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

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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.

Female 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 (McRoberts 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 (Galley and Hall 1989) and also to lack a male-specific muscle in the abdomen (Galley et al. 1991). The phenotypes of fru suggest that such downstream genes may indeed be pleiotropic.

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 (Vogel 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 danc, of which all of the available alleles disrupt associative learning and cause female sterility (although males are fertile; Belen 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 (Lischytz 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°C 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 ms2) was mobilized from 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 scp CyO; ry/ry females were used for recombination analysis. P[ry]/col cn/al nub lt stw scp; ry/ry females were crossed to al nub lt stw scp 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 scp 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, Ub6 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/ry/Col; ry/ry lines (where ry 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. These 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, w[e111;w{FlyBase 1999} or over CyO, pAct-GFP (Reichhart and Ferroday 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 (CI) for the duration 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) Electoretinograms were performed according to Minke et al. (1975).

4. Locomotion: Locomotor activity of col/Col homozygous flies was compared to that of wild-type flies according to Griffith et al. (1993).

Tests analysis: T tests were disected 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 ßZapII vector (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. The library was screened with a probe comprising a 580-bp HindIII 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 (Sambrrok et al. 1989) under stringent conditions.

Northern analysis: Poly(A)+ RNA was isolated from males and females of the following genotypes: col, col/Col, and wild type (Canton-S) using a 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 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 Engel et al. (1986). Hybridization to whole-mount embryos was essentially as described by Tautz and Pfeiff er (1989). Probes for all in situ hybridizations were labeled by alkaline phosphatase-conjugated digoxigenin (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
RESULTS

Courtship behavior is abnormal in col homoygous males: Direct screening of a large population of mutagenized flies for males displaying aberrant courtship behavior was 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)Pr[y’]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 (Hadl 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 electrotetrograms 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).
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 HindIII 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 result-
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 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).

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.
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).

**Table 1**

<table>
<thead>
<tr>
<th>Line no.</th>
<th>Size of deletion (kb)</th>
<th>col sequences deleted (kb)</th>
<th>Sequences deleted downstream of col (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-4</td>
<td>1.8</td>
<td>Entire col transcript</td>
<td>0.2</td>
</tr>
<tr>
<td>6-8</td>
<td>2</td>
<td>0.7 (3' UTR + 64 codons)</td>
<td>1.3</td>
</tr>
<tr>
<td>52-13</td>
<td>1</td>
<td>1 (3' UTR + 162 codons)</td>
<td>—</td>
</tr>
<tr>
<td>59-7</td>
<td>0.9</td>
<td>0.9 (3' UTR + 158 codons)</td>
<td>—</td>
</tr>
<tr>
<td>60-7</td>
<td>1.1</td>
<td>1.1 (3' UTR + 186 codons)</td>
<td>—</td>
</tr>
</tbody>
</table>

**Cloning of the col gene:** A genomic library was constructed from col homozygous flies and was probed with a 0.58-kb HindIII 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
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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 termini 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.

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/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, 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...
**COL**

- MAGSALRRAEYMVKQLTRLDPPEGIVAGPSREDNPEHAEALLPGTCEGCVPARLI
- MATAP-RR-S-SSSRRSESRTT-SM-FQL-FYDDSVQTV-TP-LYD-YN-N-IMSS

**Scubc7**

- FPTDYPILSPPKFTCDMFNPITADGRVCISILHAPIDDPMKYELSAERWSVPQSVKILLS
- K------LT-PSL----TPN-E------S-NN----AE-----------------
- QN---TVR-SE-W-VYP------P-N-AS---T-HT-S-V-
- CN---TVR-S-V---WS--P-S-AS---T-HT-S-M-

**Taubc7**

- VVSLAEPDSEGANYAAMKQREDFNAYADGWCAKLWWTRKMMVTVNTTFP
- M---S---I---I-I-CL--DN-P-ERQ--VKLISL-S-GF---
- FS---I-IB-KE--Q---KKVRRAVR-KSQEM---
- SG- ---F-B-KE-DK---KKVSVCRK.KSQEM---

**Atubc7**

- QHDSNSSLTHSHFLNPSLS
- -----------------------
- -----------------------

**COL1**

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.

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.
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**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.

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, col52-13 and col59-7, 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 col52-13 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 col14) 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 fruitles (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, as are 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 spermatogenetic mutants, which includes mutants in the genes for the cell cycle regulatory phosphatase twine, and the cell cycle kinase cd2 (Fuller 1998). In both mutants certain meiotic events are skipped, yet spermatid differentiation proceeds. As a result, testes of twine or cd2 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 Har even 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 (Goebel 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 col 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 mR68 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 mammals, 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 proroneuronal genes of the acent-scute complex, including Brd-box (CATGTTAA; Lai and Posakony 1997), GY-box (GTCTTCC; Lai and Posakony 1997), and K-box (TGTTGAT; 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 (Scott and Assoian 1993).

The primary role of ubiquitination is to target proteins for degradation (Her shko et al. 1984). However, existence of stable ubiquitinylated proteins has been documented (Ball et al. 1987), as well as of proteins that are reversibly ubiquitinylated (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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