm stage (*ca.* 2 hr of development), *col* transcripts are present both in the egg yolk, suggesting a maternal origin, and in the peripheral blastoderm cells, reflecting either maternal contribution or zygotic expression (Figure 8A). Subsequently (3 hr of development), they are found in the mesoderm and in the cephalic furrow (Figure 8B), and later (5 hr of development) in the extending germ band, in the neuroblasts, and in the stomodial invagination (Figure 8C). As development proceeds, in 15-hr-old embryos, it is confined to the central nervous system (Figure 8D).

COL protein expression: Class 1a transcript was modified to include a His-tag, and was expressed in *Escherichia coli*. The tagged protein was used to immunize rabbits. Western blot analysis using the polyclonal antibodies and extracts from male flies of the *col*/*col*, *col*/*CyO*, and wild-type (Canton-S) genotypes revealed elevated amounts of the COL protein in the mutant flies (Figure 9A). Homozygous and heterozygous *col* males expressed at least seven and three times, respectively, more of the COL protein than the wild type. When this experiment was repeated using extracts from embryos and larvae of one of the homozygous lethal excision lines of *col*, no difference was observed in the expression of the COL protein between the homozygous mutant and heterozygotes at the embryonic stage (Figure 9B), as expected of a transcript that is maternally deposited (Figure 8A). However, when extracts of first and second instar larvae were used, the COL protein was detected in the heterozygous larvae, but almost no protein was present in the homozygous mutant larvae (Figure 9C). The lack of the COL protein may account for the lethal phase

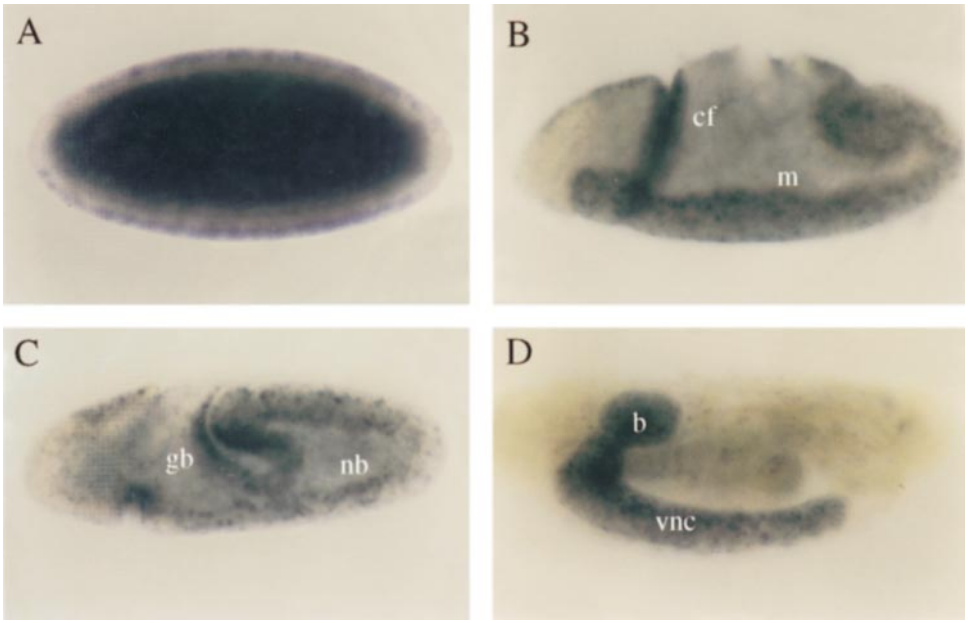

Figure 8.—Developmental expression of *col*. *In situ* hybridization of digoxigenin-labeled class 1a *col* cDNA to whole mounts of wild-type (Canton-S) embryos. (A) Maternally derived *col* transcripts are present in the egg yolk (stage 4). Note also expression in the peripheral newly formed blastoderm cells (~2 hr of development). (B) Expression in a stage 6 embryo (~3 hr of development) is seen in the mesoderm (m) and the cephalic furrow (cf). (C) A stage 10 embryo (~5 hr of development) expresses *col* in the extending germ band (gb), the neuroblasts (nb), and the remnants of the cephalic furrow. (D) Expression levels of *col* in stage 16 embryos (~15 hr of development) is exclusively in the CNS (b, brain, and vnc, ventral nerve cord). Anterior is to the left and dorsal is up.

of the excision line and provides a genetic control for the specificity of the antibodies.

The spatial distribution of the COL protein was determined using antibodies that were raised against the two different putative carboxy termini of the COL proteins (Figure 10). In general, the two antibodies revealed a similar pattern of COL expression, which was comparable to the distribution of the *col* transcript, as determined by *in situ* hybridization. However, at the blastoderm stage the COL protein is localized to the poles (Figure 10A), while the transcripts are present in the egg yolk and in all the peripheral blastoderm cells (Figure 9A).

Rescue of the *col* mutation: A 4.3-kb genomic fragment, which contains the *col* gene and ~1-kb upstream sequences and 1-kb downstream sequences, was inserted into the CaSper vector and was used to generate transgenic flies in an attempt to rescue the *col* mutation. Since homozygous *col* flies have elevated amounts of the COL protein, these transgenic flies carrying an extra copy of the normal *col* gene could not be used to rescue the original *col* mutant. Instead, the transgenic flies were used to rescue the lethality of the *col* excision lines. Two excision lines, $col^{52\text{-}13}$ and $col^{59\text{-}7}$, were crossed to three transgenic lines carrying the normal *col* gene. Two out of the three transgenic lines were able to rescue the lethality of these excision lines. Males homozygous for the $col^{52\text{-}13}$ mutation and carrying one copy of the *col* genomic transgene were tested for fertility and courtship behavior. These males courted with a C.I. value of 45%, considerably lower than wild-type (Canton-S) males (72%), but significantly higher than the C.I. of the original *col* homozygous males (12%). None of these males copulated during the observation period (30 min). The males produced no offspring and no motile sperm was observed in squashed testes preparations prepared from them.

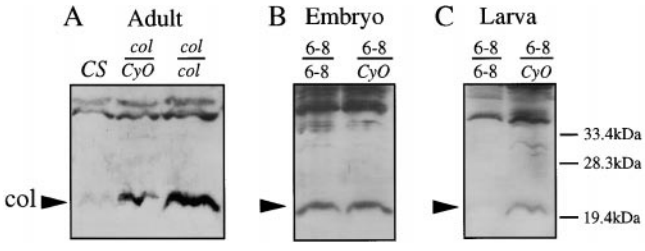

Figure 9.—Immunoblot analysis of the COL protein. (A) Protein extracts of *col*/*col*, *col*/*CyO*, and wild-type (Canton-S) adult males; or (B) extracts from embryos; and (C) larvae homozygous and heterozygous for one of the homozygous lethal excision lines of *col* (line $col^{6\text{-}8}$) were resolved on a 12.5% polyacrylamide gel and were probed with antibodies raised against the COL protein encoded by class 1a and class 1b *col* transcripts.

DISCUSSION

Genes at the top of the sex determination hierarchy participate in the control of courtship (reviewed in Yamamoto and Nakano 1998; Yamamoto *et al.* 1998). Besides these, known mutants that act earliest in the ritual are those that affect the courtship song—as in *cacophony* (Kulkarni and Hall 1987), *dissonance* (Kulkarni *et al.* 1988), and *croaker* (Yokokura *et al.* 1995). The *col* mutation blocks courtship at the very beginning of the sequence. Most homozygous *col* males do not court at all and those few that do generally fail to pro-

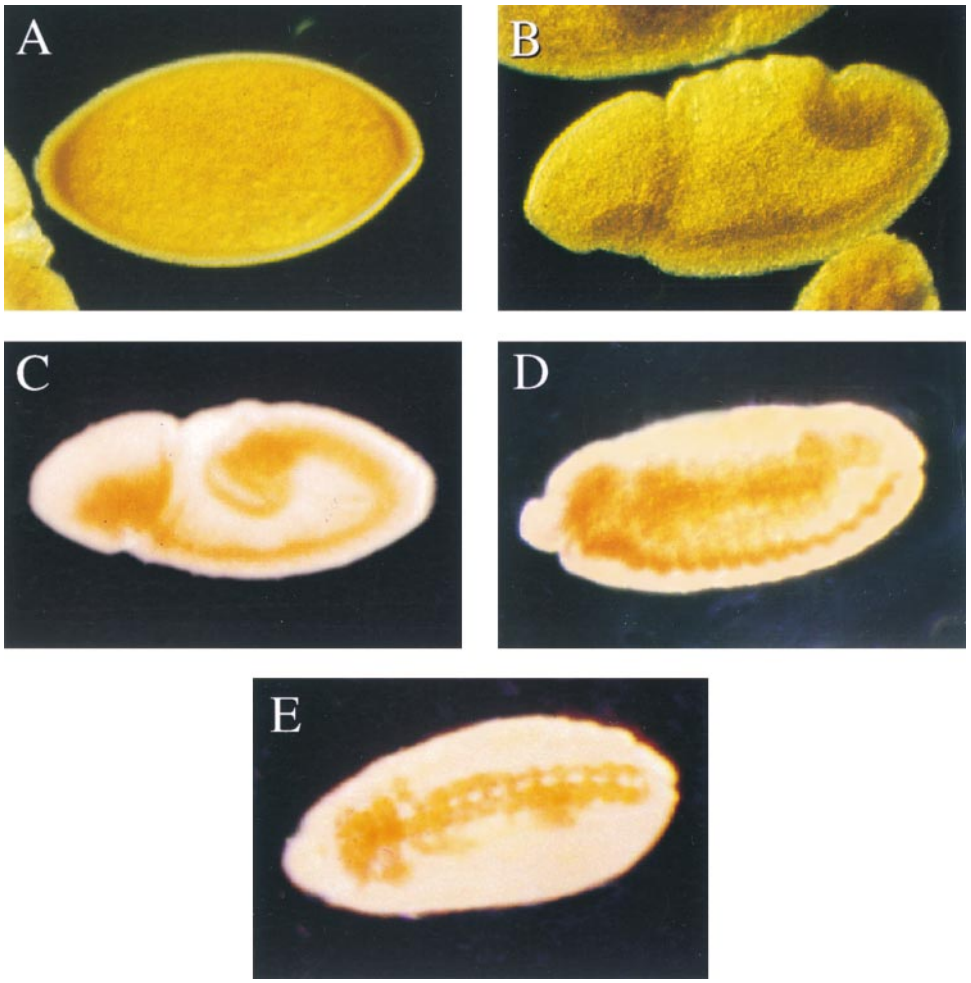

Figure 10.—Pattern of distribution of the COL protein. Antibodies raised against the 30-last carboxy-terminal amino acids of COL1 were used to determine the pattern of distribution of the COL protein in whole mounts of wild-type (Canton-S) embryos. (A) In a stage-4 embryo, the protein is localized to the poles. (B) In stage-7 and (C) in stage-8 embryos, the COL protein is apparent in the mesoderm and endoderm. (D) At stage 13, the neuromers and the supraesophageal ganglion are stained. (E) In stage-17 embryos, the COL protein is restricted to the CNS. A–D are lateral views where anterior is to the left and dorsal is up. E is a ventral view, same orientation.

gress beyond the early steps of following and wing extension. These early steps in courtship have been associated with male-specific development in the dorsal posterior brain (Hall 1979, 1994).

Mutants that are generally defective in locomotor behavior are also often defective in courtship (O'Dell 1993; Hall 1994). This sometimes manifests itself as a sluggish phenotype with a low probability of initiating courtship. However, the behavioral defect in *col* homozygous males is not due to general sluggishness since their locomotor activity is as high as that of their *col*/*CyO* sibling or wild-type males.

Visual (Cook 1980), olfactory (Markow 1987), rhythm (Gailey *et al.* 1991; Greencare *et al.* 1993), as well as learning and memory mutants (Hall 1982) have been found to court less rigorously than wild-type males and to have prolonged latency of the initiation of this behavior. However, the visual and olfactory systems in *col* homozygous flies appear to be normal and cannot account for the mutant phenotype of *col*. Therefore, the abnormal courtship behavior of the *col* mutant may be attributed to defects in those parts of the central nervous system (CNS) that are responsible for this behavior. A general feature of courtship mutants is their pleiotropic nature. However, the abnormalities they engender are usually not global but rather are restricted to a small number of particular behaviors (Hall 1994; Kyriacou and Hall 1994). For example, two mutants, *cacophony* and *dissonance*, isolated in a screen for mutants affecting the male courtship song were later, and rather surprisingly, found to be allelic to previously identified visual mutations. The former, although not visually defective, is an allele of the *nbA* gene, which, when mutated, causes poor performance in optomotor and phototactic tests (Kulkarni and Hall 1987; Smith *et al.* 1998), and the latter is allelic to the *nonA* mutation, which leads to absence of the light-on and light-off transient spikes, but does not affect the courtship song (Rendahl *et al.* 1992). Two additional examples for pleiotropy among courtship-defective mutants are *fruitless* (Wheeler *et al.* 1989) and *period* (Kyriacou and Hall 1980), both of which display at least two distinct behavioral abnormalities: one is a defect in courtship song, which is common to both mutants, and the other is abnormal circadian rhythm (in the case of *per*) or display of homosexual behavior (in *fru*). Given that courtship behavior is complex, utilizing most of the sensory modalities, it is not surprising that mutations

affecting vision, olfaction, and audition lead to aberrant courtship behavior.

The *courtless* mutation is pleiotropic too, affecting distinct systems such as the nervous system and spermatogenesis. While homozygous *col* females behave normally, are as receptive to males as wild-type females, and are fertile, homozygous *col* males hardly court virgin females and produce abnormal sperm. The defect in spermatogenesis in *col* homozygous males appears to be very similar to the phenotype reported for the *ms(1)413*, *ms(1)RD11*, and *ms(1)682* mutants (Lifschytz 1987). In all three, mitochondrial aggregation occurs prematurely, meiotic spindles are not formed, and the primary spermatocytes are transformed directly into tetraploid spermatids. Theses mutants and *col* fall into a distinct phenotypic class of spermatogenic mutants, which includes mutants in the genes for the cell cycle regulatory phosphatase *twine*, and the cell cycle kinase *cdc2* (Fuller 1998). In both mutants certain meiotic events are skipped, yet spermatid differentiation proceeds. As a result, testes of *twine* or *cdc2* males contain bundles of 16 4N-cells that grow flagellar axonemes and undergo DNA condensation and nuclear shaping, as we have observed in *col* males. A similar pleiotropy affecting both the nervous system and the reproductive tract was reported for the *dunce* mutation (Kiger *et al.* 1981). Several male sterile mutations affecting mitochondrial aggregation during spermatogenesis display in addition a common behavioral defect in that they shake their appendages abnormally (Lifschytz and Hareven 1977). An additional example of a Drosophila gene that encodes a component of the ubiquitin pathway, UbcD1, has been shown to affect meiosis in males (Cenci *et al.* 1997).

For normal courtship to occur, certain changes must take place in the CNS during its pupal-adult metamorphosis, and the subsequent reproductive maturation of the newly eclosed fly (Truman 1990) may entail specifically the dorsal posterior brain and the thoracic ganglion, known to be involved in normal sexual behavior (Hall 1979). The molecular details of these processes are largely unknown.

The *courtless* gene encodes the Drosophila homolog of the ubiquitin-conjugating enzyme UBC7. A distinctive property of the ubiquitin-mediated system is its remarkable functional diversity. It is implicated in various cellular functions including DNA repair (Jentsch *et al.* 1987), cell cycle control (Goebl *et al.* 1988), and transcription (Hochstrasser *et al.* 1991). The individual components of this system display remarkable functional specialization, suggesting narrow substrate specificities.

The *courtless* gene potentially encodes two proteins that share a conserved UBC domain, but have different carboxy extensions. The C-terminal extensions of UBCs are believed to contribute to the substrate specificity of these enzymes and to their intracellular localization (Sung *et al.* 1988). The *col* gene products are involved, at least, in two different processes, CNS development/function and spermatogenesis. Our results suggest that the level of the COL protein is critical for courtship. We found that *col*/+ males have approximately three times as much COL protein as wild-type males have and their courtship is normal. On the other hand, *col*/*col* males have at least seven times the COL protein that the wild-type males have and are highly defective in courtship. One speculation may be that excess COL protein may be compatible with normal courtship, as long as its level does not reach a certain threshold.

The *ben* product was the first to exemplify the involvement of the ubiquitin-mediated system in the development and function of the CNS of Drosophila (Muralidhar and Thomas 1993). Recently this system was implicated in the regulation of the circadian feedback loop of the fly (Naidoo *et al.* 1999). Our results suggest that the ubiquitin-mediated system is involved in additional aspects of CNS function, in those parts of the brain important for courtship behavior (mushroom bodies, antennal lobes, etc.). Our observations implicate this system, via the role of *col*, in spermatogenesis as well. They strengthen the finding that a mouse gene *A1s9*, which has been implicated in spermatogenesis, is homologous to the ubiquitin-activating enzyme E1 from yeast (Kay *et al.* 1991) and that the phenotype of mice knocked out for the mHR6B gene, the homolog of the yeast RAD6 ubiquitin-conjugating enzyme, is male infertility (Roest *et al.* 1996).

In Drosophila a single regulatory hierarchy controls all aspects of somatic sexual differentiation, including those parts of the CNS involved in sexual behavior. Differentiation of those aspects of the CNS responsible for male-specific courtship occurs during the middle of the pupal period (Belote and Baker 1987; Arthur *et al.* 1998), when the CNS undergoes extensive reorganization (Technau 1984; Truman 1990), concomitant with the accumulation of *col* transcripts. This set of genes, which determines the innate property of sexual behavior, has to be active continuously in the adult to maintain normal courtship behavior.

In adults, sexual differences were found in *col* expression, as determined by Northern blots, and the transcripts that retain the fourth intron are male specific. In embryos the *col* transcripts are found throughout the CNS with no obvious sex-specific expression. *In situ* hybridization to sectioned CNS from pupae and adults should allow us to identify whether *col* is expressed in those parts of the brain known to be involved in sexual behavior.

The *P* element in the original *col* mutant has inserted into the 3′ UTR of the *col* gene 200 bp upstream of the end of the transcription unit. The 3′ UTR of various genes is involved in regulation of the stability of the transcript as well as its translation. In mammalians, as well as in Drosophila, AU-rich sequences residing in the

3′ UTR act as negative regulatory elements to facilitate degradation of the transcript (Chen and Shyu 1995). In Drosophila, several additional motifs that negatively regulate transcript stability and its translation efficiency were identified in the 3′ UTR of various proneural genes of the acaete-scute complex, including Brd-box (CAGT TTAA; Lai and Posakony 1997), GY-box (GTCTTCC; Lai and Posakony 1997), and K-box (TGTGAT; Lai *et al.* 1998). The *col* transcript contains in its 3′ UTR two Brd-like boxes, one K-box, and several AU-rich motifs, suggesting regulation of its stability and translation efficiency. Indeed, the insertion of the *P* element in the *col* mutant into the 3′ UTR causes both reduction in the level of the *col* transcript and elevation in the level of its translation. Although we do not have an explanation for this phenomenon, such a situation has been reported for the 3′ UTR of the growth factor TGF-β1 gene. There, a CG-rich region was identified that is responsible for decrease in transcription, rather than instability of the mRNA, as well as stimulation of translation (Scotto and Assoian 1993).

The primary role of ubiquitination is to target proteins for degradation (Hershko *et al.* 1984). However, existence of stable ubiquitinated proteins has been documented (Ball *et al.* 1987), as well as of proteins that are reversibly ubiquitinated (Paolini and Kinet 1993). This suggests that the ubiquitin-mediated system plays a role also in modification of protein function. Thus, the function of the *col*-encoded UBC, in ensuring proper development and function of specific parts of the fly's CNS that are important for male sexual behavior, could conceivably be accomplished as follows. It may stably ubiquitinate a substrate protein(s) that is directly involved in patterning of the relevant connections in the CNS as was shown for *arthrin*, which is a stable actin-ubiquitin conjugate involved in the assembly or function of thin filaments in the Drosophila flight muscles (Ball *et al.* 1987). Alternatively, *col* may mark specific substrate proteins for degradation, affording the means for close regulation of the function of proteins by modulating their half-life. This may enable them to act as on/off switches of CNS functions. Overexpression of the COL protein in the *col* mutant may shift the delicate balance between COL and its specific substrate required for normal development and proper activity of the CNS, culminating in the manifested phenotype of the *col* mutant. On the other hand, loss of function of the *col* gene is fatal and causes lethality at the larval stage. Identification and characterization of genes that genetically interact with *col*, and therefore could serve as a target substrate for COL, may help in understanding the molecular mechanisms that underlie this complex behavior. It should also lead to better understanding of the increasingly sophisticated role the ubiquitin-mediated system is turning out to play in normal development and function of the CNS and of the neural diseases brought about by its malfunction (for review see Ciechanover *et al.* 2000).

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