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The three dominant female-sterile mutations of the *Drosophila ovo* gene are point mutations that create new translation-initiator AUG codons

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SUMMARY

The *Drosophila ovo* gene, which encodes a putative transcription factor (Ovo) with TFIIIA-like zinc fingers, is required for female germline survival and proper oogenesis. Three dominant female-sterile ovo^D mutations cause ovarian abnormalities that define an allelic series, with ovo^{D1} displaying the stronger phenotype and ovo^{D3} the weaker. We report here that all three ovo^D mutations are point mutations that create new in-frame methionine codons in the 5′ part of *ovo*. There are two types of overlapping *ovo* transcription units, *ovo*α and *ovo*β. By using various *ovo-lacZ* reporter genes, we determined that the long Ovo isoforms starting at methionine M1, present in transcripts *ovo*α, are expressed at low levels only in mature oocytes. Short Ovo isoforms are translated from methionine M373, the first in-frame start codon present in transcript *ovo*β, and correspond to the activity defined by recessive loss of function *ovo* mutations. The new AUGs created in ovo^D mutations all are located upstream of the M373 initiation site. Our results support the hypothesis that they can substitute for M373 as translation starts and initiate the synthesis of Ovo proteins that have extra amino acids at their N termini. We propose that premature expression of long Ovo protein isoforms occurs in ovo^D mutants and interferes with wild-type Ovo function in controlling female germline differentiation.

Key words: *ovo*, dominant female-sterile mutations, translation, *Drosophila*

INTRODUCTION

Two systems act in parallel to determine the sex differentiation pathway of *Drosophila* germ cells. An autonomous signal causes XY germ cells to develop in the male mode, irrespective of the somatic environment, while XX germ cells require both an autonomous signal determined by the X/A ratio and an inductive signal from the soma (Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1994). Systematic screens for female sterile mutations have identified a large number of genes specifically required for proper oogenesis (Gans et al., 1975; Mohler, 1977; Perrimon et al., 1986; Schüpbach and Wieschaus, 1989, 1991). Mutants belonging to the so called 'ovarian tumor class' present small ovaries with egg chambers filled with an excess of undifferentiated germ cells. The initial observation that female germline cells defective in *Sxl* also form tumorous cysts (Schüpbach, 1985) led to the idea that this phenotype identifies loci involved in germline sex determination. This was supported by the fact that, among tumorous mutants (*bam*, McKearin and Spradling, 1990; *otu*, King et al., 1986; *snf*, Oliver et al., 1988; Steinmann-Zwicky, 1988; *Sxl*, Schüpbach, 1985; and *ovo*, Busson et al., 1983), only *bam* has a function in the male germline. Identification of the genes required for germline cells to respond to the somatic feminization signal, and determination of the biochemical pathway that is involved, are of primary interest. Because it is specifically required in females and acts upstream of *Sxl* in the germline, it has been proposed that *ovo* is a key gene in this pathway (Pauli and Mahowald, 1990; Oliver et al., 1993, 1994; Horabin et al., 1995).

Recessive, null, *ovo* alleles produce rudimentary ovaries in which germ cells have degenerated, leaving only somatic tissue. Hypomorphic *ovo* alleles permit more extensive, although abnormal, development: germ cells survive but fail to differentiate, and egg chambers are filled with a large number of mitotically active, undifferentiated cells. Three dominant ovo^D mutations, ovo^{D1}, ovo^{D2} and ovo^{D3}, behaving as antimorphic alleles, have also been isolated and these, like recessive alleles, are fully penetrant for female sterility and have no effect on the male germline. In heterozygous ovo^{D3}/+ females, egg chambers complete vitellogenesis and eggs are laid, but they do not develop. In ovo^{D1}/+ females, oogenesis is arrested prior to or at stage 4. ovo^{D2} presents an intermediate phenotype with most egg chambers degenerating around stage 10 (Fig. 4D). The complete lack of vitellogenesis in ovo^{D1}/+ females has made this mutation a widely used tool for germline clonal analysis (Chou et al., 1993; Mével-Ninio et al., 1994).

Consistent with their ovarian phenotype, the antimorphic effect of ovo^D mutations seems to irreversibly affect germ cell differentiation only late during development, since elimination of the ovo^D allele by mitotic crossing over in germ cells of adult females allows normal oogenesis to proceed (Perrimon, 1984). This suggests that the *ovo* early requirement for germ cell survival (Staab and Steinmann-Zwicky, 1995) and later

function during oogenesis may involve the regulation of separate sets of genes, with only the late function being sensitive to ovo^D mutations. This led us to investigate the molecular nature of these mutations.

ovo is part of the complex locus *ovo-svb*, as it shares most of its coding sequences with *svb*, a gene involved in embryo patterning. Several predicted Ovo protein isoforms are generated from transcripts initiated at two separate sites (Mével-Ninio et al., 1995, and this report) and subject to alternative exon splicing (Garfinkel et al., 1994; Mével-Ninio et al., 1995). All isoforms are, however, predicted to include the same TFIIIA-like zinc fingers. Together the genetic and molecular data suggest that *ovo* is a transcription factor gene, controlling the expression of functions specifically required for female germline differentiation.

ovo^D mutations constitute, to our knowledge, the only existing allelic series of dominant antimorphic mutations in a transcription factor. The antimorphic effect of ovo^D mutations could be predicted to result from either *ovo* mis-expression during early stages of oogenesis or the production of proteins with altered, dominant, properties (see Little et al., 1995). We report here that all three ovo^D mutations are point mutations that create in-frame methionine codons, at sites upstream of the normal initiation site. The observed expression patterns of wild-type Ovo and Ovo^{D1} fusion proteins support the hypothesis that the neo-AUG created in ovo^{D1} is a functional translation initiator codon. Thus, the dominant-negative effects of the ovo^D mutations are likely to arise from the expression of novel protein isoforms, starting early during germline cell differentiation. The nature of revertants of ovo^{D1} provides possible mechanisms for the dominance of the ovo^D mutations.

MATERIALS AND METHODS

Drosophila stocks

Flies were raised on standard *Drosophila* medium at 23°C (Gans et al., 1975). The three *X*-linked dominant female-sterile ovo^D mutations (Busson et al., 1983) were kept as attached-*X* stocks: *C(1)DX,y f/Y* females crossed to ovo^D, v^{24}/Y males. As wild-type homologs of ovo^{D1} and ovo^{D2} on the one hand and ovo^{D3} on the other, we used the strains *fs(1)K1075* and *fs(1)K1540*, respectively, which carry isogenized X chromosomes (Busson et al., 1983). Description of balancers and mutations that are not described in the text can be found in Lindsley and Zimm (1992).

Plasmid construction and *Drosophila* transformation

Plasmids containing the 7 kb *Sal*I-*Hin*dIII *ovo* genomic fragment (previously shown to be able to rescue ovo^- but not svb^- mutations), or the homologous fragment derived from ovo^{D1} DNA, have been previously described (Mével-Ninio et al., 1991, 1994). Hybrid [ovo^+-ovo^{D1}] transgenes, depicted in Fig. 1, were cloned into the pW6 transformation vector (Klemenz et al., 1987). *ovo-lacZ* fusion genes were constructed, starting from the 7 kb *Sal*I-*Hin*dIII wild-type or ovo^{D1} genomic fragments. In-frame fusion of *ovo* and *lacZ* coding sequences was verified by DNA sequencing. The nucleotide positions (nt) refer to the EMBL database *ovo* sequence DMOVO with accession number X59772. For the p*P[ovoM1]* and p*P[ovoM-D1]* constructs, the 1.65 kb *Sal*I-*Bst*YI *ovo* fragments (nt 0-1678), issued from ovo^+ and ovo^{D1} DNA, respectively, were introduced into the pCaSpeR β-gal vector (Thummel et al., 1988) upstream of, and in-frame with, the bacterial β-galactosidase (*lacZ*) gene. The first wild-type in-frame codon for methionine (M1) is in exon 1a (nt 463-465), while the new methionine present in Ovo^{D1} is at position 79 (nt 1281-1283). In both p*P[ovoM1]* and p*P[ovoM-D1]*, fusion between *ovo* and *lacZ* is at the Ovo alanine residue 212 (nt 1677-1679). In p*P[ovoM373]*, the 2.26 kb long ovo^+ DNA fragment (nt 0-2268), was fused to *lacZ*. In this construct, the Ovo-lacZ fusion proteins initiate at either methionine M1 or M373 (see Figs 1, 2).

The pW6 and the pCaSpeR β-gal vectors contain a mini-*w* gene (Klemenz et al., 1987; Thummel et al., 1988). The hybrid p*P[*ovo^+-ovo^{D1}*]* constructs and the p*P[ovo-lacZ]* reporter constructs were injected together with the helper plasmid pUC*h*sDelta2-3 (Flybase ID:FBmc0002087) into the w^{1118} host line, and transformants were selected as described by Spradling and Rubin (1982). Several transformed lines were obtained (17 with p*P[ovoM1]*, 10 tested; 4 with p*P[ovoM373]*, 4 tested; 11 with p*P[ovoM-D1]*, 6 tested). *X-gal* staining reactions were carried out as described in Mével-Ninio et al. (1995).

Isolation and sequencing of the 800 bp *Pst*I-*Bst*XI DNA fragment from wild type and ovo^D mutant strains

Adult genomic DNA was isolated from the ovo^{D2} and ovo^{D3} mutants and from their parental strains, according to Bingham et al. (1981).

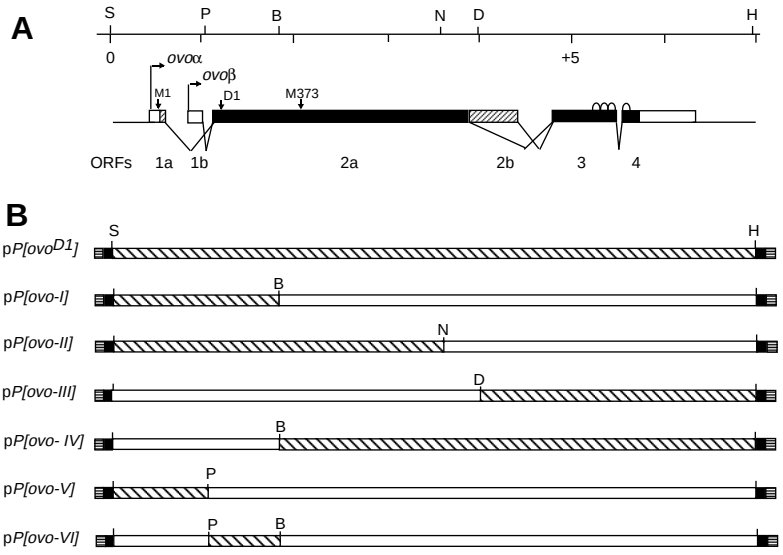

Fig. 1. (A) Molecular organization of the *ovo* locus. Top: restriction map of the 7 kb *Sal*I-*Hin*dIII genomic region sufficient to rescue *ovo* mutations in transgenic flies (Mével-Ninio et al., 1991). Coordinates are in kb from the *Sal*I site at position zero. B, *Bam*HI; D, *Drd*I; H, *Hin*dIII; N, *Not*I; P, *Pst*I; S, *Sal*I restriction sites. Bottom: structure of the *ovo* transcription units. Black boxes represent ORFs common to *ovo* and *svb*. Hatched boxes show ORFs specific to the *ovo* gene. Open boxes at the 5′ and 3′ ends of the gene correspond to untranslated mRNA regions. Positions of the four Cys2/His2 zinc-finger motifs are indicated by half circles. (B) Diagramatic representation of sequences introduced in the pW6 transformation vector and designed to map the ovo^{D1} mutation. Horizontally striped boxes represent sequences of the P-element contained in the pW6 vector and filled boxes sequences of the pBluescript KS+ polylinker. Open boxes correspond to DNA issued from the ovo^+ gene and cross-hatched boxes to DNA issued from the ovo^{D1} gene. Constructs I and III were designed to assay the presence of the ovo^{D1} mutation in the two regions in which *ovo*-specific (as opposed to *ovo-svb*) mutations are found. Constructs II and IV test the complementary regions.

The 800 bp *Pst*I-*Bst*XI DNA fragment was amplified using specific primers: Primer 1, 5′-AGTTGCTGCAGCGTTTGACACCAA-3′ (nt 1087-1110); Primer 2, 5′-GAGCAGAATTCGTGCGGCCAAAATG-3′, (nt 1934-1909). A single nucleotide change was introduced into primer 2 at nt 1926 to create an *Eco*RI restriction site. PCR reactions were performed as described in Sambrook et al. (1989) in 50 μl solution containing 300 ng genomic DNA and 10 pmoles oligomers. The amplified DNA was recovered after phenol-chloroform treatment, digested by *Eco*RI and *Pst*I and cloned into pBluescript KS+. Sequencing was performed using the dideoxy-chain termination method (Sanger et al., 1977). Two independent PCR amplifications from *ovo*^{D2} and *ovo*^{D3} DNAs were sequenced.

RESULTS

Mapping the *ovo*^{D1} mutation by using chimaeric transgenes causing female sterility

ovo^{D} mutations behave genetically as typical dominant antimorphic mutations (Busson et al., 1983), suggesting that they encode proteins that interfere with the activity of wild-type Ovo in a dose-dependent manner. Preliminary molecular analyses suggested that *ovo*^{D} are point mutations, since no differences were detected between the restriction patterns of *ovo*^{D} and wild-type genomic DNAs (Mével-Ninio et al., 1989). The *ovo*$^{+}$ function was restricted to a 7 kb *Sal*I-*Hin*dIII fragment by transformation experiments (Mével-Ninio et al., 1991). The corresponding fragment from *ovo*^{D1} conferred a dominant-sterility phenotype (Chou et al., 1993; Mével-Ninio et al., 1994), showing that the *ovo*^{D1} mutation lies within this fragment.

We first mapped the *ovo*^{D1} mutation by constructing hybrid transgenes in which different regions of the *ovo*$^{+}$ gene have been replaced by the homologous regions taken from *ovo*^{D1} (Fig. 1). Two properties of these transgenes were examined when injected into flies. First, since the *ovo*^{D1} mutation resulted in an arrest of oocyte development, we expected that

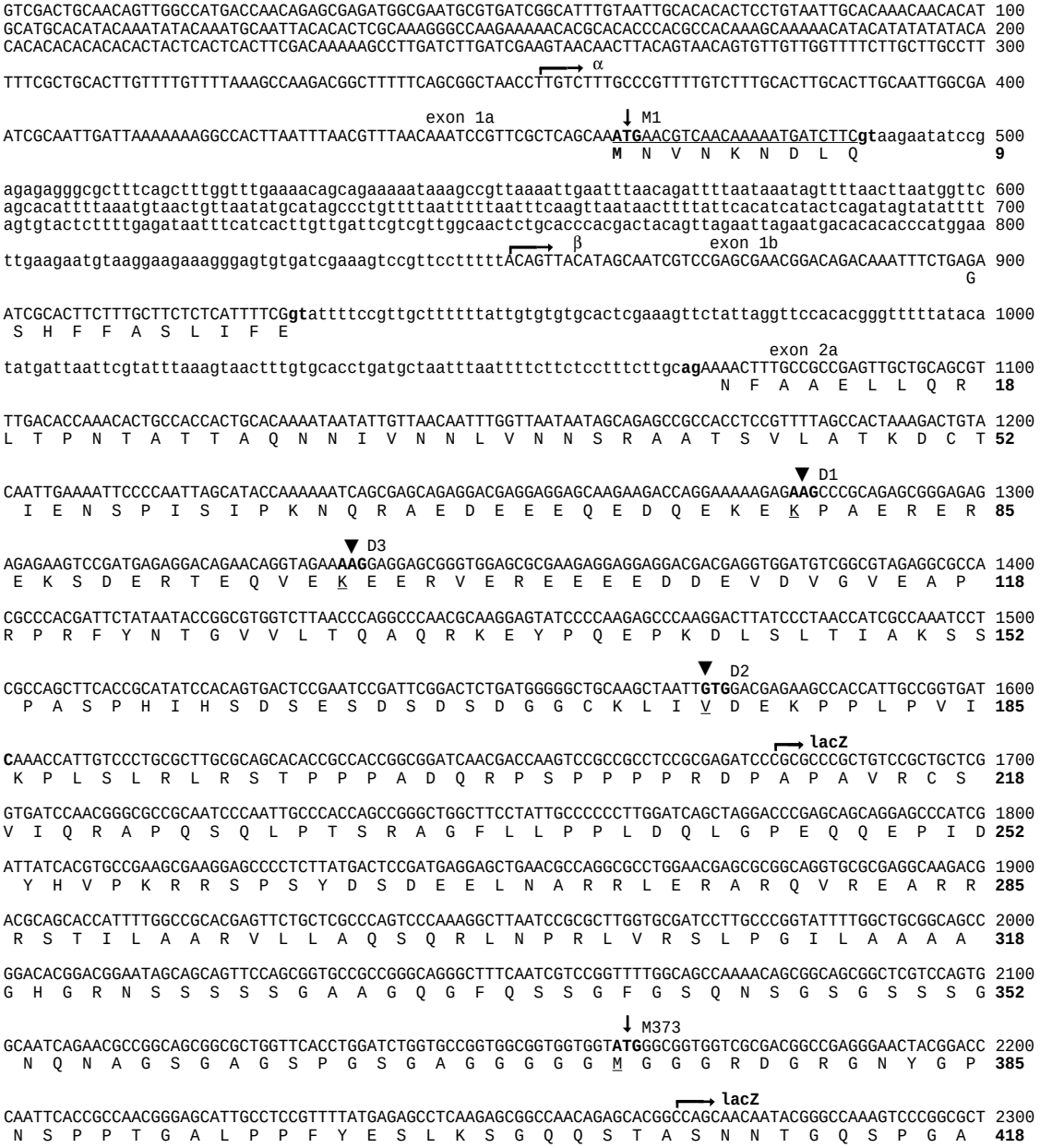

Fig. 2. Nucleotide and protein sequence of the 5′ region of the *ovo* gene with introns in lower case letters. The nucleotide sequence starts at the *Sal*I site position 1 and includes exons 1a, 1b and part of exon 2a. Positions of the initiation sites of the *ovoα* and *ovoβ* transcripts are given by the bent arrows. Positions of the wild-type M1 (transcripts *ovoα*) and M373 (transcripts *ovoβ*) initiator methionines are indicated by small downwards-pointing arrows. Positions of the AUG initiator codons created by *ovo*^{D} mutations are indicated by large arrowheads. The positions where *ovo* and *lacZ* sequences are fused in the *ovoM1* (or *ovoM-D1*) and *ovoM373* reporter genes are also indicated. Numbering of amino acids starts at methionine M1, the translation initiator site present in transcripts *ovoα*.

transformed germ cells of injected female embryos (G0) would degenerate, giving no transformant. Second, we expected that these transgenes would mimic the ovo^{D1} phenotype in the female progeny of transformed males.

ovo-specific (as opposed to *ovo-svb*) mutations have been mapped in two separate regions of the *ovo-svb* locus (Mével-Ninio et al., 1991, 1995; Garfinkel et al., 1992). *P[ovo*$^{+}$*-ovo*^{D1}*]* hybrid transgenes I to IV were designed to assay separately each of these two regions (Fig. 1). Table 1 shows that these transgenes fall into two classes. For transgenes III and IV, transformed progeny were obtained from G0 females, and transformed females in the next generation were fully fertile. We further verified that these two transgenes carry an ovo^{+} function by complementation tests using ovo^{D2} (Busson et al., 1983). In contrast, transgenes I and II led to defective oogenesis, as previously observed for *P[ovo*^{D1}*]*, a transgene that contains the whole 7 kb ovo^{D1} fragment (Mével-Ninio et al., 1994). These results mapped the ovo^{D1} mutation to within 1.8 kb in the 5′ region of the gene. Two additional transgenes *P[ovoV]* and *P[ovoVI]* were then tested (Table 1), allowing us to map the ovo^{D1} mutation within a 800 bp *Pst*I-*Bst*XI genomic fragment (nt 1092-1869) (Fig. 1).

Each of the three ovo^{D} mutations creates an in-frame AUG codon with the potential to generate new Ovo isoforms

To determine the nucleotide position of the ovo^{D1} mutation, we compared the sequences of the *Pst*I-*Bst*XI fragment from both the ovo^{D1} and its parental ovo^{+} chromosome (from strain *fs(1)K1075*). While the sequence of *fs(1)K1075* proved to be identical to the published *Oregon R* sequence (Mével-Ninio et al., 1991), a single difference was found between ovo^{D1} and wild-type DNA: the A at nucleotide position 1282 in wild type is replaced by a T in ovo^{D1}. This substitutes a methionine for a lysine at position 79 in the open reading frame in exon 2 (Fig. 2).

The fact that all three dominant ovo^{D} mutations display similar sterility phenotypes, albeit of different severity, led us to predict that the position in the Ovo protein is similar. Starting from this hypothesis, we sequenced the *Pst*I-*Bst*XI fragment from the ovo^{D2} and ovo^{D3} mutant genes, and the ovo^{D3} parental gene (*fs(1)K1540* strain). As in ovo^{D1}, a single nucleotide change was detected in either ovo^{D2} or ovo^{D3}. In ovo^{D2}, the G at nucleotide position 1572 in wild type is replaced by an A. This substitutes a methionine for a valine at position 176. In ovo^{D3}, the A at nucleotide position 1336 is replaced by a T. This creates a methionine in place of a lysine at position 97 (Fig. 2). Therefore, in all three ovo^{D} mutations, a new in-frame AUG codon is created in the open reading frame in exon 2.

Two separate AUGs are differentially used for wild-type Ovo protein synthesis

The unexpected finding that all three dominant ovo^{D} mutations create new in-frame AUG codons raised the possibility that these codons are used as bona fide translation initiators, for the synthesis of novel Ovo isoforms with antimorphic activity. We therefore first determined which of two possible methionine codons is used for initiation of the wild-type Ovo protein(s). Indeed, there is evidence for two types

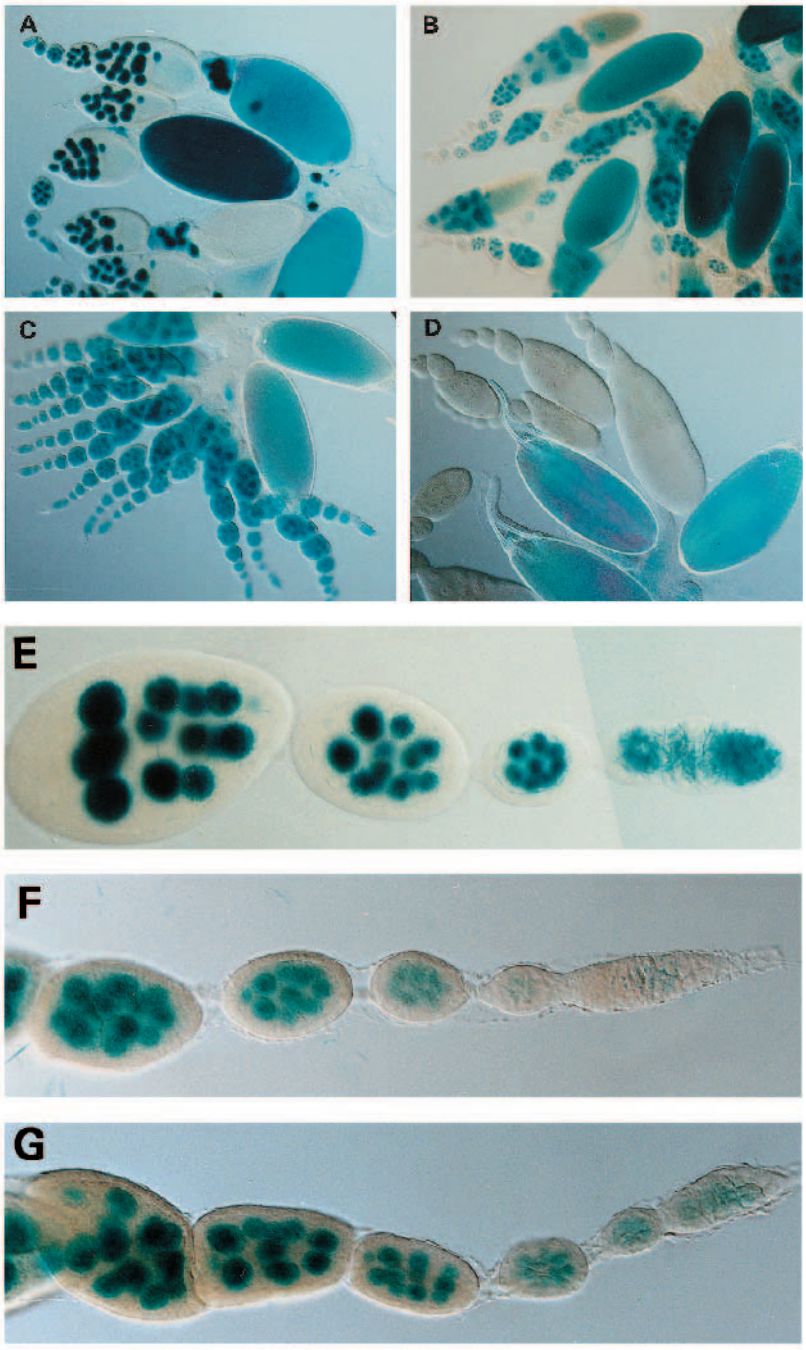

Fig. 3. Expression of *ovo-lacZ* reporter genes during oogenesis. (A) *ovoB*, which encodes the almost entire Ovo protein, is expressed in the nuclei of germ cells throughout oogenesis and accumulates in the cytoplasm of mature oocytes. Expression of *ovoM373* (B) and *ovoM-D1* (C) is similar to that of *ovoB* except that staining is weaker and both nuclear and cytoplasmic. (D) *ovoM1* expression is detected exclusively in the cytoplasm of mature oocytes. (E-G) Enlargements showing expression of *ovoB* (E), *ovoM373* (F) and *ovoM-D1* (G) in the germarium and early egg chambers.

of *ovo* transcripts, differing in their 5′ ends and referred to below as *ovoα* and *ovoβ*, respectively (Mével-Ninio et al., 1995; Figs 1, 2). The first in-frame methionine codon present in transcript *ovoα* is methionine M1, while the first in-frame AUG in transcript *ovoβ* codes for methionine M373. M373 is located in exon 2a, an exon shared by transcripts *ovoα* and *ovoβ*. Initiation of translation at M1 and M373 would therefore result in two different proteins, designated below as OvoM1 and OvoM373, differing at their N termini by the 372 amino acids present solely in OvoM1. To determine at what stage during development M1 and M373 are used for initiating Ovo protein synthesis, we made translational fusions between different parts of *ovo* and *lacZ* coding sequences, to serve as reporter genes in transgenic lines. Transgenes *ovoM1* and *ovoM373* are *lacZ* fusions upstream and downstream of M373, respectively (see Fig. 2 and Materials and Methods). Developmental expression of *ovoM1* and *ovoM373* was examined and compared to expression of *ovoB*, a *lacZ* fusion gene that contains almost the entire *ovo* coding region, with only the two carboxy-terminal codons missing (Mével-Ninio et al., 1995).

Similar to *ovoB*, *ovoM373* expression is detected in the nuclei of germline cells throughout oogenesis before accumulating in the mature oocyte (Fig. 3B,F). Expression of *ovoM373* is also detected in germ cells of female larval gonads (not shown). The only difference with *ovoB* is in the intensity of staining, which is weaker in case of *ovoM373*, making its detection in the germarium variable from line to line. This difference may, at least partly, reflect different stability of the OvoM373 and OvoB proteins, possibly due to the difference in their intracellular localization; OvoB is strictly nuclear while Ovo M373 is both nuclear and cytoplasmic. Possibly for the same reason, *ovoM373* is not detected in male larval gonads or testes. In contrast to *ovoM373*, *ovoM1* expression is detected only late during oogenesis, in the ooplasm of mature oocytes (Fig. 3D). Furthermore, this late expression is weak in every transformant line tested.

Notwithstanding minor quantitative differences between different lines, the main conclusion that could be drawn from comparing the expression of *ovoM1*, *ovoM373* and *ovoB* is that the Ovo protein(s) present during early germline cell differentiation is initiated at methionine M373. The in-frame methionine codons created by *ovo*^{D} mutations therefore represent potential start codons for the synthesis of proteins larger than wild-type OvoM373 during these early stages.

Table 1. Transformation with hybrid *[ovo*$^{+}$*-ovo*^{D1}*]* genes

Insert	A=%transformed G0 males (N_t/N)	B=%transformed G0 females (N_t/N)	B/A	Genotype
P[ovo^{D1}*]*	29 (11/38)	1.8 (1/56)	0.06	*ovo*^{D1}
P[ovo-I]	4.6 (3/65)	0 (0/60)	0	*ovo*^{D1}
P[ovo-II]	17.1 (12/70)	2.6 (2/75)	0.15	*ovo*^{D1}
P[ovo-III]	4.8 (4/83)	10.3 (6/58)	2.1	*ovo*$^{+}$
P[ovo-IV]	10.4 (10/96)	25.3 (24/95)	2.4	*ovo*$^{+}$
P[ovo-V]	10 (16/160)	20 (32/160)	2	*ovo*$^{+}$
P[ovo-VI]	27.2 (21/77)	2.4 (2/82)	0.09	*ovo*^{D1}

G0 adult flies derived from injected embryos were crossed individually and grown at 23°C. From each cross, a mean number of 250 emerging flies were examined for the pigmented eye phenotype. N is the total number of fertile G0 flies and N_t the number of G0 flies giving rise to transformed progeny. The hybrid *[ovo*$^{+}$*-ovo*^{D1}*]* transgenes that were tested are shown in Fig. 1.

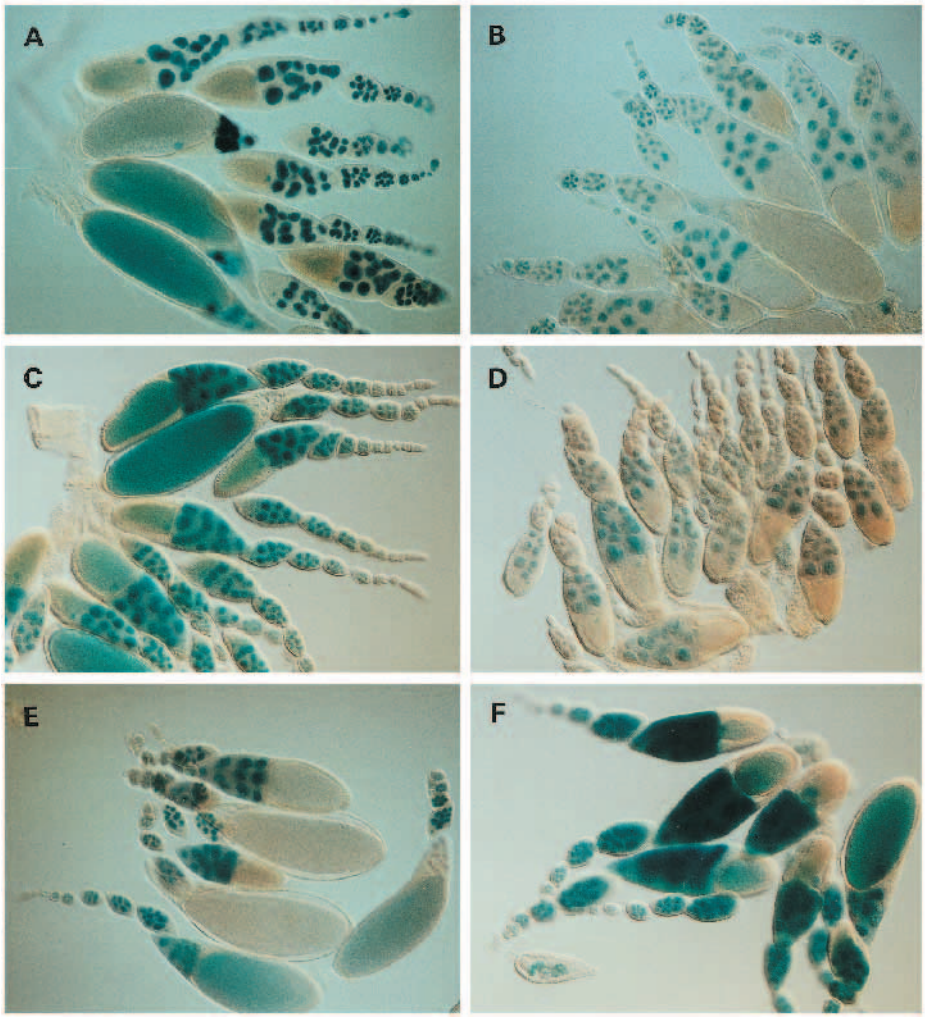

Fig. 4. *ovo* reporter gene expression in ovaries of wild type (A,C,E) and *ovo*^{D2} (B,D,F) flies. Expression of *ovoB* (A,B) or *ovoM373* (C,D) is significantly weaker in *ovo*^{D2} than in wild-type ovaries. Contrary to *ovoB*, *ovoM373* does not rescue the *ovo*^{D2} sterility and egg chamber development is arrested prior to, or at stage 10 (D). *ovoM-D1* exhibits more extensive staining in *ovo*^{D2} than in wild-type egg chambers, although staining in *ovo*^{D2} is mostly cytoplasmic (E,F). Like *ovoB*, *ovoM-D1* partially rescues the *ovo*^{D}*2* oogenesis defects, as attested by the presence of stage 12 oocytes in B and F.

The AUG codon created by the *ovo^{D1}* mutation is most probably a new translation start

To test for translation initiator activity of the neo-AUG codon at position 79 in *ovo^{D1}*, we made an *ovoD1-lacZ* reporter construct, *P[ovoM-D1]*, representing the mutant equivalent of *P[ovoM1]* (Fig. 2). The presence of the *ovo^{D1}* mutation in the *ovoM1* fusion transgene resulted in a spectacular change in the transgene expression pattern. While *ovoM1* is expressed at very low levels, exclusively in mature oocytes, *ovoM-D1* is expressed in germline cells throughout oogenesis, starting in the germarium (Fig. 3C,G). In mature oocytes, strong staining is observed, uniformly distributed in the ooplasm. As a whole, the expression patterns of *ovoM-D1* and *ovoM373* are indistinguishable. A complete transition from the *ovoM1* to the *ovoM373* expression pattern therefore results from the presence of the Ovo^{D1} methionine at position 79. This allowed us to conclude that the AUG created by the *ovo^{D1}* mutation is most likely to be a functional start codon. Because *ovo^{D2}* and *ovo^{D3}* also create new in-frame AUGs upstream of M373, it seems very likely that the same mechanism, i.e. initiation of translation at the newly created AUG, is used in all three *ovo^{D}* mutations. The antimorphic effect of the *Ovo^{D}* mutations is therefore probably due to the synthesis of novel Ovo protein isoforms with NH_2-terminal extensions of 294, 197 and 276 amino acids in Ovo^{D1}, Ovo^{D2} and Ovo^{D3}, respectively.

Expression of the *ovo-lacZ* reporter genes in *ovo^{D}* females

In order to test whether Ovo^{D} interferes with expression of wild-type Ovo, we compared the expression of the *ovoM1, ovoM373, ovoB* and *ovoM-D1* reporter genes in wild-type and *ovo^{D}* ovaries. Whereas no detectable change of *ovoM1* expression was observed, (i.e. it remained very weak and only detectable in mature oocytes), expression of *ovoM373* (or *ovoB*) was significantly weaker in *ovo^{D2}* (Fig. 4,A-D) and *ovo^{D3}* (not shown), compared to wild-type ovaries. In contrast, expression of *ovoM-D1* appeared stronger in *ovo^{D2}* than in wild-type ovaries (Fig. 4E,F). In *ovo^{D1}*/+ females, oogenesis is arrested prior to or at stage 4 and reporter gene activity was either not detected (*ovoM373*) or only detected in a few nuclei (*ovoB* and *ovoM-D1*; data not shown), consistent with a previous report suggesting that Ovo^{D} may negatively regulate wild-type *ovo* expression (Oliver et al., 1994). We noted, however, that the presence of either *ovoB* or *ovoM-D1* appeared to improve significantly the fertility of the dominant-sterile *ovo^{D2}* females. The average number of eggs laid per day per *ovo^{D2}* female rose from 2 to 30 and 24, in the presence of *ovoB* and *ovoM-D1*, respectively. We noted that *ovoB* could allow the development up to the adult stage of more than 10% of the eggs. This rescue is somewhat more efficient than what we observed in a previous study (Mével-Ninio et al., 1995) and is due to the use of a different *ovoB* strain producing higher levels of OvoB protein. In the case of *ovoM-D1*, a very small fraction of laid eggs (fewer than 1/1000) could develop. By contrast, the presence of *ovoM373* did not detectably change the *ovo^{D2}*/+ phenotype. These results indicate that the relative expression of the Ovo^{+} and Ovo^{D} proteins, which is critical for the strength of the *ovo^{D}* phenotype, is very sensitive to the number and nature of the *ovo* genes or transgenes present in *ovo^{D}* mutant cells, even though these transgenes lack most of the Ovo protein-coding information.

DISCUSSION

ovo is required in XX germline cells for both viability and proper differentiation. Because it is specifically required in females and acts upstream of *Sxl* in the germline, *ovo* has been proposed to be a germline target of the somatic feminization signal (Pauli and Mahowald, 1990; Oliver et al., 1993). The exact role of *ovo* has proved difficult to assess, due in part to the loss of germ cells in amorphic alleles (Oliver et al., 1987; Staab and Steinmann-Zwicky, 1995) and the genetic and molecular complexity of the *ovo-svb* locus (Mével-Ninio et al., 1991, 1995; Garfinkel et al., 1994). We report here a molecular characterization of the dominant *ovo^{D}* mutations and show that they correspond to the creation of new in-frame AUG codons, which most probably initiate the synthesis of novel Ovo isoforms. Expression of these novel isoforms with antimorphic activity would antagonize the function of wild-type Ovo, leading to abnormal differentiation of the oocyte.

Different functions for different Ovo protein isoforms?

ovo is required during larval stages for survival of the female germline (Staab and Steinmann-Zwicky, 1995) and later for proper oogenesis. Furthermore, the existence of a large pool of maternal *ovo* transcripts inherited by the embryo, a fraction of which is incorporated into pole cells (Mével-Ninio et al., 1991, 1995), suggests a possible maternal function of *ovo*, a role which cannot easily be assessed because all *ovo* mutations lead to defective oogenesis. Distinct *ovo* functions could possibly be fulfilled by different *ovo* isoforms, since a diversity of Ovo protein products results from the use of two separate transcription start sites, generating two types of transcripts, *ovo*α and *ovo*β, and the alternative splicing of one protein coding exon, exon 2b (Mével-Ninio et al., 1991, 1995; Garfinkel et al., 1994). Our results, using various *ovo-lacZ* reporter genes, support the hypothesis that the developmental expression of only one class of Ovo isoforms, starting at methionine M373, correlates with the *ovo* functional requirement, based on the phenotypes of *ovo* mutations. A longer isoform, starting at the upstream methionine M1, is only detected in mature oocytes and early embryos. This could indicate that Ovo isoforms initiating at methionine M1 have no role in oogenesis per se, but may provide maternal information to the embryo. The *ovo^{D}* ovarian phenotype that is likely to result from misexpression of long Ovo isoforms (although different from those initiated at M1) predicts distinct functions for OvoM1 and OvoM373. A somewhat related situation, where two protein isoforms are synthesized by alternative start codons, has recently been described for the gene *oskar*; only the short isoform has full *oskar* activity (Markussen et al., 1995). A perhaps more closely related situation (although in that case the use of two different AUGs involves a leaky ribosome scanning mechanism) is the translation of the liver-enriched LAP transcriptional activator and the LIP transcriptional inhibitor from two separate AUGs on the same mRNA (Descombes and Schibler, 1991). Because long Ovo isoforms may be synthesised late in oogenesis, from transcripts *ovo*α, we propose that the *ovo^{D}* phenotype is due to the premature expression of such long isoforms due to the creation of upstream AUGs in transcripts *ovo*β.

The *Ovo*^{D} mutations create new initiator AUGs, which are likely to result in the production of novel Ovo protein isoforms

The dominant female-sterility phenotype of *ovo*^{D} mutations is strictly cell autonomous and can be reversed up to the adult stage by induction of +/+ germline clones (Perrimon et al., 1984). This indicates that mitotically active germline stem cells are present in *ovo*^{D} ovaries and remain competent for undergoing oogenesis and suggests that the Ovo^{D} proteins antagonize *ovo* function during oogenesis. *ovo*^{D} mutations are point mutations that create in-frame methionine codons upstream of the wild-type translation start M373 and the expression pattern of wild-type Ovo and Ovo^{D} fusion proteins supports the hypothesis that the *ovo*^{D1} neo-AUG is a functional initiator codon. By analogy, we postulate that the AUGs present in *ovo*^{D2} and *ovo*^{D3} are also new initiator codons. Based on these results, we favor the interpretation that the dominant effect of *ovo*^{D} mutations is due to the synthesis of novel forms of the Ovo protein, rather than to the consequence of single amino acid changes.

The observation that the relative dominance and severity of *ovo*^{D} ovarian abnormalities do not correlate with the relative lengths of the N-terminal extensions potentially present in the different Ovo^{D} proteins, is rather intriguing. It is possible that the respective strengths of the *ovo*^{D1}, *ovo*^{D2} and *ovo*^{D3} phenotypes reflect different rates of protein synthesis initiated at the different neo-AUGs present in each mutant.

Possible mechanisms for the antimorphic properties of the Ovo^{D} proteins

Because *ovo*^{D} mutations create new AUGs, their antimorphic effects are likely to arise from the expression of Ovo proteins that differ from wild-type Ovo by N-terminal extensions. Several observations indicate that these extensions have no antimorphic activity by themselves, but only within the context of an otherwise normal Ovo protein. First, the 133-amino-acid long N-terminal Ovo^{D1} region (from methionine 79 to proline 211), expressed as part of the Ovo^{D1}-lacZ fusion protein, is not antimorphic since *ovoM-D1* transgenic lines are fully fertile. Second, insertions of transposable elements in *ovo*^{D}, downstream of the M373 codon (but either upstream of or within the zinc-finger coding region) completely reverse the *ovo*^{D} mutations (Mével-Ninio et al., 1989; Garfinkel et al., 1992). These data suggest that the zinc-finger DNA binding domain is required in Ovo^{D} mutant proteins for their dominant-negative activity. This contrasts with the situation observed for the WT1 protein encoded by the Wilm's tumour suppressor gene, in which either point mutations or deletions in the zinc finger region are dominant-negative and lead to the Denish-Drash syndrome (Coppes et al., 1993; Little et al., 1995). Since Ovo^{D1} requires the presence of the zinc-finger domain, the most straightforward interpretation to explain its antimorphic activity is a direct competition between the mutant and Ovo^{+} proteins for occupying target sites on the DNA (assuming, as predicted from its zinc-finger structure, that Ovo is a sequence-specific transcription factor), with the Ovo^{D} proteins being inactive. An equally possible mechanism would be interference by squelching (Ptashne and Gann, 1990). That is, the Ovo^{D} proteins may be titrating a limiting factor, different from Ovo, but necessary for Ovo function. A third possibility is that Ovo forms homodimers. Dimer formation between one Ovo^{+} and one Ovo^{D} molecule could result in an inactive complex sequestering the functional Ovo protein in a dose-dependent manner. In either case, competition or squelching, the resulting phenotype should be close to that of *ovo* hypomorph alleles. This appears to be the case, since among the diverse ovarian abnormalities observed in *ovo*^{D1} and *ovo*^{D2} females, tumorous cysts are frequently found as they are in recessive hypomorphic alleles.

A strong inhibition of the expression of transgenes *ovoB, ovoM373* and *ovoM-D1* is observed in the germline of *ovo*^{D1} females. A similar observation was previously made by Oliver et al. (1994), using transcriptional rather than translational *ovo-lacZ* fusion genes, and leading to the interpretation that the Ovo^{D1} product is a negative trans-regulator of *ovo*$^{+}$, (even though the presence of *ovo*$^{+}$ is not absolutely required for *ovo-lacZ* expression; Oliver et al., 1994). We have now extended this observation to *ovo*^{D2} females. Expression of the transgenes *ovoM373* and *ovoB* is down-regulated in *ovo*^{D2} compared to wild-type ovaries. Yet, although unable to substitute for the wild-type *ovo* gene in rescuing the lack of function *ovo* phenotype, the *ovoB* transgene significantly improves fertility of *ovo*^{D2} females (Mével-Ninio et al., 1995 and this report). This observation supports the existence of a direct interaction between OvoB and Ovo^{D2} (or Ovo^{+}), shifting the equilibrium between inactive (Ovo^{+}/Ovo^{D2}) and active (Ovo^{+}/Ovo^{+} and, possibly Ovo^{+}/OvoB) forms towards more of the active forms.

In contrast to *ovoM373* and *ovoB*, *ovoM-D1* leads consistently to more intense lacZ staining, although mostly cytoplasmic, in *ovo*^{D2} compared to wild-type females. However, as is the case for *ovoB*, the presence of *ovoM-D1* significantly improves the *ovo*^{D2} phenotype. One possible interpretation of these data is that a direct interaction occurs between the OvoM-D1 and Ovo^{D2} proteins to form a stable complex sequestering Ovo^{D2} in the cytoplasm. This interaction would, however, have to be different from the interaction between Ovo^{D2} and either OvoB or Ovo^{+}, since the 132 amino acid region of Ovo^{D1} expressed from the *ovoM-D1* transgene is absent from the short Ovo isoform while it overlaps by 35 amino acids with Ovo^{D2}. Another possibility is that a bias in the relative translation of the native and different ovo^{D} proteins (or lacZ fusion proteins) due to the different AUG contexts is exacerbated in competition conditions, suggesting the existence of a limiting factor for *ovo* translation (that remains to be identified). Such a bias in translation efficiency would account for both the high sensitivity of the *ovo*^{D} phenotype to the gene copy number and the differential expression of Ovo-lacZ and OvoD1-lacZ in different *ovo*^{D} mutant contexts.

The developmentally regulated production of distinct transcription factor isoforms by alternative use of promoters and/or splicing is a widespread phenomenon (review by Lopez, 1995). The alternative use of different AUGs of unequal strength from the same mRNA has recently emerged as an addition to this potential regulatory complexity. Whereas dominant-negative mutations of WT1 have previously been shown to map in zinc-finger domains and affect DNA binding (Little et al., 1995), the molecular basis of ovo^{D} dominance is unprecedented. A functional dissection of the mechanism of this dominance will certainly provide deeper insight into the role of *ovo* in the female germline differentiation pathway and the control of this pathway at the level of transcription in *Drosophila*.

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