Developmental Biology **226,** 1–17 (2000)
doi:10.1006/dbio.2000.9811, available online at http://www.idealibrary.com on IDEAL®

The Mutation *without children*^{rgl} Causes Ecdysteroid Deficiency in Third-Instar Larvae of *Drosophila melanogaster*

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Larvae homozygous for the recessive lethal allele *without children*^{rgl} (*woc*^{rgl}) fail to pupariate. Application of exogenous 20-hydroxyecdysone elicits puparium formation and pupation. Ecdysteroid titer measurements on mutant larvae show an endocrine deficiency in the brain–ring gland complex, which normally synthesizes ecdysone, resulting in a failure of the larvae to achieve a threshold whole body hormone titer necessary for molting. Ultrastructural investigation revealed extensive degeneration of the prothoracic cells of the ring gland in older larvae. The *woc* gene, located in polytene chromosomal region 97F, consists of 11 exons. A 6.8-kb transcript is expressed throughout development but is absent in the mutant *woc*^{rgl} larvae. The *woc* gene encodes a protein of 187 kDa. Eight zinc fingers of the C2–C2 type point to a possible function as a transcription factor. The *woc* protein shows considerable homology to human proteins which have been implicated in both mental retardation and a leukemia/lymphoma syndrome.

Key Words: without children; **ecdysone; ring gland; zinc finger; mental retardation.**

INTRODUCTION

Since Stefan Kopeč (1922) discovered that the larval brain functions as an endocrine organ controlling pupation in the gypsy moth and thus founded the field of neuroendocrinology, the hormonal control of insect development has been studied intensively. It is now clear that the brain hormone (prothoracicotropic hormone) activates another endocrine organ, the prothoracic gland, to synthesize and secrete a polyhydroxylated steroid prohormone which is then converted to the major molting hormone of insects, 20-hydroxyecdysone, by peripheral tissues (see Gilbert, 1989). A second group of hormones, the juvenile hormones, modulate the character of a developmental stage as exemplified by comparing the larval–larval molt to the larval–pupal or pupal–adult molt (Gilbert *et al.*, 1980; Thummel, 1996).

20-Hydroxyecdysone is the regulatory agent in virtually all aspects of insect development although its precursor, ecdysone, may have roles of its own. The absence of 20-hydroxyecdysone in some larval or pupal insects results in a halt to normal development termed diapause, a "resting" stage that persists until the ecdysteroid titer increases to a critical level. Several mutants of *Drosophila melanogaster* which appear to lack 20-hydroxyecdysone have been described. Further, 20-hydroxyecdysone-induced gene activity, as seen in the puffing pattern of salivary gland polytene chromosomes, has made *Drosophila* an excellent model system to study the developmental gene network controlled by 20-hydroxyecdysone (Becker, 1959; Ashburner *et al.*, 1974; see Thummel, 1996; Henrich *et al.*, 1999), as well as the gene network controlling and modulating ecdysteroid synthesis during different developmental stages in a tissue-specific manner (see Henrich *et al.*, 1999). The first solid evidence showing that steroid hormones act at the level of the nucleus was the classic study of the effects of 20-hydroxyecdysone on chromosome puffing by Clever and Karlson (1960). More recent work has demonstrated that 20-hydroxyecdysone triggers metamorphosis and the activation of several members of the nuclear hormone receptor family, which then act as transcription

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0012-1606/00 $35.00

factors directing the expression of cascades of secondary genes (e.g., Guay and Guild, 1991; Karim *et al.*, 1993; for reviews see Thummel, 1996; Henrich *et al.*, 1999).

The stage-specific expression of these regulatory genes seems to be dictated, at least in part, by dynamic changes in the ecdysteroid titer during the early and late stages of larval–pupal metamorphosis (Henrich *et al.*, 1999). Thus, identifying genes that regulate the gene network controlling and modulating ecdysteroid synthesis, and therefore the ecdysone titer, is equally important in understanding the hormonal control of development. A comparatively small number of so-called ecdysoneless mutants have been isolated, all of which show developmental arrest at the late larval stage and fail to form a puparium (Rayle, 1967; Kiss *et al.*, 1978; Garen *et al.*, 1977; Holden and Ashburner, 1978; Klose *et al.*, 1980; Henrich *et al.*, 1987b; Sliter *et al.*, 1989; Sliter and Gilbert, 1992; Warren *et al.*, 1996). In these mutants the ring gland fails to secrete normal levels of ecdysone. The ring gland, most prominent in the larva, is a composite endocrine organ, containing the cells of the prothoracic gland, corpus allatum (the source of juvenile hormone), and corpus cardiacum (the source of peptide hormones) (King *et al.*, 1966; Dai and Gilbert, 1991). Studies *in vitro* have shown that the ring gland is the source of both ecdysone and juvenile hormone (see Gilbert *et al.*, 1996).

To gain further insights into the control mechanism by which some mutants lost the ability to synthesize ecdysone, we have analyzed a mutant allele of the *without children* (*woc*) gene located in polytene chromosomal region 97F. Homozygous mutant woc^{rgl} larvae fail to form a puparium and die as larvae within 2 to 3 weeks. They exhibit an up to 20 times enlarged ring gland, which we show here is incapable of normal ecdysone biosynthesis.

MATERIAL AND METHODS

Isolation of woc P-lacW Insertional Mutations

To obtain *P-lacW* insertional mutations, flies from the *P-lacW* transposon strain *y w P-lacW* (Bier *et al.*, 1989) were crossed with flies from the strain *w; Sb Δ2-3/TM3* providing the transposase (Robertson *et al.*, 1988). The resulting jumpstarter males, phenotypically characterized by stubble bristles and white, variegated eyes, were mass crossed to *w/w, TM3/TM6B* balancer females. Approximately 33,500 *P-lacW* mutagenized balanced males were tested for complementation with the deficiency $Df(3R)mbt^{P}$ (Fig. 1A), which represents a deletion of 29 kb in the salivary gland polytene chromosome region 97F3–11 (Gateff *et al.*, 1993).

EMS Mutagenesis for Chromosome III

For EMS mutagenesis, 2- to 4-day-old wild-type Oregon R males were fed on an EMS–sugar solution using the method of Lewis and Bacher (1968). The mutagenized males were mass crossed to females from the third chromosome double-balancer strain *CxD/TM3* (Lindsley and Zimm, 1992). To obtain strains from each single, mutagenized third chromosome, the heterozygous balanced animals were pair crossed with animals from the double-balancer strain *TM3/TM6B*. In the next cross heterozygous balanced animals were inbred. Among 5000 mutagenized chromosomes 577 mutational events were detected in the progeny of the third generation as evidenced by the absence of homozygous adults.

Isolation of woc cDNAs

A cDNA phage library from 0- to 3-h-old embryos (Poole *et al.*, 1985) was kindly provided by H. Jäckle and was screened. Two genomic *Sal*I fragments (Fig. 1B) were used as hybridization probes. The probes were labeled following the protocol of the random-primed DNA labeling kit (Boehringer Mannheim) and hybridized to the plaque lifts (Benton and Davis, 1977). A total of 10^5 plaques were screened. Phage DNA was isolated after the method of Zabarovsky and Turina (1988). Seven cDNAs were isolated, three of which were used for further analysis, cDNA B3 containing the poly(A) tail; cDNA 6, which extended the farthest in the 5′ direction; and cDNA 11, which contained the largest insert. The 5′ region of the *woc* transcript was identified by different RT-PCRs using poly(A)$^+$ mRNA from adult females.

Northern Blot Analysis

Total RNA was extracted as described previously (Sass, 1990) from wild-type Oregon R larvae (L1, L2, L3), L3 mutant woc^{rgl} larvae, and L3 larvae of the woc^{rgl} transformant line 15-45. Approximately 30 μg of each RNA was separated on a 1% agarose/5% formaldehyde gel, transferred to a nylon membrane (Pall Biodyne A), and hybridized with an [α-^{32}P]CTP-labeled single-stranded antisense RNA probe derived from the *woc* cDNA 11. The cDNA 11 was transcribed *in vitro* with the help of the Riboprobe Gemini System (Promega). Hybridization was carried out at 68°C in 50% formamide buffer (Maniatis *et al.*, 1982). Blots were washed at 78°C in 2× SSC and 0.1% SDS.

DNA Sequence Analysis

Overlapping restriction fragments of the *woc* genomic region (Fig. 1B) were cloned into the pBluescript KS(+) vector and sequenced on both strands by the dideoxy termination method (Sanger *et al.*, 1977) using the pBluescript KS(+) reverse and universe primer as well as specific *woc* primers ordered from Eurogentec and MWG Biotech. The different *woc* cDNAs and the 5′ RT-PCR products were sequenced by primer walking. For automated sequence analysis (ABI; Model 373A) the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) was used.

Construction of the Plasmid pHS85-15 for Germ-Line Transformation

Based on sequence data we chose a 15-kb *Sal*I–*Kpn*I genomic fragment (Fig. 1B, asterisks) encompassing the coding region and, in addition, sufficiently long 5′ and 3′ sequences for cloning into the germ-line transformation vector. For the pHS85-15 construction the 8.5-kb genomic *Sal*I fragment was cloned into the *Sal*I site of the P-element transformation vector pHS85 (Sass, 1990), which provides a constitutive source of antibiotic G418 resistance. The orientation of the inserts was analyzed via restriction with *Kpn*I. Next, the distal *Sal*I site was removed from the construct by *Kpn*I digest and religation. Thereafter the 6.8-kb *Sal*I fragment was

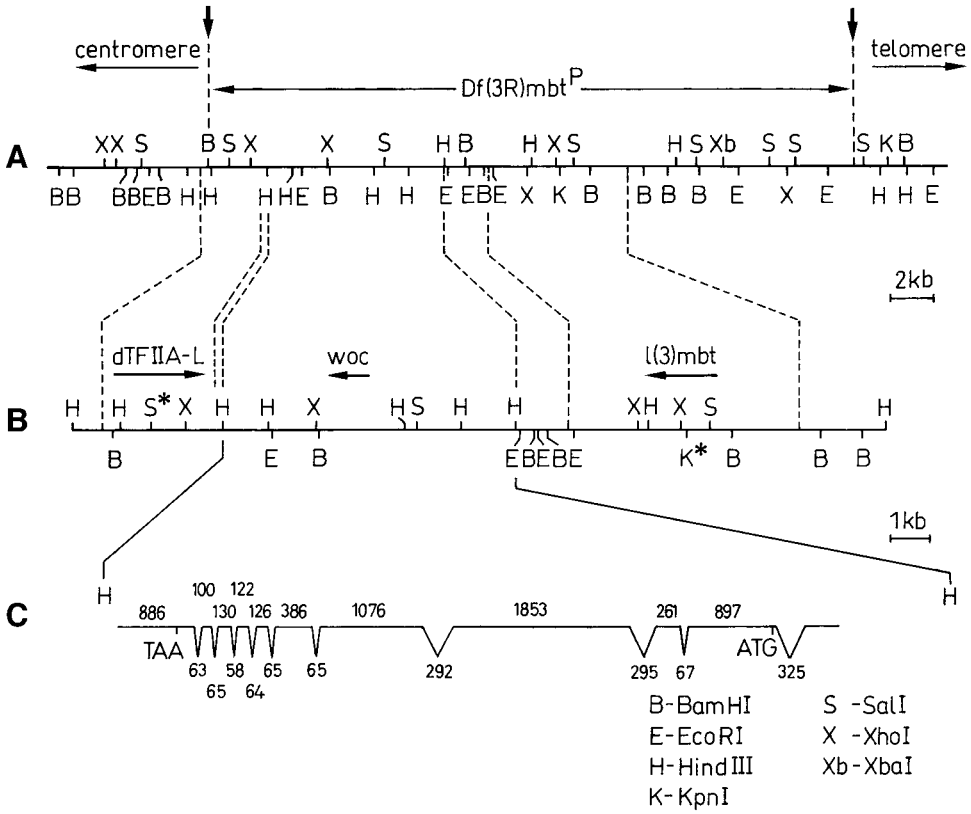

FIG. 1. (A) Restriction map of deficiency *Df(3R)mbt*^{P} and the flanking regions. Arrows indicate the breakpoints. *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Sal*I (S), *Xba*I (Xb), *Xho*I (X). (B) Localization of the *woc* gene in respect to the adjacent genes *dTFIIA-L* and *l(3)mbt*. The arrows indicate the transcriptional direction. Asterisks note the genomic fragment present in the rescue construct pHS85-15. (C) Exon–intron structure of the *woc* gene. The potential initiation codon ATG and the termination codon TAA are indicated. The numbers represent the lengths of the exons and the introns in base pairs.

cloned into the remaining *Sal*I site. The orientation was verified by a *Bam*HI digest.

P-Element-Mediated Germ-Line Transformation

P-element-mediated germ-line transformation was performed according to the method of Rubin and Spradling (1982). DNA derived from the germ-line transformation plasmid (see above) and the helper plasmid pπ25.7Δ2-3 (Karess and Rubin, 1984) was purified on CsCl–EtBr equilibrium density gradients (Sambrook *et al.*, 1989). Plasmid DNA (0.8 mg/ml) was co-injected with helper plasmid DNA (0.2 mg/ml) into *woc*^{st1}/TM3 embryos. Transgenic flies of the F1 generation were selected on unyeasted instant *Drosophila* food containing 0.8 mg/ml G418 by their capacity to survive. For the rescue of the *woc*^{rgl} allele one of the *woc*^{st1} transformant stocks containing the rescue construct inserted on chromosome II was crossed with the balancer stock *CyO/Pm; TM3/TM6b*. The same was done with the *woc*^{rgl}*/TM6B* stock. *PHS85-15/Pm; +/TM6B* females were crossed with *+/CyO; woc*^{rgl}*/TM3* males. From the progeny *pHS85-15/CyO; woc*^{rgl}*/TM6B* animals were inbred and analyzed.

In Situ Localization of the woc Transcripts

In situ hybridizations to whole-mount wild-type Oregon R embryos were performed according to the protocol of Tautz and Pfeifle (1989). As the *woc* probe, cDNA 11 was labeled by random priming with digoxigenin–dUTP (Boehringer Mannheim). For detection of the hybridized probe we used an alkaline phosphatase-conjugated digoxigenin monoclonal antibody diluted 1:2000 in PBST. The alkaline phosphatase reaction was carried out using 4.5 ml NBT and 3.5 ml X-phosphate (Genius Detection kit; Boehringer Mannheim). Photographs were taken using Leitz optics. The embryos were staged according to Campos-Ortega and Hartenstein (1985).

20-Hydroxyecdysone Feeding Experiment

Fifty third-instar mutant larvae were placed on 1 g of *Drosophila* dry food (Carolina Biological Supply) moisturized with 3.5 ml of *Drosophila* Ringer's solution containing 250 μg of 20-hydroxyecdysone. Fifty wild-type and mutant third-instar larvae were fed on the same amount of dry food moisturized with Ringer's solution devoid of 20-hydroxyecdysone and served as controls. The

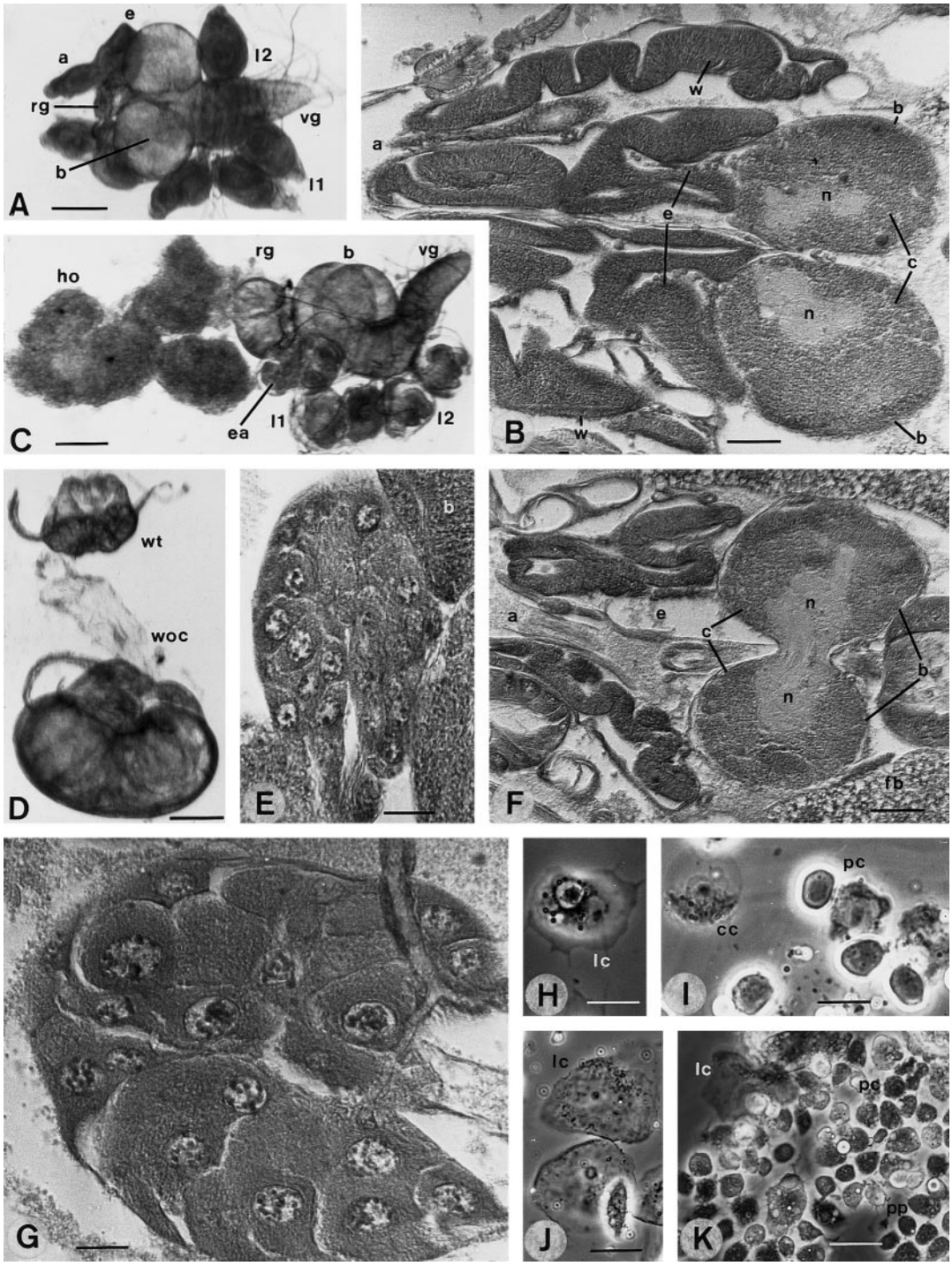

FIG. 2. Histology of the brain–ventral ganglion complex, imaginal discs, ring glands, hematopoietic organs, and hemocytes of wild-type and *woc*^{rgl} third-instar mutant larvae. (A) Whole mount of the wild-type brain (b)–ventral ganglion (vg) complex with eye (e)–antennal (a) and first (l1) and second (l2) leg imaginal disc pairs. Note the ring gland (rg) between the two eye–antennal imaginal discs. (B) Longitudinal histological section through wild-type brain hemispheres (b), the eye (e), the antennal (a), and the wing (w) imaginal discs. (C) Whole mount of the *woc* brain (b)–ventral ganglion (vg) complex, the leg imaginal discs (l1, l2), and the overgrown hematopoietic organs (ho). Compare the size of the mutant ring gland (rg) with that of the wild-type ring gland in A. Note also the smaller and abnormally shaped imaginal discs. (D) Whole mount of the wild-type (wt) and mutant *woc*^{rgl} ring gland. (F) Longitudinal histological section through the *woc*^{rgl} mutant brain (b) hemispheres and the eye (e), antennal (a), and wing (w) imaginal discs. Note the smaller imaginal discs in comparison to B. (E, G)

number of mutant larvae undergoing pupation was noted and compared with the control animals.

Transmission Electron Microscopy

Freshly dissected brain–ventral ganglion complexes from "old" wandering dauer *woc* larvae were fixed immediately in a mixture of 2% glutaraldehyde, 2% paraformaldehyde, and 1% tannic acid in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C and processed for postfixation, sectioning, and electron microscopy as described previously (Dai and Gilbert, 1997).

Quantification of Ecdysteroids

Whole body ecdysteroid titers of wild-type and heterozygous or homozygous *woc* mutant third-instar larvae were determined by RIA (Warren *et al.*, 1984; Warren and Gilbert, 1986, 1988) employing the SHO-3 antibody (courtesy of Dr. S. Sakurai, Kanazawa University, Japan), which is insensitive to ecdysteroid metabolites with side-chain alterations (Kiriishi *et al.*, 1990). As soon as they left the food, early wandering larvae (10 each) were homogenized thoroughly in methanol (1.5 ml) and the mixture was centrifuged. The solids were reextracted once with ethanol (1.5 ml) and the solvents were pooled and evaporated prior to assay. Titers in "late" homozygous *woc* dauer larvae (those wandering for 2–3 days) and "old" larvae (those still wandering after 1–2 weeks) were also determined.

The secretory activities of brain–ring gland complexes from early wandering third-instar wild-type, heterozygous, or homozygous *woc* mutant larvae in addition to late and old wandering homozygous *woc* dauer larvae were also determined by RIA employing the SHO-3 antibody. Glands were dissected under ice-cold *Drosophila* Ringer's and placed into individual drops of Grace's medium (70 μl) contained in 96-well plastic petri dishes. After incubation at 26°C for 2 h with gentle agitation, the medium containing secreted ecdysteroids was carefully separated from the glands and added to individual RIA tubes. The remaining incubated glands, in pools of 20, were extracted with methanol (1.5 ml), the extracts evaporated, and the residues assayed for ecdysteroids synthesized in the glands but not secreted into the medium.

RESULTS

Origin of the without children Alleles

In a *P-lacW* insertional mutagenesis we identified among 33,500 mutagenized, balanced males a mutant strain leading to male as well as female sterility in the homozygous adults. This first allele was named *without children* (*woc*^{st1}) based on the sterile phenotype. The gene was mapped by Southern blotting to a 6.8-kb *Sal*I genomic fragment near the centromeric breakpoint of the deficiency *Df(3R)mbt*^{P} (Fig. 1A) in the salivary gland chromosome region 97F3–11. This region also contains the brain tumor suppressor gene *lethal(3)malignant brain tumor* (*l(3)mbt;* Gateff *et al.*, 1993) and the *dTFIIA-L* gene (Habtemichael *et al.*, personal communication; Fig. 1B). In a further EMS-mutagenesis screen, we detected among 577 third-chromosome recessive-lethal mutations a second *woc* allele, which showed third-instar larval lethality. In complementation with the first recessive-sterile allele it also exhibits sterility in both sexes. The new *woc* allele, which will be the subject of this paper, was designated *woc*^{rgl} because of the highly enlarged third-instar larval mutant ring gland. Both *woc* alleles are able to totally complement all alleles of the two adjacent genes *dTFIIA-L* and *l(3)mbt*.

Phenotype of the Homozygous woc^{rgl} *Larvae*

The homozygous *woc*^{rgl} animals fail to pupariate and die as third-instar larvae after an extended larval life of up to 3 weeks. Mutant larvae exhibit extremely enlarged ring glands compared to the wild type (Figs. 2C and 2D). The wild-type ring gland, the source of the molting prohormone ecdysone, represents a small, flat ring (Fig. 2D) located anterior to the brain hemispheres (Fig. 2A). The mutant ring gland, in contrast, is ball-like and about 10 times larger than the wild-type counterpart. It often almost reaches the size of a brain hemisphere (Fig. 2C). As shown in histological preparations, the enormous size of the mutant ring gland is due to the enlargement of the individual ecdysone-secreting, prothoracic gland cells (compare Fig. 2E with 2G). Associated with cell enlargement is also the increased size of the nucleus. Under phase-contrast examination of ring gland preparations, the giant chromosomes can be discerned easily within the nucleus, which often reaches the size of salivary gland polytene chromosomes (data not shown).

Mutant larvae of advanced age are characterized by an up to 100-fold increase in the size of their hematopoietic organs (Fig. 2C), which occupy large portions of the body cavity. Correspondingly, there is a considerable increase in the number of free hemocytes compared to the wild type (data not shown). The wild-type hematopoietic organs harbor two types of hemocyte precursors, the proplasmatocytes and the procrystal cells. Upon release into the hemolymph, proplasmatocytes differentiate into the phagocytic plasmatocytes (Fig. 2I), which can become podocytes and

Histological sections through wild-type and mutant ring glands, respectively. Compare the sizes of the individual prothoracic gland (ecdysone-secreting) cells and their nuclei in the wild-type and mutant ring gland. (H) Whole mount of wild-type lamellocytes (lc) from the hemolymph. (I) Whole mount of wild-type hemocytes, showing plasmatocytes (pc) and a crystal cell (cc). (J) Whole mount of two large lamellocytes (lc) from the hematopoietic organ. (K) Whole mount of hemocytes in the mutant hematopoietic organ showing proplasmatocytes (pp), plasmatocytes (pc), and lamellocytes (lc). c, cortex; f, fat body; n, neuropile. Histology: hematoxylin, eosin. Bars in A and C 150 μm; B and F 40 μm; D 50 μm; E and G 20 μm; H, I, and J 15 μm; K 10 μm.

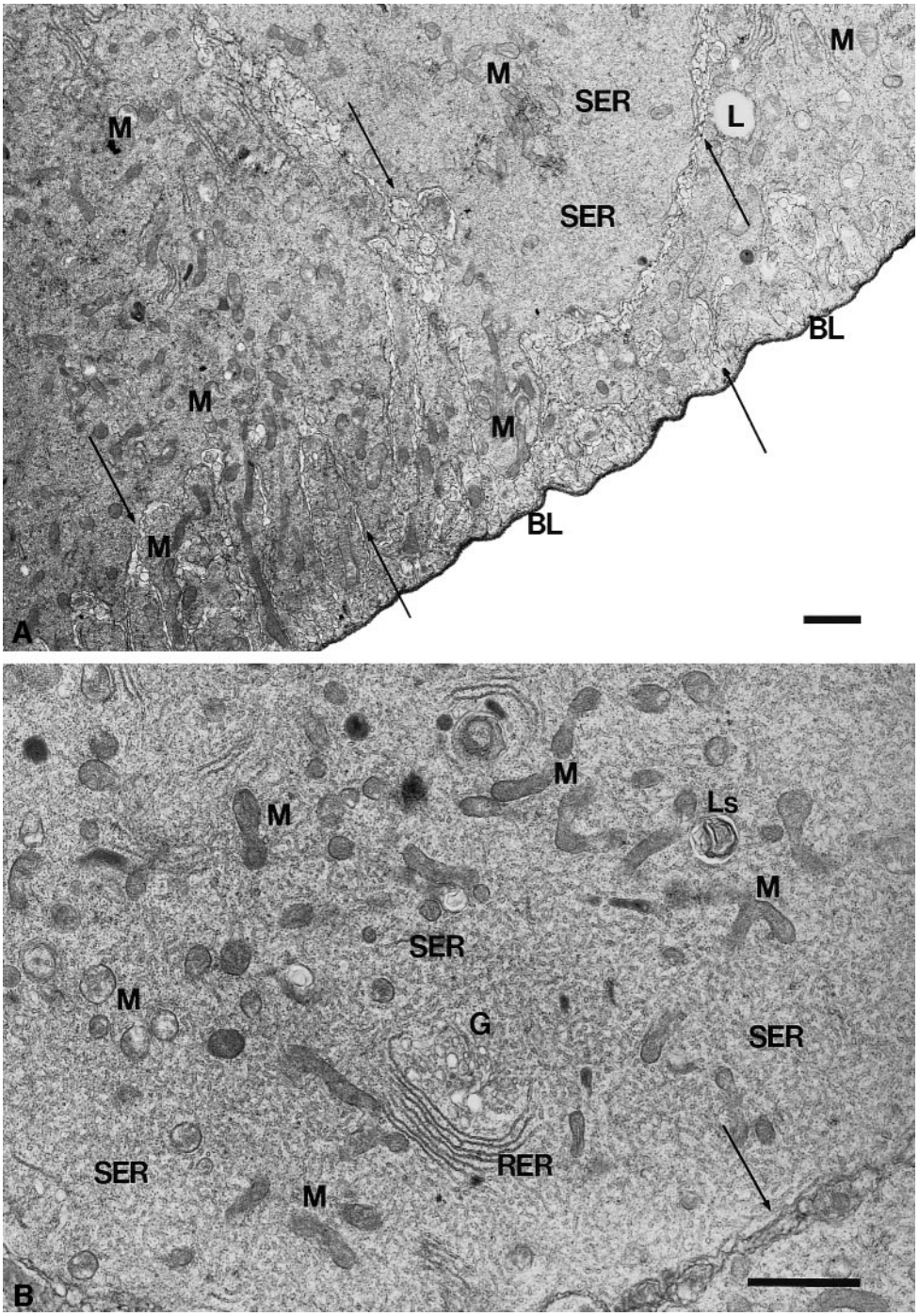

FIG. 3. Electron micrographs of a prothoracic gland cell from a heterozygous (*woc*/+) third-instar wandering larva. (A) Note the fully developed membrane invagination system. (B) A higher magnification showing various-shaped mitochondria and large amounts of smooth endoplasmic reticulum in the cytoplasm. BL, basal lamina; G, Golgi complex; L, lipid droplet; Ls, lysosome or lysosome-like structure; M, mitochondrion; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; Arrow, membrane invagination. Bar 1 μm.

lamellocytes (Fig. 2H; Shrestha and Gateff, 1982). Procrystal cells give rise to crystal cells (Fig. 2I). They are highly fragile cells whose crystals contain prophenoloxidase (T. Trenczek, R. Poppe, and E. Gateff, unpublished) and are involved in hemolymph coagulation and melanization. The cell population in the mutant hematopoietic organs consists of

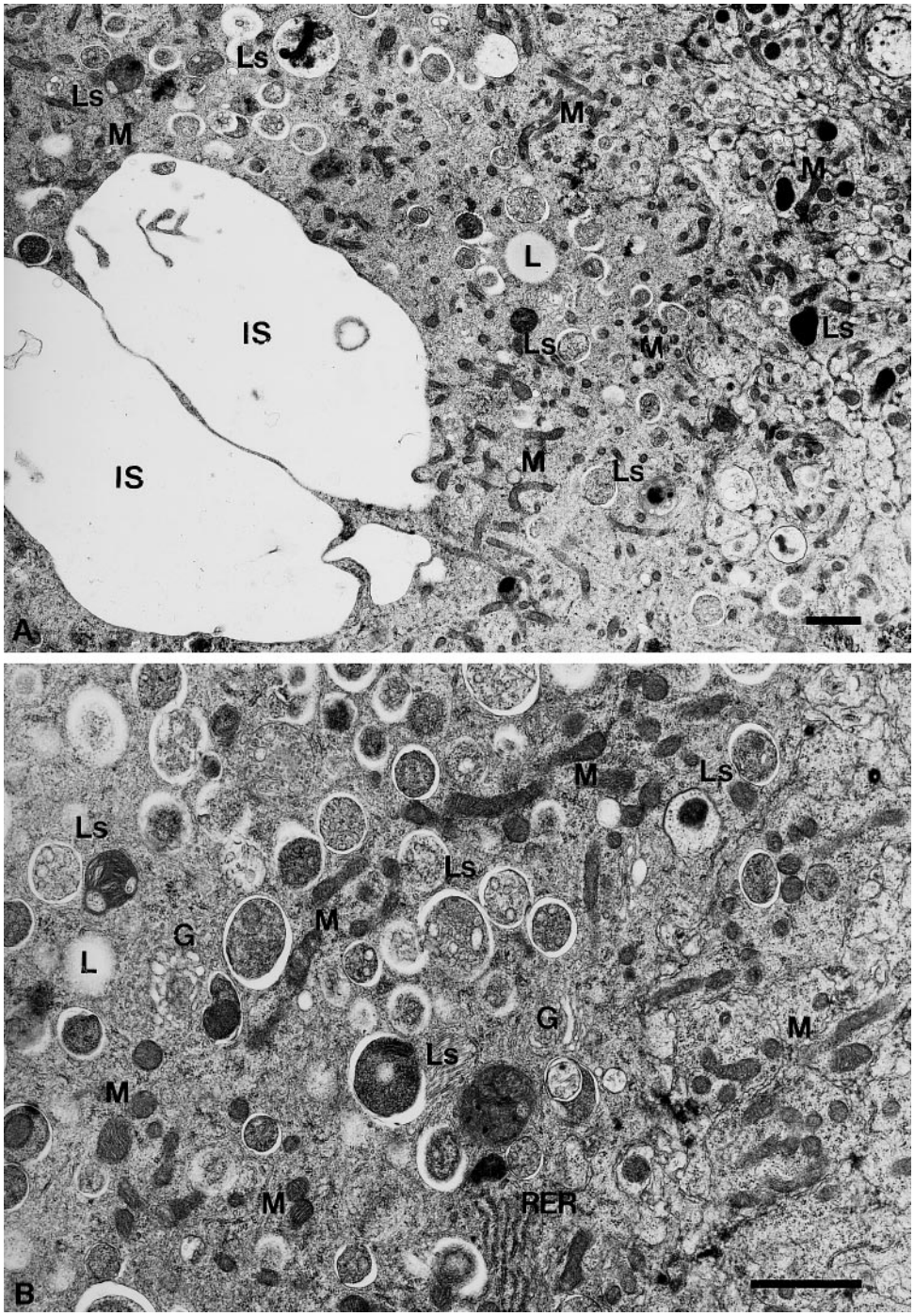

FIG. 4. Electron micrographs of a prothoracic gland cell from an old homozygous (*woc*) third-instar wandering larva. (A) Note the giant intracellular spaces in the cytoplasm. (B) A higher magnification showing a large amount of lysosomes or lysosome-like structures in the cytoplasm. Same abbreviations as in Fig. 3, plus IS, intracellular space. Bar 1 μm.

up to 80% proplasmatocytes and about 20% plasmato- and lamellocytes (Figs. 2J and K), the latter often forming melanized masses. Few crystal cells, recognizable by their crystalline inclusions, are also encountered (data not shown). Upon release into the hemolymph, plasmatocytes transform into lamellocytes, which often conglomerate to

TABLE 1

Whole Body Ecdysteroid Titers and *in Vitro* Brain–Ring Gland Complex Ecdysteroid Secretion during the Third Larval Instar in Wild-Type and *woc* Mutant *Drosophila*

Animal	Stage	Whole body titer (pg/animal)[a]	Ecdysteroid production *in vitro* (pg/gland/h)[b]
Wild type	Early wandering 3rd-instar larvae	27.0 ± 2.1 (n = 12)	60.0 ± 3.5 (n = 57)
Heterozygous *woc*^{rgl}	Early wandering 3rd-instar larvae	18.0 ± 2.4 (n = 8)	29.0 ± 2.9 (n = 14)
Homozygous *woc*^{rgl}	Early wandering 3rd-instar larvae	10.0 ± 0.7 (n = 12)	10.0 ± 0.5 (n = 48)
Homozygous *woc*^{rgl}	Late wandering larvae (2–3 days)	10.0 ± 0.9 (n = 11)	10.0 ± 0.4 (n = 46)
Homozygous *woc*^{rgl}	Old wandering larvae (1–2 weeks)	<1.6 (n = 10)[c]	<5.0 (n = 24)[d]

Note. Numbers in parentheses indicate time from the beginning of wandering.

[a] By RIA, extracts of groups of 10 animals.

[b] By RIA, media from individual gland incubations.

[c] Lower limit of assay sensitivity for whole body extracts is 16 pg/assay.

[d] Lower limit of assay sensitivity for *in vitro* production is 10 pg/assay.

form melanotic masses (data not shown). In the wild-type hematopoietic organs, differentiation of proplasmatocytes into plasmatocytes and lamellocytes never occurs; this differentiation instead takes place in the hemolymph (Shrestha and Gateff, 1982). The highly enlarged mutant hematopoietic organs and increased hemocyte count suggest that the mutant hemocytes divide faster than wild-type hemocytes. To test the ability of these hemocytes for autonomous, malignant growth, small pieces of mutant hematopoietic organs or free hemocytes were transplanted into the body cavity of wild-type female flies. The transplantation test revealed no autonomous malignant growth of the mutant hemocytes in the wild-type environment.

Mutant imaginal discs are smaller, misshapen, and lack the characteristic folding pattern of the epithelial monolayer, compared to their wild-type counterparts (compare Figs. 2A and 2C with 2B and 2F). In about 20% of the larvae, imaginal discs either are not present or are present in a rudimentary condition (data not shown).

The mutant brain hemispheres are slightly smaller than in wild-type larvae but do not show obvious histological differences from wild-type brains (compare Figs. 2A and 2C with 2B and 2F). Another phenotypic aberration is noted in the cardia, which appear to have lost their muscle mass and instead are filled with fluid (data not shown).

Ultrastructural Abnormalities of the woc^{rgl} Mutant Ring Glands

The normal prothoracic gland portion of the ring gland of wandering third-instar wild-type larvae displays several ultrastructural features that reflect increased steroidogenic activity, including extensive invaginations of the plasma membrane and increases in both the concentration of smooth endoplasmic reticulum (SER or transitional ER) and elongated mitochondria (Dai and Gilbert, 1991). The prothoracic cells of *woc*^{rgl} heterozygotes also show these ultrastructural features (Fig. 3) as might be expected of glands capable of actively synthesizing ecdysteroids. However, the prothoracic glands of old homozygous *woc*^{rgl} larvae not only fail to produce sufficient ecdysteroids at this stage (see below), but also acquire abnormal morphological features that reflect the disruptive effects of the mutation (Fig. 4). These abnormalities include an accumulation of a large amount of lysosomes or lysosome-like structures and a drastically reduced concentration of SER in the cytoplasm, indicating that the glands are undergoing early degeneration prior to the failure to pupariate. The other distinct feature is the appearance of giant intracellular spaces in the cytoplasm similar to previous findings with the *ecdysoneless* (*l(3)ecd*^{1ts}) ring gland at the same developmental stage (Dai *et al.*, 1991). No obvious ultrastructural abnormalities were noted in the corpus allatum portion of the ring glands of either homozygous or heterozygous larvae (data not shown), indicating that *woc*^{rgl} is primarily a prothoracic gland-disruptive phenotype, although juvenile hormone plays a prothoracic gland maintenance role (Gilbert, 1962) and a gonadotropic role in many insects. Although appearing normal at the ultrastructural level, a defect in juvenile hormone synthesis in the *woc*^{rgl} mutant cannot be ruled out.

Ecdysone Feeding Experiments

To demonstrate that the inability of mutant larvae to pupariate is a consequence of a lack of ecdysteroid, feeding experiments were performed. All 50 third-instar mutant larvae placed on 20-hydroxyecdysone-containing medium pupated within 1 to 2 days and then died, while the untreated mutant control larvae died 2 to 3 weeks later without pupariating.

Whole Body Ecdysteroid Titers in Third-Instar Larvae

By RIA (SHO-3 Ab), the whole body ecdysteroid titer (Table 1) in early wandering heterozygous *woc* mutant

larvae, 18 pg/animal, is about 66% of that found in wild-type larvae of the same age, i.e., 27 pg/animal. In comparable early wandering homozygous *woc* mutant larvae, whole body titers are only 37% of wild type (10 pg/animal). After wandering for an additional 2–3 days, by which time all wild-type and heterozygous larvae have pupated, these remaining homozygous late dauer larvae still contain 10 pg/animal. However, after 1–2 weeks of wandering, the whole body titers of these old homozygous *woc* larvae are below the level of detectability, i.e., <1.6 pg/animal (16 pg/assay tube containing a crude extract of 10 animals).

Ecdysteroid Biosynthesis by Third-Instar woc Brain–Ring Gland Complexes in Vitro

To determine the functional basis of the *woc* mutation, the *in vitro* rate of synthesis and secretion of ecdysteroids into the medium by brain–ring gland complexes over 2 h was measured by the SHO-3 antibody and is shown in Table 1. Wild-type glands from early wandering larvae release about 60 pg/gland/h. Heterozygous *woc* mutant glands are about 50% as active as wild-type glands (29 pg/gland/h). Homozygous *woc* mutant brain–ring glands show only 16% of the activity of wild-type glands (10 pg/gland/h). After 2–3 days of wandering, the *in vitro* activity of glands from these late dauer *woc* mutants remains constant. However, it falls below the level of detectability in old wandering dauer *woc* larvae, i.e., <5 pg/gland/h (10 pg/assay tube containing the medium following the incubation of one gland for 2 h). The sensitivity here is greater than before because these samples contain fewer trace contaminants than whole body extracts.

It is possible that the homozygous *woc* ring gland within the complex is able to synthesize ecdysone in a normal fashion, but is unable to secrete this newly made ecdysone into the medium. To rule out this possibility, following the incubation of individual brain–ring gland complexes, the glands alone were pooled and extracted and the extracts subjected to RIA using the SHO-3 antibody. The level of ecdysteroids remaining in the brain–ring gland complex after incubation was quite variable, but it was generally 3 pg/gland or below in the wild-type as well as the homozygous and heterozygous *woc* mutant tissues.

Germ-Line Transformation

The successful rescue of a mutant phenotype by P-element-mediated germ-line transformation is the final proof for the cloning of a gene. Following germ-line transformation with the pHS85-15 vector containing the wild-type *woc* gene region, 20 independent *woc*^{st1} stocks carrying the *woc* transgene have been isolated. In all these stocks we observed that the homozygous *woc*^{st1} adults recover their fertility. The same rescue vector was also able to rescue the *woc*^{rgl} allele. This allele is balanced with *TM6B*. The Tubby marker leads to short thick heterozygous larvae. Therefore, the homozygous mutant larvae can be recognized by their body size. In the presence of the *woc* vector pHS85-15 (proved by G418 selection) these larvae pupate and lead to viable fertile adults with a wild-type-like phenotype. Thus the transformant *woc*^{rgl} strain (15-45) can be bred without a balancer.

Sequence and Structure of the woc Gene

The genomic organization of the *woc* gene was investigated by sequencing three overlapping *woc* cDNAs (B3, 6, 11; see Material and Methods) and the corresponding genomic region. Only the cDNA B3 contained a poly(A) tail. Since all cDNAs proved to be incomplete in the 5′ direction, the first three exons were identified by RT-PCR (Figs. 1C and 5). The translation initiation site was identified by the presence of a *Drosophila* TS consensus sequence (Cavener, 1987). The gene has a single ORF of 5064 bp defining a putative 187-kDa protein of 1688 amino acids (Fig. 5). The protein contains eight zinc fingers of the C2–C2 type, two AT-hook DNA binding motifs which include the core sequence RGRP (Chuang and Kelly, 1999), two proline repeats (Xiao *et al.*, 1998), and a putative bipartite nuclear localization signal (Robbins *et al.*, 1991).

Homology searches using the Blast program identified three human homologues designated DXS6673E/ZNF261 (van der Maarel *et al.*, 1996; Nagase *et al.*, 1997), ZNF198/FIM/RAMP (Xiao *et al.*, 1998; Popovici *et al.*, 1998; Smedley *et al.*, 1998), and ZNF262 (Ishikawa *et al.*, 1997; Sohal *et al.*, 1999). Comparing all the zinc fingers in the *Drosophila* WOC and the human WOC homologues we found a striking consensus sequence, $CX_2CX_{13\text{-}18}GX_4FCSX_2CX_3Y/F$, which defines a new zinc finger-related motif designated winc finger (woc zinc finger). An interesting characteristic of the winc fingers is the conservation of phenylalanines surrounding the second C–C motif. The eight zinc fingers in the *Drosophila* WOC protein also match the described consensus sequence despite the glycine, which is not conserved (Fig. 5).

All four proteins of the woc family contain two proline

FIG. 5. Sequence of the *woc* genomic region and amino acid sequence of the WOC protein (Accession No. AJ276394). The putative ATG start codon is in bold letters. The two asterisks above the sequence define the part of the 5′ region not present in the cDNAs, but identified by RT-PCR. The putative zinc fingers, the two AT-hook DNA binding motifs, and the proline repeat domains are boxed. The stop codon TAA is indicated by an asterisk. The polyadenylation signal and the polyadenylation site are shown in bold letters. Exon sequences are in uppercase; intron sequences are in lowercase. The predicted protein sequence is given using the one-letter code. Note that most of the eight zinc fingers follow the formula $CX_2CX_{13\text{-}18}GX_4FCSX_2CX_3Y/F$ found for the three human homologues.

```
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cgtgggctcagtgtgtgtgtgtgcgtcggtgtgcctgtgcgtgtgtgtgtacacattttaggcggcaatggtgtggggccctggccagccgatagtccgccgcgccgcgaatgttatc

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                                                                                                                M  E  E  I  S   5

CAGTTTGGACTCGTTCGGCGCCGATTCCAATGCGCGACCGGAGTCGGCCACCCCCAGGTCGGAGGAACAACAGGATCAGGATCGACAACAGACTGCTCCGGCGGCCAGTGAAACTCTGGA
 S  L  D  S  F  G  A  D  S  N  A  R  P  E  S  A  T  P  R  S  E  E  Q  Q  D  Q  D  R  Q  Q  T  A  P  A  A  S  E  T  L  D  45

TACGCCCGCCTCGCCGCCAGCGGAGGATGCAGCTGCGCAGGAAAGCAAACCGCCTCCTTCCAAGGAGGCGGATGACGACTTCGAACAGATCTCCGAGGGATCATTAGACGCCGATGATAA
 T  P  A  S  P  P  A  E  D  A  A  A  Q  E  S  K  P  P  P  S  K  E  A  D  D  D  F  E  Q  I  S  E  G  S  L  D  A  D  D  N  85

CCAGTCGCGGGATAAGGCGGCTAGTACACCAGCTGATCCCGTGGATGCGGAGGAGCCCGCCGAGGAGCCAACGCCCAACGAGGAGCCGCAAGGCGGTGAGGATGAGCAGCCACAAACAGA
 Q  S  R  D  K  A  A  S  T  P  A  D  P  V  D  A  E  E  P  A  E  E  P  T  P  N  E  E  P  Q  G  G  E  D  E  Q  P  Q  T  E  125

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 A  V  R  V  D  P  Q  E  E  P  E  E  A  E  E  E  T  D  A  L  Q  A  T  A  E  E  V  E  A  A  A  A  E  A  D  A  V  D  G  G  165

TCCCGCTTCACCCGAAGCAATGGATGTGGATGAAGGCGAGGTGGATGCAGAAGGTGAAGCTGAGCAGGAAGCAGAAGACGACGCAGCTCTGGATCGAGAGGCGACAGATGATGTAGATAT
 P  A  S  P  E  A  M  D  V  D  E  G  E  V  D  A  E  G  E  A  E  Q  E  A  E  D  D  A  A  L  D  R  E  A  T  D  D  V  D  M  205

GCAGTCCGTGGGCGCAGATTCACACCAGGCGGAGGATGCAGAGCCACCGGAGGGCCGAGATGAGGTGGACACAAGCTTAGAGGAGCAGGAAAATGGCCAAGATCCTGATGAACGGGAGAA
 Q  S  V  G  A  D  S  H  Q  A  E  D  A  E  P  P  E  G  R  D  E  V  D  T  S  L  E  E  Q  E  N  G  Q  D  P  D  E  R  E  K  245

AGATGCGGCCGCCGATGAAGATGATCCGGATGCAGAAGGAGATGCGGAATCGGAGCAGGTGGATGAAAATGCCGAGGAAACTGGTGAGGCCGAACATGGCGATGgtaagtcttagtactt
 D  A  A  A  D  E  D  D  P  D  A  E  G  D  A  E  S  E  Q  V  D  E  N  A  E  E  T  G  E  A  E  H  G  D  D                280

ctttgatgcatgtttataaaagactaaatgaatgaactatttacattatagACACAGTGGAAAATGTTTTGGAGCCAGAGGAATCAGATGTTTGCCTTATTCCCGATGATCAAGAAACGG
                                                                                                  T  V  E  N  V  L  E  P  E  E  S  D  V  C  L  I  P  D  D  Q  E  T  E  303

AAGTGACTGAAGCTGAGAAGGAACTGGCCCGCGAGAATGCCGAGAAGGCCGCCGAAGAGGAGGAAGCCGAGGAGCGGCAAGCCAAGACACCCGATAACGATAGAAAGTCCCCGGCAGATG
  V  T  E  A  E  K  E  L  A  R  E  N  A  E  K  A  A  E  E  E  E  A  E  E  R  Q  A  K  T  P  D  N  D  R  K  S  P  A  D  A  343

CCGCCGCCGATCCAGAAGACGACAGTGCAGCTGCCGTTGACGCCACCGACGTATCTCCAACCACGCCAACTGgtaagtgctccacactaagacttgaaatgccctatcccacatctactc
  A  A  D  P  E  D  D  S  A  A  A  V  D  A  T  D  V  S  P  T  T  P  T  A                                            367

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GAGAAGGAAAACGATAAGGCAGAGGAGAACGCCGAGGAGGGGGAGGAGGAGGAGGCTCGGCAAAAGCCTGCTGCTCCCAGTGGGTTCGAGTACATCTGCGATACCGCCTGTGTCGACGCC
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L  L  R  D  Q  P  G  K  Y  F  V  R  R  K  K  F  L  V  E  E  V  V  R  E  E  G  A  A  E  A  E  A  S  A  D  G  E  G  G  D   524

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F  N  L  L  N  A  E  E  P  D  K  F  K  L  K  R  H  S  I  R  V  R  N  I  G  A  T  T  L  Q  P  P  Q  N  S  P  T  K  R  T   604

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C  V  R  F  G  F  D  V  R  Q  F  C  C  A  G  C  L  N  T  F  K  K  G  L  K  T  C  S  C  C  Q  K  D  I  N  G  G  Q  E  G   724

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R  H  I  V  S  C  Q  W  C  K  V  K  K  Y  N  F  D  M  I  Y  Q  I  G  G  P  H  D  Q  E  T  L  T  C  S  I  N  C  L  T  M   884
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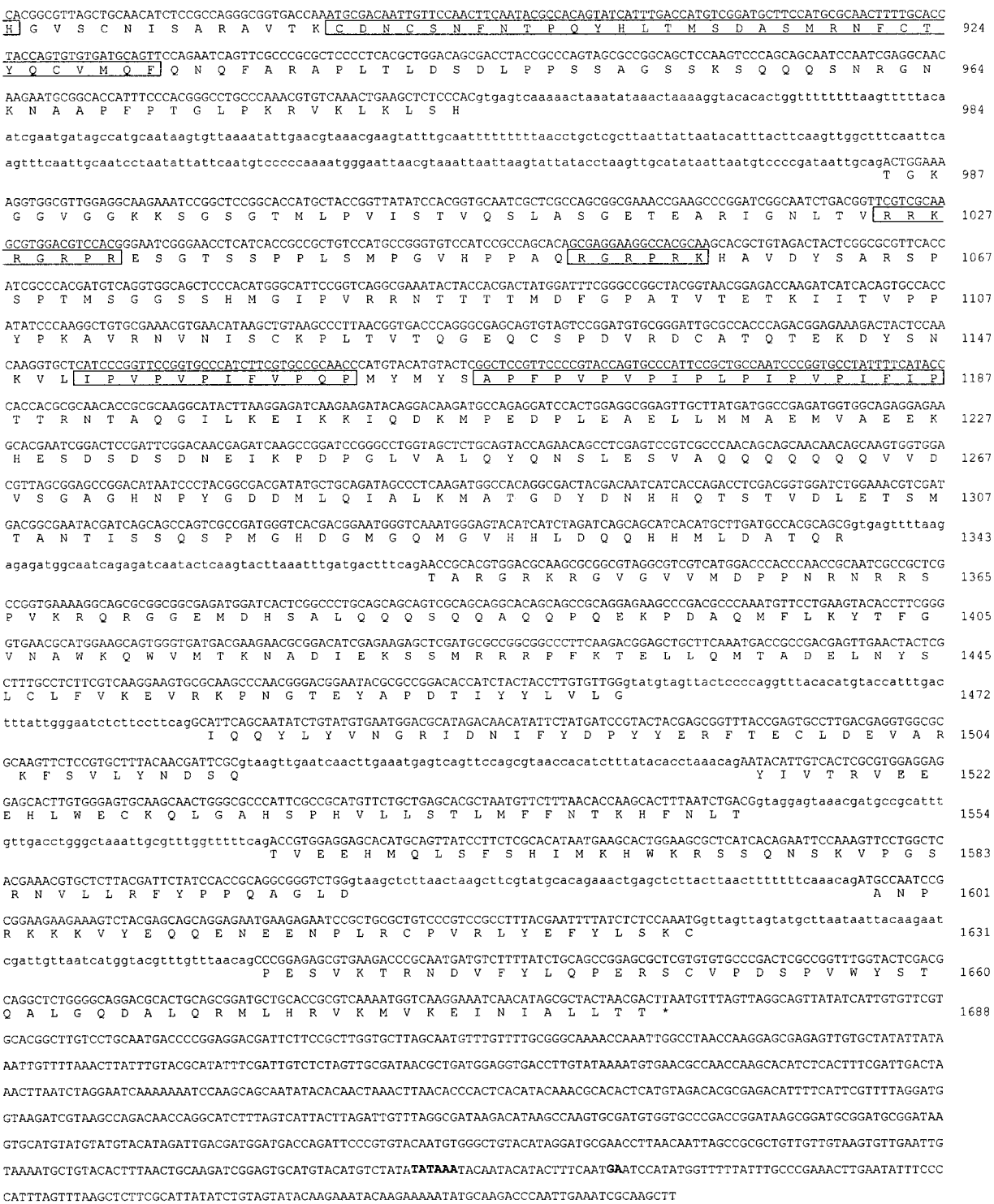


FIG. 5—*Continued*

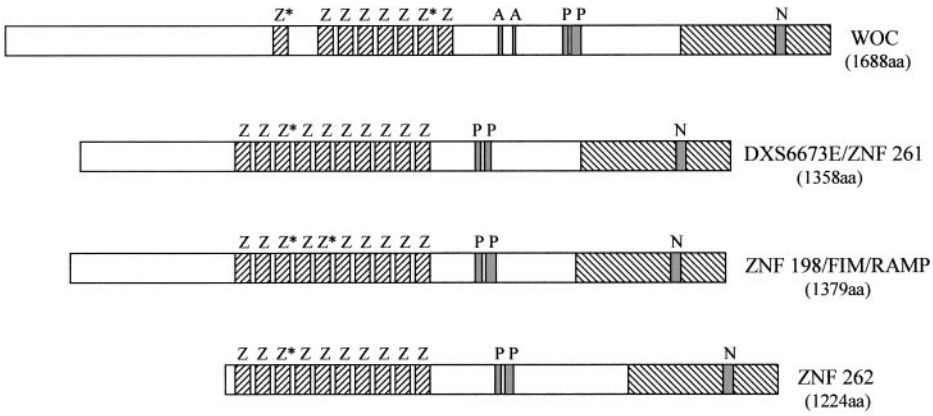


FIG. 6. Comparison of the *Drosophila* WOC protein structure and the three human homologues. The putative zinc fingers of the C2–C2 type are indicated by Z. The asterisks indicate the zinc fingers where the third cysteine is not preceded by phenylalanine. The proline-repeat domains are shown as P, the AT-hook DNA binding motifs as A, and the putative bipartite nuclear localization signal as N. The hatched part at the C-terminus represents a highly conserved region.

repeats first described by Xiao *et al.* (1998) in the ZNF198 protein. This highly hydrophobic region was mentioned by van der Maarel *et al.* (1996) as putative transmembrane domain for the DXS6673E protein. The putative bipartite nuclear localization signal described by Popovici *et al.* (1998) for the FIM (ZNF198/RAMP) protein is conserved in the *Drosophila* WOC as well as in the two other human homologues (Fig. 6). In contrast the two AT-hook DNA-binding motifs are present exclusively in the *Drosophila* WOC protein. In addition to the above-described motifs it is of note that the C-terminal part (about 300 aa) is highly conserved in the human WOC proteins as well as in the *Drosophila* WOC (e.g., WOC-DXS6673E 49% identity, ZNF198-DXS6673E 47% identity).

Northern Analysis

The expression of the *woc* gene was analyzed first for all three wild-type larval stages and a single transcript of 6.8 kb was identified throughout larval development (Fig. 7, lanes L1–L3). This transcript is missing in the woc^{rgl} mutant third-instar larvae (Fig. 7), while in the rescued woc^{rgl} third-instar transformant larvae containing the *woc* genomic region on the second chromosome, the expression is comparable to that of wild type (Fig. 7, lane 15-45). A very long exposure (4 weeks) revealed a faint signal in woc^{rgl} mutant third-instar larvae, indicating a strong reduction in expression rather than its complete absence. Northern analysis using adult females as well as 0- to 2-h embryos revealed in addition to the 6.8-kb transcript a second alternatively spliced maternal transcript of 6.3 kb which cannot be detected at any other stage (not shown).

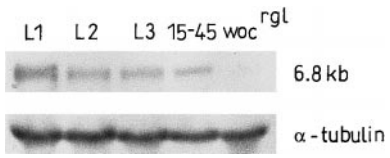


FIG. 7. Northern blot of RNA from first- (L1), second- (L2), and third- (L3) instar wild-type larvae; from L3 larvae of the woc^{rgl} transformant strain 15-45; and from homozygous woc^{rgl} mutant larvae probed with the radiolabeled cDNA 11. The α-tubulin control is shown below.

In Situ RNA Expression during Wild-Type Embryogenesis

Wild-type stage 1 to 4 preblastoderm embryos exhibited large amounts of maternal *woc* mRNA in the cortical cytoplasm and throughout the yolk (Fig. 8A). In blastoderm embryos (stage 5), *woc* mRNA was found throughout the cytoplasm. During gastrulation *woc* mRNA could be seen in the cephalic region, the anterior and the posterior transverse furrows, and the proctodeum, as well as in the presumptive germ band (Fig. 8B). In the following developmental stages up to the fully extended germ band (stage 11), *woc* mRNA was present in the mesoderm and neuroectoderm (Fig. 8C). With the beginning of germ-band retraction *woc* mRNA starts to accumulate in the supraesophageal ganglion (Figs. 8D and 8E). At stage 14 the central nervous system exhibits strong *woc* mRNA expression while the visceral mesoderm revealed only faint expression (Figs. 8F and 8G).

In Situ RNA Expression in Third-Instar Larvae

Woc mRNA expression was analyzed in several wild-type tissues and was positive in midgut, cardia, and cecae (data not shown) as well as in the brain–ventral ganglion complex

and the ring gland. Expression was extremely high in all imaginal discs and the ring gland (Fig. 9). In the brain hemispheres, the presumptive adult optic centers exhibit the highest *woc* expression. Within the ventral ganglion, expression seems to be restricted to the thoracic region (Fig. 9A).

DISCUSSION

The new *woc*^{rgl} mutation described here is another "low-ecdysteroid" *Drosophila* mutation affecting both *in vivo* and *in vitro* molting hormone biosynthesis during the third larval instar. The whole body ecdysteroid titer (Table 1) measured at the early wandering stage of development in wild-type larvae, 27 pg/animal, is consistent with the work of others (Borst *et al.,* 1974; Hodgetts *et al.,* 1977). In comparable early wandering homozygous *woc* mutant larvae, titers are only 37% of those in wild-type larvae and identical to the ecdysteroid level of newly molted wild-type animals at the beginning of the third instar (Garen *et al.,* 1977). It may represent ecdysteroids synthesized at the end of the second instar, i.e., those required for the molt to the third instar, but which have not yet been degraded and/or excreted. After wandering for an additional 2–3 days, by which time all wild-type and heterozygous larvae have pupated, the titer in the remaining homozygous late dauer larvae remains constant, a reflection of the low level of ecdysteroidogenesis occurring in the glands at this stage of development (see below). However, after 1–2 weeks of wandering, the whole body titers of these homozygous old dauer larvae fall below the level of sensitivity in this particular assay format, i.e., $<$1.6 pg/animal.

In vitro, wild-type brain–ring gland complexes release ecdysteroids into the medium at a rate of 60 pg/gland/h, consistent with previous data (Henrich *et al.,* 1987a). The low *in vitro* ecdysteroid secretory activity of brain–ring gland complexes dissected from early third-instar homozygous *woc* larvae, 16% of wild type, is consistent with their respective low whole body titers (Table 1). While glands from late homozygous dauer *woc* larvae still retained this low rate of ecdysteroid production, the activity of glands from old homozygous *woc* larvae was below the limit of detectability in this particular assay format, i.e., $<$5 pg/gland/h.

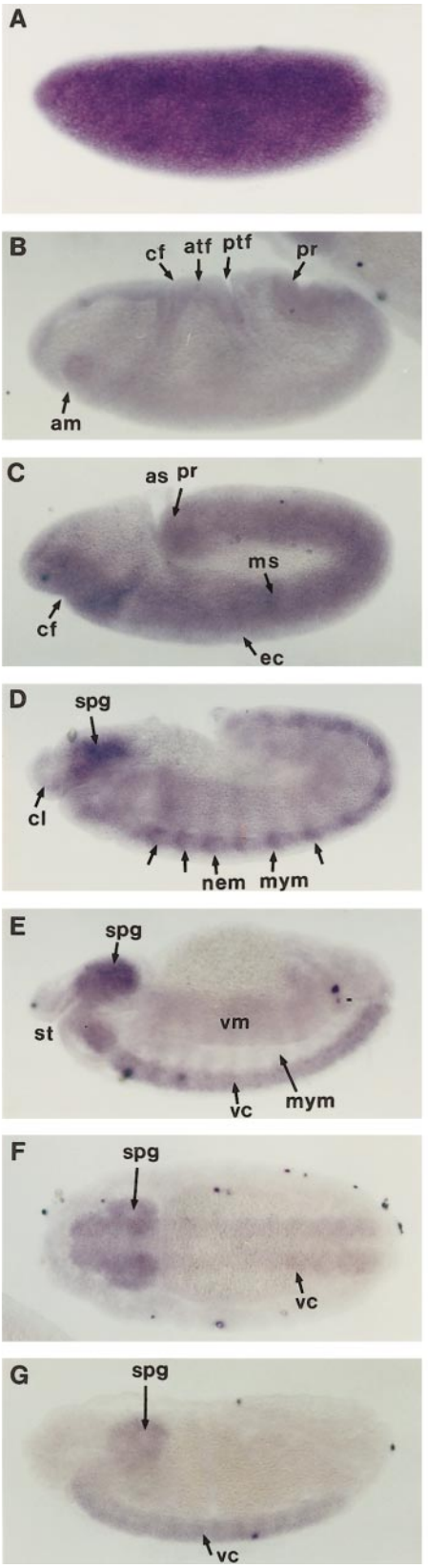
The level of ecdysteroids synthesized by the glands but not yet secreted into the medium following *in vitro* incubation of both heterozygous and homozygous *woc* mutant glands was highly variable in the range of 3 pg/gland or less, similar to glands from wild-type larvae (Redfern, 1983). The data indicate that the low *in vitro* brain–ring gland complex ecdysteroid secretory activity of both heterozygous and homozygous individuals is not due to a failure to secrete ecdysteroids, but rather is due to the failure to synthesize a critical quantity of ecdysteroids. This failure ultimately results in the lower whole body titers found in these animals. Being a reflection of active ecdysteroid concentrations in the target tissues, the whole body titer in homozygous *woc* mutant larvae during the third instar apparently never attains a threshold value that is required for normal molting.

Interestingly, only six such mutations are currently known. The first extensively studied ecdysone-deficient mutant, the *lethal(3)ecdysoneless*^{1ts} (*l(3)ecd*^{1ts}), was first described by Garen *et al.* (1977). This conditional larval lethal mutation disrupts ecdysone synthesis by the ring gland at the restrictive temperature and can be rescued by exogenous 20-hydroxyecdysone. Subsequently, Warren *et al.* (1996) showed that the defect in ecdysteroidogenesis does not affect any of the enzymes involved in either the 7,8-dehydrogenation of cholesterol or 25-hydroxycholesterol or the subsequent oxidation of 7-dehydrocholesterol or 7-dehydro-25-hydroxycholesterol to ecdysone. Instead, the *ecd*^{1ts} mutation appears to affect the expression of a gene responsible for the efficient transport of sterol intermediates from the site of synthesis in the microsomal compartment to the site of subsequent oxidation to ecdysteroids.

The present *woc*^{rgl} mutation also forms a puparium and pupates after administration of exogenous 20-hydroxyecdysone. This suggests that third-instar mutant larvae are capable of responding to 20-hydroxyecdysone and that the inability of the larvae to pupariate/pupate is due to a failure to achieve a threshold hemolymph titer necessary for molting.

On the fine-structural level, *woc*^{rgl} prothoracic gland cells from old homozygous *woc* wandering larvae exhibit large amounts of lysosomes and lysosome-like structures, which point to an early degeneration process in contrast to the wild-type ring gland. In addition there is a drastic reduction of peripheral membrane invaginations and SER (compare Figs. 3 and 4). The fine-structure preparation of *ecd*^{1ts} prothoracic cells, in contrast, shows no sign of degeneration (Dai *et al.,* 1991), although as in *woc*^{rgl} prothoracic gland cells, membrane invaginations and the SER in *ecd*^{1ts} also are reduced. The accumulation of lipid droplets (large intracellular spaces) in both *ecd*^{1} and *woc*^{rgl} mutants, presumed to contain sterol precursors of ecdysteroids, suggests problems with sterol transport or sterol utilization, respectively. Similar to the *ecd* gene (Henrich *et al.,* 1993), the *woc* gene is expressed in the ring gland and in other larval tissues such as the imaginal discs, the brain–ventral ganglion complex, and others. Especially severe defects can be observed in *woc*^{rgl} imaginal discs, which show an aberrant folding pattern and smaller size (Fig. 2C). The *woc*^{rgl} imaginal disc abnormalities are in accord with other studies which showed that 20-hydroxyecdysone is an essential regulator of imaginal disc growth (Fristrom *et al.,* 1970). It is noteworthy that in the ecdysteroid-deficient conditional *dre4* mutant larvae, imaginal disc growth is not affected (Redfern and Bownes, 1983; Sliter and Gilbert, 1992). It is suggested that the *dre4* gene may regulate steroidogenesis during most, or all, stages of postembryonic development.

Ecdysteroids are involved in the control of female reproduction as well (see Hagedorn, 1985) and *Drosophila* oogen-



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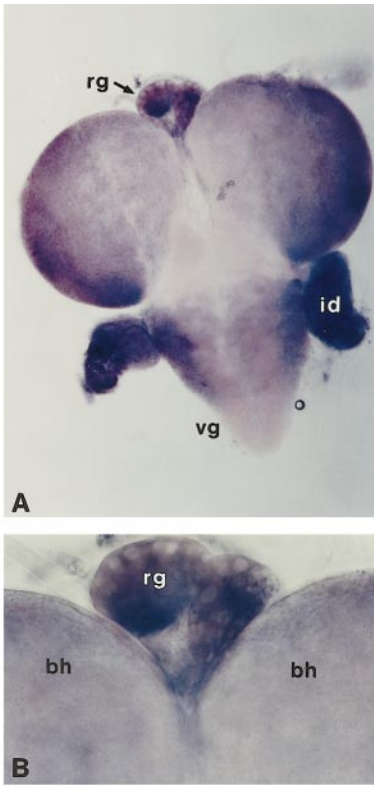

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esis is also hormonally controlled. Ecdysteroid-deficient mutations consistently affect oogenesis (Garen *et al.*, 1977; Redfern and Bownes, 1983; Henrich *et al.*, 1993; Warren *et al.*, 1996; Richard *et al.*, 1998). The viable *woc* allele *woc*^{st1} shows not only female, but also male sterility, indicating the involvement of ecdysteroids in spermiogenesis as well.

Since neither the *ecd*^{1} nor the *dre4* gene causing ecdysteroid deficiency has been cloned, a molecular analysis of their function has not been completed. The cloning of the *woc* gene should allow functional analyses, and such analyses are now proceeding (J. T. Warren *et al.*, unpublished information). The amino acid sequence of the putative *Drosophila* WOC protein reveals the presence of eight zinc fingers of the C2–C2-type (Figs. 5 and 6). Thus, the putative WOC protein may function as a transcription factor. *In situ* RNA–RNA hybridizations to wild-type larval tissues allows speculation on the multiple functions of the WOC protein in the ring gland, the brain–ventral ganglion complex, the imaginal discs (Fig. 9), the midgut, the cardia, and the cecae. Northern blot analysis of wild-type larvae revealed a continuously expressed *woc* gene (Fig. 7). Nevertheless, variations during the instars, for instance higher expressions at molts than in the intermolts, cannot be excluded. In older mutant larvae the *woc* mRNA can be barely detected. In the *woc*^{rgl} rescued strain 15-45, mRNA is expressed in amounts comparable to those in the wild type (compare Fig. 7, lanes L3 and 15-45). The rescue of the mutant phenotype by P-element-mediated germ-line transformation together with the reappearance of the *woc* transcript shows unequivocally that the lack of *woc* gene activity is the causal event in the inhibition of ecdysteroid biosynthesis.

Sequence homology searches using the Blast program identified three human genes homologous to *woc*. The mutated human DXS6673E gene, described by van der Maarel *et al.* (1996), was purported to be a candidate gene for nonspecific, X-linked mental retardation. The DXS6673E gene consists of 25 exons defining an open reading frame of 4074 bp. In comparison, the *Drosophila woc* gene with only 11 exons shows an open reading frame of 5064 bp (Fig. 5). Despite the fact that most of the exon–intron splice sites in the 5′ region of the gene are different, the last two exon–intron splice sites are identical. Figure 6 shows that the structures of the *Drosophila* WOC and the human DXS6673E proteins are highly conserved although the DXS6673E protein contains two more zinc fingers than the WOC protein and lacks the AT-hook domains (Reiter *et al.*, 1998). The second human gene, ZNF198/FIM/RAMP, shows substantial homology with DXS6673E (Popovici *et al.*, 1998). It is fused with FGFR1 in the t(8;13) in the leukemia/lymphoma syndrome. All the translocation events described so far had occurred within the conserved proline-repeat region. The ZNF198/FIM/RAMP protein, like DXS6673E, contains 10 zinc fingers which have been previously mentioned as five MYM domains each consisting of two zinc fingers. We do not use this designation because each of the individual zinc fingers follows the same consensus sequence, $CX_2CX_{13\text{-}18}$-$GX_4FCSX_2CX_3Y/F$. It is of note that in all the human proteins the C-terminal 300 aa are highly conserved, even in comparison to the *Drosophila* WOC. The considerably high conservation of the *Drosophila* WOC and the ZNF198/FIM/RAMP, ZNF261/DXS6673E, and ZNF262 proteins suggests strongly that the human homologues may also play a developmental role, perhaps via steroid hormone synthesis. Future germ-line transformation of the *woc*^{rgl} mutant animals with one of the human *woc* genes should test this assumption. The possibility of the WOC protein regulating the expression of an enzyme catalyzing the very first step in ecdysteroid biosynthesis (J. T. Warren *et al.*, unpublished information) leads to the intriguing possibility that a disorder in steroid hormone synthesis can be causally related to mental retardation in humans.

ACKNOWLEDGMENTS

We thank Erika Jost for her able technical assistance, Ursula Böll for excellent photographic work, and Regina Gramsch for the graphic representations. We are grateful to Uta Kestner and Pat Cabarga for clerical assistance. The genetic and molecular work was supported by the Deutsche Forschungsgemeinschaft. Research from the Gilbert laboratory was supported by Grants DK-30018 from the NIH and IBN-9603710 from the NSF.

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FIG. 8. *In situ* RNA expression of the *woc* gene during embryogenesis. Anterior is left. (A–E, G) Lateral view; (F) dorsal view. (A) Preblastoderm embryo exhibiting large amounts of maternal *woc* mRNA. (B) Gastrulation embryo showing *woc* mRNA in the cephalic region(cf), the anterior (atf) and the posterior (ptf) transversal furrow, the anterior midgut (am) and the proctodeum (pr). (C) Stage 10 embryo *woc* mRNA was found in the mesoderm (ms) and the ectoderm (ec). The amnioserosa (as) shows no staining. (D) Stage 12 embryo. Staining is seen in the supraesophageal ganglion (spg), the neuromeres (nem), and the myomeres (mym). (E) Stage 14 embryo, with prominent staining in the spg and reduced expression in the visceral mesoderm (vm), the mym and the ventral nerve cord (vc). (F) The same is seen in a dorsal view of a stage 15 embryo. (G) Stage 17 embryo, only the ventral nervous system (spg and vc) shows *woc* mRNA expression.
FIG. 9. *In situ* RNA expression of the *woc* gene in wild-type larvae. (A) Brain–ventral ganglion (vg) complex with the imaginal discs (id) and ring gland (rg). (B) Higher magnification of the brain hemispheres (bh) and the ring gland (rg).

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Received for publication November 9, 1999
Revised March 28, 2000
Accepted June 3, 2000