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Core Promoter Sequences Contribute to *ovo-B* Regulation in the *Drosophila melanogaster* Germline

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ABSTRACT

Utilization of tightly linked *ovo-A vs. ovo-B* germline promoters results in the expression of OVO-A and OVO-B, C_2H_2 transcription factors with different N –termini, and different effects on target gene transcription and on female germline development. We show that two sex-determination signals, the X chromosome number within the germ cells and a female soma, differentially regulate *ovo-B* and *ovo-A*. We have previously shown that OVO regulates *ovarian tumor* transcription by binding the transcription start site. We have explored the regulation of the *ovo-B* promoter using an extensive series of transgenic reporter gene constructs to delimit *cis*-regulatory sequences as assayed in wild-type and sex-transformed flies and flies with altered *ovo* dose. Minimum regulated expression of *ovo-B* requires a short region flanking the transcription start site, suggesting that the *ovo-B* core promoter bears regulatory information in addition to a "basal" activity. In support of this idea, the core promoter region binds distinct factors in ovary and testis extracts, but not in soma extracts, suggesting that regulatory complexes form at the start site. This idea is further supported by the evolutionarily conserved organization of OVO binding sites at or near the start sites of *ovo* loci in other flies.

GERMLINE sex determination in Drosophila requires *ovo*$^+$ (Oliver 2002). There are two primary germline sex-determination signals, an autonomous X chromosome karyotype signal (2X; the Y chromosome is not sex determining in Drosophila) and a nonautonomous inductive signal from the surrounding soma. The *ovo* gene acts downstream of these primary sex-determination signals to control 2X germ cell differentiation functions via *ovarian tumor* (*otu*) and ultimately *Sex-lethal* (*Sxl*) and to provide for 2X germ cell viability through an undefined pathway.

The *ovo* genes of Drosophila and mice encode C_2H_2 zinc-finger transcription factors (Mevel-Ninio *et al.* 1991; Garfinkel *et al.* 1992) required for germ cell and epidermal development (Oliver *et al.* 1987; Dai *et al.* 1998; Payre *et al.* 1999). Understanding the regulatory circuits upstream and downstream of *ovo* is complicated by the alternative mRNA the locus produces. Transcription from two closely linked start sites, *ovo-A* and *ovo-B*, gives rise to mRNA encoding two major C_2H_2 transcription factor isoforms and multiple variants due to alternative splicing (Mevel-Ninio *et al.* 1996; Andrews *et al.* 1998, 2000; Salles *et al.* 2002). The choice of promoters used is critical, as OVO-A is a negatively acting and OVO-B is a positively acting transcription factor (Andrews *et al.* 2000). Either the absence of OVO-B encoding transcripts (Andrews *et al.* 2000) or excessive and/or precocious expression of OVO-A encoding transcripts (Mevel-Ninio *et al.* 1996; Andrews *et al.* 1998, 2000) results in female sterility due to various degrees of defective oogenesis, including the complete absence of germ cells. Production of positive and negative transcription factors from *ovo* loci may also be conserved, as dual *ovo* promoters and ORFs are found in the distantly related olive fruit fly, *Bactrocera oleae* (Khila *et al.* 2003).

Ovo-A and *ovo-B* promoters are active in the female germline, but show differences in overall expression levels and perhaps pattern—the *ovo-A* promoter being considerably weaker and perhaps active later in oogenesis. RT-PCR and reporter gene expression show that both promoters are also active in the male germline (Mevel-Ninio *et al.* 1996; Andrews *et al.* 2000; Andrews and Oliver 2002). However, male germline development requires neither OVO-A nor OVO-B encoding transcripts (Andrews *et al.* 2000). The direct downstream target locus, *otu*, is strongly upregulated by OVO-B expression and strongly downregulated by OVO-A expression (Lu and Oliver 2001). The *otu* locus is, in turn, required for the regulation of germline sexual identity and oogenesis by *Sxl* (Oliver 2002).

While we know that the *ovo* locus (the sum of *ovo-A* and *ovo-B* expression) responds to sex-determination

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signals and regulates a downstream sex-determination pathway, we are just beginning to explore the specific roles of the *ovo-A* and *ovo-B* isoforms in germline sex determination. For example, the *ovo* locus (Oliver *et al.* 1994; Hinson and Nagoshi 1999; Waterbury *et al.* 2000), and the *ovo-B* promoter in particular (Andrews and Oliver 2002), is regulated by both a 2X germline karyotype (Oliver *et al.* 1994; Hinson and Nagoshi 1999; Andrews and Oliver 2002) and a female soma (Waterbury *et al.* 2000; Andrews and Oliver 2002). The regulation of the *ovo-A* promoter by sex-determination signals is unexplored. In this article we present evidence that the two alternative *ovo* promoters are differentially controlled by primary sex-determination signals.

Regulated transcription of genes typically depends on a basal core promoter and *cis*-regulatory modules that enhance or silence transcription from that core promoter. To better understand the *cis*-regulation of the *ovo-B* promoter, we compare the transcriptional activity of a series of deletion constructs stably introduced into flies by *P*-element-mediated transformation. In wild-type flies, a compact region surrounding the *ovo-B* core promoter is sufficient for the correct pattern of *ovo-B* expression, although flanking regions clearly augment *ovo-B* expression. Thus, the required sequences for *ovo* expression are very close to, or indeed at, the transcription start site, as is the case with the similarly organized *otu* locus (Lu *et al.* 1998; Lee and Garfinkel 2000; Lu and Oliver 2001). There are OVO DNA-binding sites at +1 of *ovo-B* and OVO footprints completely occlude the transcription start sites of both *ovo-B* and *otu* (Lu *et al.* 1998; Lee and Garfinkel 2000). This is an unusual location for a *cis*-regulatory element, as the polymerase complex must occupy this same region. We provide reporter, biochemical, and comparative genomic evidence suggesting that this binding site is functional.

MATERIALS AND METHODS

Drosophila culture and histology: We used standard Drosophila techniques throughout (Ashburner 1989). Flies were grown at 25° ± 0.5° on PB or Gif media (KD Medical, Columbia, MD). Reporter gene expression was monitored in fixed gonads of flies heterozygous for the reporter that were stained with X-Gal as described by Pauli *et al.* (1993), except that the gonads where preincubated in staining buffer at ~22° overnight. Alleles (with FlyBase identifications) used in this study were: ovo^{D1rv23} (FBal0012400), ovo^{D1} (FBal0013375), $ovo^{\Delta ap}$ (FBal0104461), *Df(2R)Trix* (Fbab5072), $tra\text{-}2^{B}$ (FBal0017022), $tra^{hs.PB}$ (FBal0035817), and $tra^{Hsp83.PS}$ (FBal0044393). See FlyBase (2003) for details and additional references. All DNA positions in the reporter constructs follow convention (Mevel-Ninio *et al.* 1991). The *ovo-A* start site is at +361 and the *ovo-B* start site is at +853. We also refer to positions relative to the specific transcription start sites in the text.

Transgenes: Standard molecular biology techniques were use throughout (Sambrook *et al.* 1989). We used a series of transgenic flies bearing deletions of the ~1-kb region, which replicates the wild-type pattern of *ovo-B* and *ovo-A* expression in wild-type adult gonads (Table 1). New reporter genes designed to map *cis*-regulatory regions were derived from previously described reporters (Andrews *et al.* 2000). They are: $ovo::lacZ^{1.1}$ (FBal0104821), $ovo::lacZ^{\Delta a}$ (FBal0104823), *ovo-B::lacZ* (FBal0123190), and $ovo::lacZ^{\Delta bp}$ (FBal0104822). Deletions in the pCaSper-βgal-based plasmids were generated by removing the *ovo Bam*HI fragments. Site-directed deletions were then introduced by PCR. Amplicons with deletions were directionally recloned at unique *Bgl*II and *Age*I sites. We verified deletion boundaries by sequencing with fluorescent dye terminators (ABI-PRISM, dRhodamine Terminator cycle sequencing, and an ABI-377, Perkin-Elmer, Norwalk, CT). Completed constructs were stably introduced into flies using *P*-element-mediated transformation.

TABLE 1

***ovo* reporter genes**

Reporter	Lines examined	Deletion[a] (bp)
$lacZ^{\Delta ap}$	9	345–493
$lacZ^{\Delta ap\Delta 1}$	1	1–100, 345–493
$lacZ^{\Delta ap\Delta 2}$	3	1–228, 345–493
$lacZ^{\Delta ap\Delta 3}$	5	1–327, 345–493
$lacZ^{\Delta ap\Delta 4}$	2	1–589
$lacZ^{\Delta ap\Delta 5}$	3	1–728
$lacZ^{\Delta ap\Delta 6}$	6	1–814
$lacZ^{\Delta ap\Delta 7}$	3	1–100, 345–493, 971–1082
$lacZ^{\Delta ap\Delta 8}$	4	1–100, 345–493, 887–1082
ovoB::lacZ	3	1–781, 911–1082
$lacZ^{\Delta bp}$	3	831–978
$lacZ^{\Delta bp\Delta 1}$	2	1–100, 831–978
$lacZ^{\Delta bp\Delta 3}$	3	1–228, 831–978
$lacZ^{\Delta bp\Delta 5}$	3	831–978, 987–1082
$lacZ^{\Delta bp\Delta 6}$	2	631–1082
$lacZ^{\Delta bp\Delta 8}$	3	416–1082
$lacZ^{\Delta bp\Delta 9}$	1	536–730, 831–978

[a] Relative to $lacZ^{1.1}$.

Gel shifts: Gel mobility shift assays of mutated OVO binding sites with bacterially expressed OVO DNA-binding domain polypeptides were performed according to Lu *et al.* (1998). Mutated binding sites were embedded in a 23-bp double-stranded oligonucleotide from the *otu* core promoter. For gel shifts using protein from fly tissues, the wild-type *ovo-B* transcription start site oligo was TCCTTTTTACAGTTACA TAGCAA and the competing oligo was CTTAATTTAACGTT TAACAAATC (the nonamer corresponding to the putative site is underlined). The ovaries, testes, and carcasses of male and female adult flies were dissected and frozen in liquid nitrogen and stored at −70°. The tissues were homogenized in high-salt buffer (one volume tissue in three volumes buffer; 20 mm HEPES, pH 7.9, 25% glycerol, 800 mm KCl, 0.2 mm EDTA, 0.2 mm PMSF, 0.5 mm DTT) on ice. Following centrifugation (15,000 rpm in a microfuge for 15 min at 4°), the supernatants were collected and diluted with KCl-free high-salt buffer to a final KCl concentration of 100 mm. Diluted protein extract was centrifuged again, the supernatants were collected, and aliquots were stored at −70°. Protein (4 μg, as determined by Bradford assays) was used in the gel-shift assays with 1 ng of labeled oligo in the reaction buffer [10 mm Tris, pH 7.5, 2 μg of poly(dI-dC), 50 mm NaCl, 50 μm $ZnCl_2$, 1 mm DTT, 2.5% Ficoll 400, 0.1% NP-40, and 50 μg BSA].

Informatics: All sequences were obtained from GenBank (Benson *et al.* 2004). The *Drosophila melanogaster* sequence

(Celniker *et al.* 2002) was AE003433 gi:22831713. Although the *ovo* gene sequence of several other Drosophila species is available, noncoding regulatory region sequence is not included. To obtain these sequences from other species, the putative regulatory region of *ovo* (−1 kb from the start of exon 2, which is common to both *ovo-A* and *ovo-B* transcripts) was compared with blastn (Altschul *et al.* 1990) against contigs from preliminary assemblies for *D. pseudoobscura*, *D. virilis*, *D. yakuba*, and *D. simulans*. While we were able to locate good hits to *D. virilis ovo* (tblastn, $e = 1e\text{-}056$) in the Agencourt provisional assembly (http://rana.lbl.gov/drosophila/assemblies/dvir_agencourt_arachne_12jul04.tar.gz), we failed to find significant matches within the noncoding region upstream of the *ovo* ORF and could not unambiguously identify the putative transcription start site for alignment with the *ovo-B* promoter of other *ovo* genes. We therefore excluded *D. virilis* from this study. Sequence 1 kb upstream of the start of exon 2 of *B. oleae ovo* (Khila *et al.* 2003) was extracted from BOL535757, accession AJ535757, gi:27656719. VISTA sequence alignments were used to compare each species to *D. melanogaster* (Mayor *et al.* 2000).

We compiled a list of OVO binding sites on the basis of previous DNAse protection "footprint" assays and SELEX (Lu *et al.* 1998; Lee and Garfinkel 2000), as well as derivative binding sites analyzed by DNA mobility shifts in this report. The resulting sites (36 of which are unique) were aligned and a position-specific scoring matrix was calculated. A pseudocount of 0.01 was added to each cell (King and Roth 2003). Additionally, we generated a position weight matrix (PWM) with background base frequency corrections of 0.3 (T, A) and 0.2 (C, G; Lenhard *et al.* 2003; Sandelin and Wasserman 2004). We wrote a perl script to calculate the PWM score of each possible nonamer in the promoter regions of *ovo* loci or non-*ovo* control sequences. To examine the significance of putative binding site enrichment, we compared results from the 5′-untranslated region (UTR), *D. melanogaster* core promoters, and random sequence. Raw and UTR sequences were downloaded from FlyBase (release 3.2.1). Random 100-bp sequences were extracted from the raw sequence via a perl script that uses the function "rand" to select random positions along the sequence. Core promoter sequences (Ohler *et al.* 2002) were downloaded from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/seq_tools/datasets/Drosophila/promoter/prom/promoter_all_1941.fa). *P*-values are the frequency of control 100-mers showing equal or greater numbers of high-scoring nonamers when compared to the *ovo* sequences.

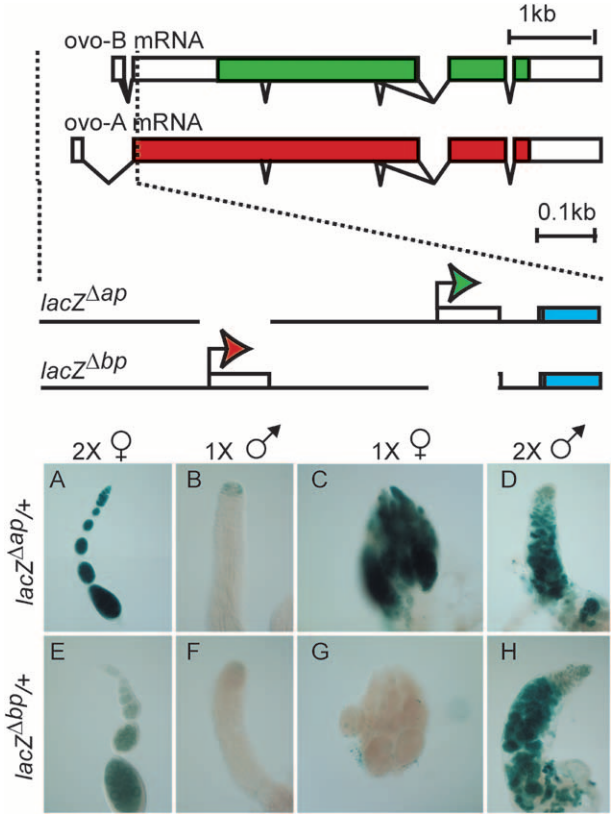

Figure 1.—Expression of *ovo-B*- and *ovo-A*-specific reporters. The organization of the *ovo* locus and germline transcripts (top) and an expanded view outlining the structure of the base *ovo-A*- and *ovo-B*-specific reporters (bottom) are shown above the gonad photos. Shown are: *ovo* promoters (solid bent arrows), exons (bars), introns (bent lines), open reading frames (solid bars) encoding repressive (red) and activating (green) OVO isoforms, and lacZ encoding fragments (blue bars). The alternative exons defining the *ovo-A* and *ovo-B* mRNA are spliced to a common exon 2. Photomicrographs show expression of *ovo-B* and *ovo-A* reporters in wild-type and sex-transformed gonads. The sex chromosome karyotype and somatic differentiation phenotype are shown at the top and the reporter genotype is shown at the left. (A and E) Wild-type females. (B and F) Wild-type males. (C and G) Males transformed into females with $tra^{Hsp83.PS}$. (D and H) Females transformed into males due to absence of *tra-2* [$tra\text{-}2^{B}/Df(2R)Trix$].

RESULTS

Expression of ovo-B and ovo-A promoters in wild-type and sex-transformed gonads: We know little about promoter selection at the two closely linked promoters at the *ovo* locus. To test the hypothesis that primary sex-determination signals control the use of alternative transcription start sites, we compared the expression of the *ovo-A* and *ovo-B* promoters in response to a 2X karyotype and the sexual identity of the soma (Figure 1).

The functional unit of the ovary is the ovariole. From anterior to posterior, the ovarioles contain stem cells, dividing cystocytes, and young 16-cell egg chambers in the germarium, followed by progressively more advanced egg chambers and ultimately eggs, all arranged along the length of the ovariole (Spradling 1993). Wild-type females bearing the *ovo*-B reporter $ovo^{\Delta ap}$*::lacZ* ($lacZ^{\Delta ap}$) showed robust expression in all the germline cells from the germarium to differentiated egg chambers, with later stages showing the strongest staining (Figure 1). The expression of the *ovo-A* reporter $ovo^{\Delta bp}$*::lacZ* ($lacZ^{\Delta bp}$) was much weaker than that of $lacZ^{\Delta ap}$ with no overt staining in the anterior third of the germarium, light staining in middle regions of the germarium, and decreased staining in early egg chambers, followed by increased staining in later stages (Andrews *et al.* 2000; Andrews and Oliver 2002).

Germ cells in the testis are also arranged from anterior to posterior, with stem cells arranged around the hub at the apex; dividing cystocytes are also found within the apex, with growing spermatocytes, spermatids, and

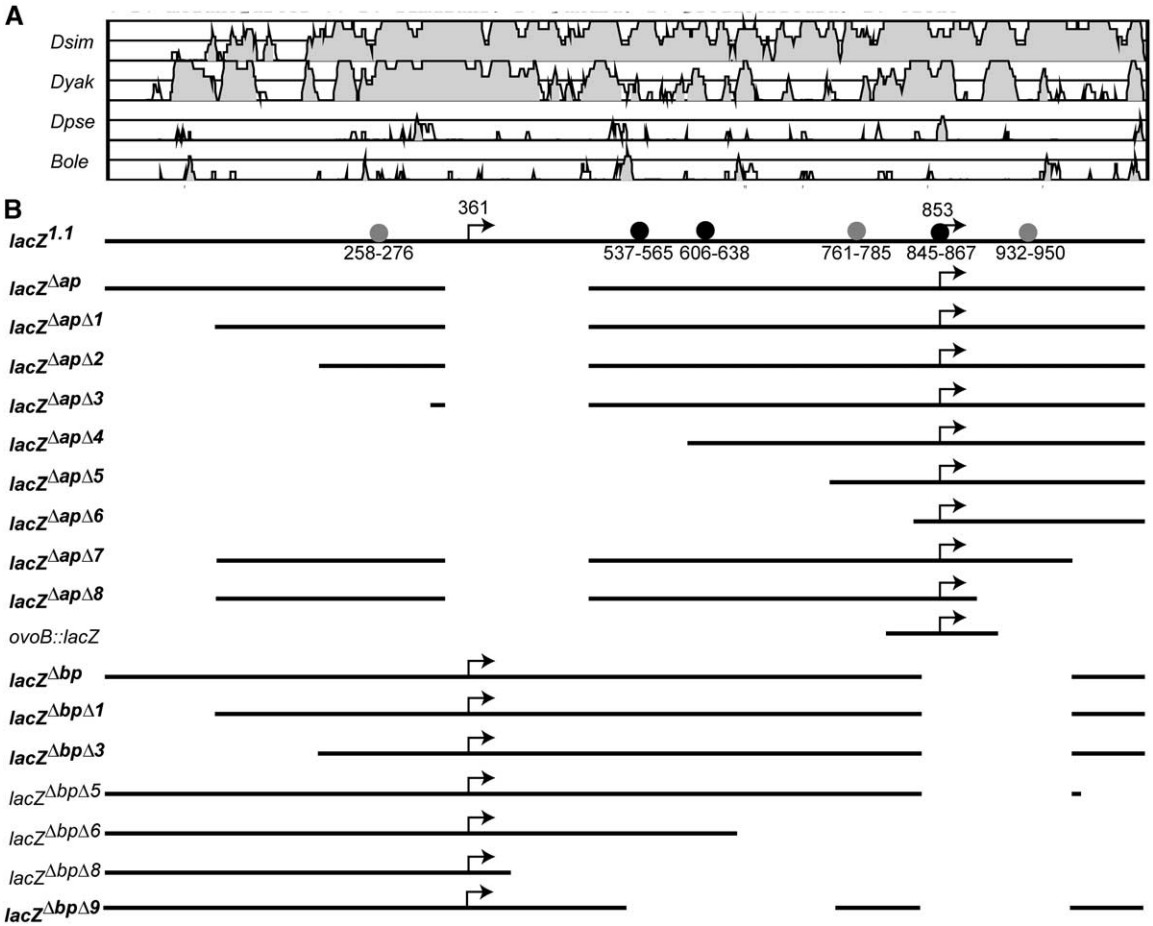

FIGURE 2.—Genomic alignments and reporters for *ovo* expression. (A) VISTA plots showing the alignments between the 1-kb *ovo* control region of *D. melanogaster* aligned against *D. simulans* (*Dsim*), *D. yakuba* (*Dyak*), *D. pseudoobscura* (*Dpse*), and *B. oleae* (*Bole*). For each plot the lowest mapped score is 50% and the maximum is 100%. The line bisecting the plots is 75% and regions showing >80% homology are shaded. (B) *D. melanogaster* reporter genes are also shown. The names of the reporters are shown (left) and those showing detectable expression are indicated (boldface type). DNA present in the reporter is indicated by lines. Positions of the transcription start sites are indicated (bent arrows). On the base construct $lacZ^{1.1}$, the positions of OVO footprints (the entire protected region, not simply the consensus sites) are indicated (circles). Strong footprints (solid circles) and weak footprints (shaded circles) are also indicated, along with the coordinates.

sperm arranged to the posterior (FULLER 1993). Expression of the $lacZ^{\Delta bp}$ or $lacZ^{\Delta ap}$ reporters in the testis is weak and restricted to the apex (Figure 1) as previously reported (ANDREWS *et al.* 2000; ANDREWS and OLIVER 2002).

To examine the effect of a female soma on reporter expression we utilized either of two constitutive *transformer* (*tra*) alleles, $tra^{Hsp83.PS}$ and $tra^{hs.PB}$, both of which encode the female-specific TRA protein. Constitutive expression of TRA transforms 1X animals into somatic females with ovarian tumors (STEINMANN-ZWICKY *et al.* 1989; FLYBASE 2003). The precise sexual nature of the tumor cells is controversial (OLIVER 2002), but they are certainly poorly differentiated. Both $lacZ^{\Delta ap}$ and the endogenous *ovo-B* promoter show clear, but weak, expression of *ovo-B* in 1X $tra^{hs.PB}$ females (ANDREWS and OLIVER 2002), but in 1X $tra^{Hsp83.PS}$ females we find moderate to strong expression of $lacZ^{\Delta ap}$ (Figure 1). In contrast to the *ovo-B* reporter $lacZ^{\Delta ap}$, which is clearly expressed in 1X males transformed into females, the *ovo-A* reporter $lacZ^{\Delta bp}$ showed no overt staining in 1X female flies (Figure 1). Thus, a female soma supports the expression of *ovo-B*, but not *ovo-A*.

To examine the effect of a 2X karyotype on *ovo* reporter expression, we transformed females into anatomical males using loss-of-function alleles of *transformer-2* (*tra-2*). This results in a 2X male with a spermatogenic germline, although the number of germ cells is greatly reduced (NÖTHIGER *et al.* 1989). These 2X males showed strong expression of $lacZ^{\Delta ap}$ (Figure 1). Interestingly, 2X males bearing $lacZ^{\Delta bp}$ also showed very strong staining (Figure 1) that exceeded the staining seen in the egg chambers of wild-type 2X females and dramatically exceeded the expression in the 2X germarium. In summary, we observed strong *ovo-B* expression in all 2X germ cells regardless of somatic phenotype, while we observed stronger *ovo-A* expression in 2X males than in 2X females. The strong expression of *ovo-A* in 2X females transformed into somatic males along with the weak expression in 1X males transformed into females

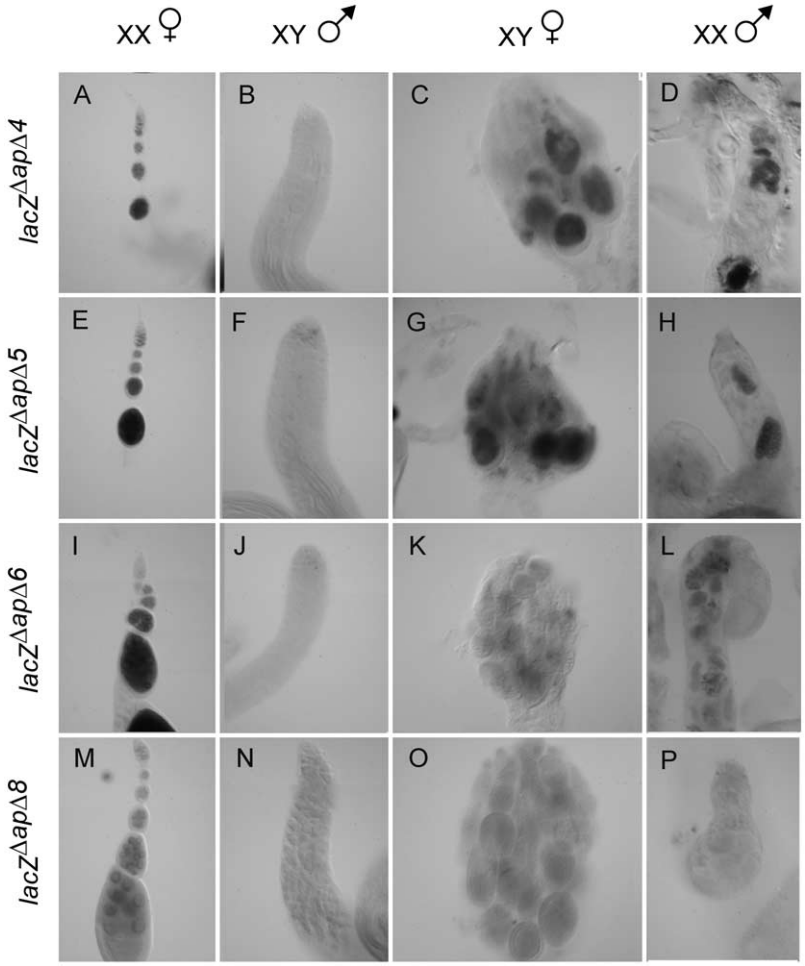

FIGURE 3.—Examples of *ovo-B* reporter expression in wild-type and sex-transformed flies. The sex chromosome karyotype and differentiation phenotype are shown at the top and the reporter genotype is shown at the left. (A–D) Heterozygous for $ovo^{\Delta ap\Delta 4}$. (E–H) Heterozygous for $ovo^{\Delta ap\Delta 5}$. (I–L) Heterozygous for $ovo^{\Delta ap\Delta 6}$. (M–P) Heterozygous for $ovo^{\Delta ap\Delta 8}$. (A, E, I, and M) Wild-type females. (B, F, J, and N) Wild-type males. (C, G, K, and O) Males transformed into females with $tra^{Hsp83.PS}$. (D, H, L, and P) Females transformed into males due to absence of *tra-2* [$tra\text{-}2^{B}/Df(2R)Trix$].

suggests that sex-determination signals differentially regulate *ovo-A vs. ovo-B* promoters.

Mapping *cis*-regulatory domains: To identify *cis*-regulatory regions directing *ovo* expression, we used a computational approach coupled with a deletion analysis. We compared the sequences of the ~1-kb *ovo* promoter region from *D. melanogaster*, *D. simulans*, *D. yakuba*, *D. pseudoobscura*, and *B. oleae* and made a series of constructs in the *D. melanogaster* base reporters, $lacZ^{\Delta ap}$ and $lacZ^{\Delta bp}$, introduced them into the genome of *D. melanogaster* by *P*-element-mediated transformation, and then tested them in wild-type and sex-transformed flies (Figure 2).

Sequences of different species of flies were compared to the *D. melanogaster* sequence (Figure 2A) by using VISTA plots (MAYOR *et al.* 2000). These data show that the flies more closely related to *D. melanogaster*, *D. simulans* [~5.4 million years ago (MYA)], and *D. yakuba* (~22.8 MYA; TAMURA *et al.* 2004) have not diverged sufficiently to highlight discrete regions. Sequence alignments of the more distantly related flies, *D. pseudoobscura* (~54.9 MYA; TAMURA *et al.* 2004) and *B. oleae* (80–100 MYA; KHILA *et al.* 2003), reveal limited homology to *D. melanogaster* in the *ovo* promoter region. However, given that the *B. oleae* sequences in question are able to support the expression of a YFP reporter gene in the *D. melanogaster* ovary, it is clear that sequences critical for *ovo* regulation are present (KHILA *et al.* 2003). There are two short regions of homology between *B. oleae* and *D. melanogaster* upstream of *ovo-A*, two more between the *ovo-A* and *ovo-B* promoters, and one between *ovo-B* and the exon 2 junction. There is some homology between *D. pseudoobscura* and *D. melanogaster* in the vicinity of the *B. oleae* and *D. melanogaster* homologies, but the most striking *D. pseudoobscura* and *D. melanogaster* homology is at the *ovo-B* transcription start site.

The deletion series built from $lacZ^{\Delta ap}$ revealed that important *cis*-regulatory regions for expression are near the *ovo-B* start site. Females bearing any of the six 5′ deletions ($lacZ^{\Delta ap\Delta 1}$ through $lacZ^{\Delta ap\Delta 6}$) showed at least some expression in the germline (Figure 2B; Figure 3, A–L; additional data not shown), indicating that no regions upstream of −39 bp from the *ovo-B* start site are obligatory for female germline expression. Similarly, the $lacZ^{\Delta ap\Delta 8}$ reporter was expressed to a clearly detectable extent even though all sequences 3′ of +35 bp from the *ovo-B* start site were removed (Figure 2B; Figure 3, M–P). These data indicate that the −39- to +34-bp region bears information required for at least some expression of *ovo-B* in the appropriate spatial and tem-

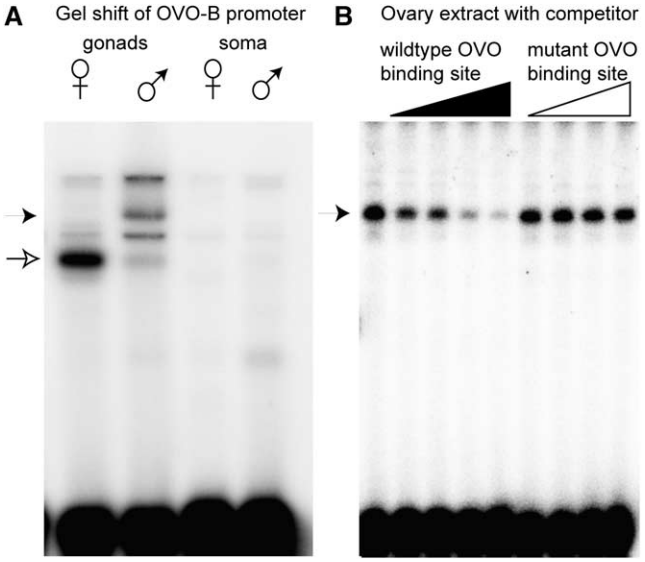

FIGURE 4.—Gel mobility shifts using the *ovo-B* core promoter and extracts from gonadal and somatic tissues. (A) Shifting activities from proteins extracted from female or male gonads or nongonadal soma were used. The source of protein for the gel shifts is indicated above the lane. Bands highly enriched in shifts using ovarian extracts (open arrow) or testis (solid arrow) are indicated. (B) Similar gel shifts performed with increasing amounts of unlabeled wild-type OVO binding site oligo or mutated oligo. The mobility of the shifted band in the absence of competitor is indicated (arrow).

poral pattern. The −71- to + 58-bp core promoter construct *ovo-B::lacZ* showed no detectable expression, as previously reported (LU and OLIVER 2001), clearly indicating that the 129-bp core promoter cannot function in the absence of any flanking *cis*-regulatory regions. Therefore, we conclude that *ovo-B* expression requires either upstream or downstream elements and the core promoter.

We applied the same deletion strategy to exploring the regulation of the *ovo-A* promoter (Figure 2B; data not shown). The results were straightforward. The 5′ deletions in the $lacZ^{\Delta bp}$, $lacZ^{\Delta bp\Delta 1}$, or $lacZ^{\Delta bp\Delta 3}$ constructs had no effect on *ovo-A* expression in 2X females. Similarly, the deletion of sequences between the *ovo-A* and *ovo-B* transcription start sites ($lacZ^{\Delta bp\Delta 9}$) did not abolish transcription, whereas all the 3′ deletions greatly reduced or abolished expression, indicating that the region downstream of the *ovo-B* promoter (+126 to +230 bp from the *ovo-B* start site) is required for proper expression from *ovo-A*.

All of the *ovo-B* reporters (other than the core promoter construct *ovoB::LacZ*, which was not detectably expressed) were expressed in germ cells with either a 1X or a 2X karyotype residing in either a female or a male soma (Figure 3; additional data not shown). Similarly, 1X flies transformed from males into somatic

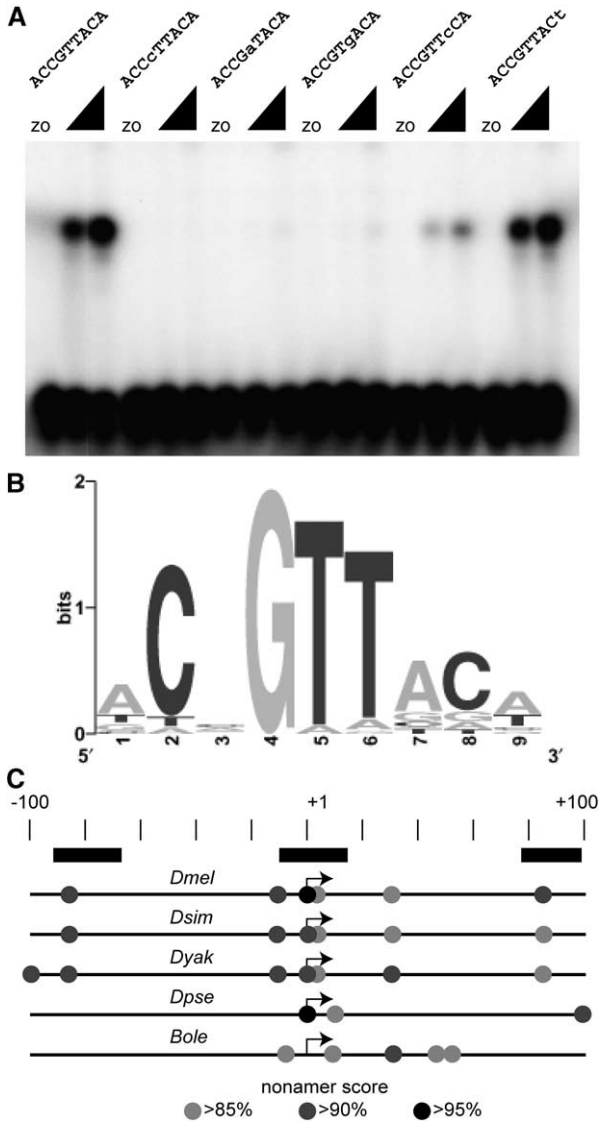

FIGURE 5.—OVO binding sites. (A) Examples of gel shifts used to test for binding activity of mutated strong OVO binding sites. The sequences of the binding sites are shown. Altered residues are in lowercase type. Two concentrations of OVO DNA-binding domain were used as well as extracts from bacteria expressing a control transcript (zo). (B) Sequence Logo showing the refined OVO binding site model. (C) The position of potential OVO binding sites in *D. melanogaster* (*Dmel*), *D. simulans* (*Dsim*), *D. yakuba* (*Dyak*), *D. pseudoobscura* (*Dpse*), and *B. oleae* (*Bole*) relative to the known (*Dmel* and *Bole*) or predicted *ovo-B* transcription start site (scale in base pairs shown). Nonamer scores are indicated by shading (see key). The extent of OVO footprints in *D. melanogaster* is shown (solid rectangles).

females expressed none of the *ovo-A* reporters, except for the single $lacZ^{\Delta bp\Delta 9}$ line. Thus, the deleted reporters show a wild-type response to sex-determination signals. While there may be sequences responding preferentially to a 2X karyotype and a female soma, these are not neatly delimited modules—one responding to karyotype and another responding to somatic sexual identity.

TABLE 2

OVO binding sequences

Putative binding site	Method	Score (−47.5 to 13.6)	% of maximum	Reference
ACTGTTACG	SELEX	10.0	94.19	Lee and Garfinkel (2000)
ACTGTTACT	SELEX	11.1	96.01	Lee and Garfinkel (2000)
ACGGTTACA	SELEX	11.8	97.12	Lee and Garfinkel (2000)
ACAGTTGCT	SELEX	10.0	94.22	Lee and Garfinkel (2000)
ACAGTTACA	SELEX	12.8	98.75	Lee and Garfinkel (2000)
TCTGTTAAG	SELEX	4.6	85.31	Lee and Garfinkel (2000)
TCGGTTGCT	SELEX	7.2	89.64	Lee and Garfinkel (2000)
TCGGTTTCT	SELEX	6.1	87.73	Lee and Garfinkel (2000)
GCTGTTCGT	SELEX	4.8	85.74	Lee and Garfinkel (2000)
TCCGTTACT	SELEX	10.8	95.51	Lee and Garfinkel (2000)
GCCGTTAGA	SELEX	9.7	93.68	Lee and Garfinkel (2000)
CCGGTTACG	SELEX	7.1	89.46	Lee and Garfinkel (2000)
GCTGTTAAT	SELEX	6.1	87.77	Lee and Garfinkel (2000)
TCGGTTTCT	SELEX	6.1	87.73	Lee and Garfinkel (2000)
ACAGTTATA	SELEX	8.8	92.15	Lee and Garfinkel (2000)
ACAGTTAGT	SELEX	9.4	93.19	Lee and Garfinkel (2000)
GCAGTTACT	SELEX	10.5	94.9	Lee and Garfinkel (2000)
CCCGTTACG	SELEX	8.9	92.34	Lee and Garfinkel (2000)
ACTGTTTCT	SELEX	8.1	91.11	Lee and Garfinkel (2000)
ACTGTTCGA	SELEX	7.2	89.59	Lee and Garfinkel (2000)
ACTGTTTCA	SELEX	9.1	92.65	Lee and Garfinkel (2000)
ACAGTTACA	Gel shift, footprint	12.8	98.75	Lu *et al.* (1998)
GCCGTTAAA	Gel shift, footprint	8.5	91.77	Lu *et al.* (1998)
ACAGTAACA	Gel shift, footprint	9.0	92.54	Lu *et al.* (1998)
ACAGTTAGA	Gel shift, footprint	10.3	94.74	Lu *et al.* (1998)
TTCGTTGCC	Gel shift, footprint	3.5	83.59	Lu *et al.* (1998)
ACCGTTACA	Gel shift, footprint	13.6	100	Lu *et al.* (1998)
CCTGTTATC	Gel shift, footprint	3.3	83.29	Lu *et al.* (1998)
GCCGTAGTA	Gel shift, footprint	2.5	81.89	Lu *et al.* (1998)
AACGTTACA	Gel shift	8.6	91.94	This study
ACCGATACA	Gel shift	9.1	92.73	This study
ACCGTGACA	Gel shift	8.8	92.17	This study
ACCGTTCCA	Gel shift	11.2	96.06	This study
ACCGTTACT	Gel shift	12.6	98.46	This study
TTAGTTGCC	Gel shift	2.8	82.34	This study
ACCGTTACT	Gel shift	12.6	98.46	This study
GCAGTTAAA	Gel shift	7.8	90.52	This study
ACAGTAACA	Gel shift	9.0	92.54	This study
TTAGTTGCC	Gel shift	2.8	82.34	This study
ACAGTTACA	Gel shift	12.8	98.75	This study
ACAGTTAGA	Gel shift	10.3	94.74	This study
AACGTTACA	Gel shift	8.6	91.94	This study
ACCGATACA	Gel shift	9.1	92.73	This study
ACCGTTCCA	Gel shift	11.2	96.06	This study

Distinct gonadal proteins bind at the *ovo-B* transcription start site: The results of the reporter deletion study suggests that the *ovo-B* core promoter region (the ~60-bp region that is occupied by initiating RNA polymerase) bears regulatory information and is not simply a basal promoter depending only on more distant instruction by enhancers. This same region was previously studied in the context of the *otu* locus, where the *ovo-B* core promoter could substitute for the *otu* promoter, while core promoters without OVO binding sites could not (Lu and Oliver 2001). If the core promoter has some regulatory function, we can predict that it will be bound by regulatory proteins to mediate activation or derepression in the female germline. To test for such binding activities, we performed DNA mobility shift assays on the *ovo-B* core promoter using proteins extracted from ovaries and testes, as well as female and male nongonadal soma (Figure 4A). Striking differences were observed in these DNA mobility shift assays. A very strong shift is observed following incubation of the *ovo-B* core

TABLE 3

OVO binding site matrices

	Position								
Base	1	2	3	4	5	6	7	8	9
	Position frequency matrix								
A	28	2	15	0	2	3	32	4	25
C	3	41	17	0	0	0	4	33	4
G	7	0	5	46	0	1	6	6	4
T	8	3	9	0	44	42	4	3	13
	Position weight matrix								
A	1.02	−2.78	0.12	−10.43	−2.78	−2.20	1.21	−1.78	0.86
C	−1.51	2.16	0.89	−9.85	−9.85	−9.85	−1.20	1.84	−1.20
G	−0.390	−9.85	−0.88	2.32	−9.85	−3.19	−0.52	−0.515	−1.20
T	−0.79	−2.20	−0.52	−10.43	1.57	1.51	−1.78	−2.20	−0.09

promoter with ovary extract, and there was a different pattern of strong shifts in extracts from testis. The female shifting activity is consistent with positive action at the *ovo-B* core promoter. The strikingly different pattern of mobility shifts using testis extracts raises the possibility that there are distinct core promoter-binding complexes in the two tissues. The testis complex may be repressive or weakly activating, while the ovarian complex is strongly activating. There was little shifting activity from extracts from either male or female soma. This is consistent with the idea that the absence of *ovo-B* reporter expression in the soma (data not shown) is due to the absence of activation at the core promoter, rather than repression. While we have been unable to determine if the proteins binding to the core promoter are derived from *ovo* (further purification has not been successful), it is clear that wild-type, but not mutant, OVO binding sites compete for the binding activity (Figure 4B). This raises the possibility that OVO binding at the *ovo-B* core promoter is important. Thus, looking for this site in other species could be informative.

Comparative genomic analysis of OVO binding sites: Before the comparative genomic analysis, we refined the OVO binding site definition and made new scoring matrices on the basis of the previous footprinting and SELEX studies (Lu *et al.* 1998; Lee and Garfinkel 2000), augmented with analysis of OVO binding to mutated sites (Figure 5; additional data not shown). The new gel-mobility shift data confirm the importance of the central GTT core of previously identified sites. For example, if the G residue in the fourth position of the strongly binding ACCGTTACA motif is changed to C, binding is abolished and mutating either of the T's in fifth and sixth positions greatly reduces OVO binding (Figure 5A). We have now generated a list of confirmed OVO binding sites. The resulting 9-bp consensus motif is similar to those previously reported (ACNGTTACA; Figure 5B; Table 2).

To query DNA sequences for the presence of OVO binding sites, we generated a scoring matrix (Table 3). Applying this matrix to any nonamer results in a score between −47.5 and 13.6, which we express as a percentage of the maximum score. The 46 confirmed binding sites have an average score of 92.3% with a standard deviation of 5%. The minimum score of a confirmed OVO binding site is 81.9%. We determined the frequency of high-scoring nonamers in several sets of control sequences (Table 4). One set of 5000 control sequences is from random segments of the genome within each Muller element (the *ovo* locus is X linked). The second set of 9958 control sequences is from 5′-UTRs (some of the potential OVO binding sites are within the 5′-UTR). The third set of control sequences is −50- to +50-bp segments from a core promoter database (Ohler *et al.* 2002). These sequences allow us to determine the significance of high-scoring nonamers in the *ovo-B* core promoters of different species of Drosophila.

If OVO binding sites at the *ovo-B* core promoter are important for *ovo-B* expression, then we expect that those sites will be enriched in that region in multiple species. We therefore asked if there was significant enrichment for high-scoring (≥85%) nonamers in the 100-bp region flanking the *ovo-B* transcription start sites as compared to control sequences. The *ovo-B* core promoter regions of *D. melanogaster*, *D. simulans*, *D. yakuba*, and *B. oleae* each have four such high-scoring nonamers in the 100-bp core promoter region ($P < 0.01$ *vs.* random sequences, <0.01 *vs.* 5′-UTRs, and <0.03 *vs.* core promoters). *D. pseudoobscura* shows three high-scoring nonamers ($P < 0.04$ *vs.* random sequences, <0.04 *vs.* 5′-UTRs, and <0.09 *vs.* core promoters). Furthermore, there was a nearly identical arrangement of high-scoring OVO binding sites in *D. melanogaster*, *D. simulans*, and *D. yakuba* (Figure 5C). In all three cases there are binding sites ~85 bp upstream and downstream of the *ovo-B* start site, which correspond to two regions that are pro-

TABLE 4

High-scoring (>85%) OVO nonamers in control sequences

Chromosome arm	*N* sampled	Average	SD	Maximum	Median
Random 100-bp sequences					
X	1000	0.67	0.98	11	0
2L	1000	0.64	0.89	6	0
2R	1000	0.69	0.87	5	0
3L	1000	0.64	0.92	7	0
3R	1000	0.66	0.89	5	0
5′-UTR sequences[a]					
X	1748	0.85	0.64	4.2	0.8
2L	1757	0.77	0.61	4.3	0.7
2R	2051	0.84	0.68	5.4	0.8
3L	1921	0.80	0.66	5.2	0.7
3R	2481	0.83	0.65	5.8	0.8
Core promoter sequences[b]					
All[c]	1941	1.02	1.05	6	1

[a] Adjusted for 100-bp length, sequences <100 bp excluded.
[b] Trimmed to 100 bp centered on the transcription start site.
[c] Pooled to increase sample size.

tected by OVO protein in DNA footprint experiments (Lu *et al.* 1998). There are also nearly identical clusters of three overlapping OVO binding sites at the transcription start site, where a very strong OVO footprint was observed (Lu *et al.* 1998). Thus, the organization of OVO binding sites is conserved between these species. The conserved binding sites at the predicted start site of the *D. pseudoobscura ovo-B* transcript are even more striking (Figure 5C) given the low overall conservation between *D. melanogaster* and *D. pseudoobscura ovo* promoter regions (Figure 2A). The region flanking the predicted *ovo-B* start site of *B. oleae* has only one nonamer scoring above 90% and does not show the −80-bp and +100-bp sites found in the other species. Perhaps the supernumerary motifs compensate for weaker binding to individual sites. It has been shown that *cis*-regulatory modules can retain function across species as an intact module, while still diverging to the point that chimeric modules derived from two species fail to function (Ludwig *et al.* 2000). It is therefore possible that function of the *B. oleae ovo-B* core promoter in a *D. melanogaster* ovary (Khila *et al.* 2003) is maintained using a slightly different transcription factor solution.

Response of reporters to OVO-A and OVO-B: At least some of the OVO binding sites in the *ovo* promoter region are functional, but the effect of OVO expression on the *ovo* promoter has not been mapped to any particular OVO binding sites (Lu *et al.* 1998; Andrews *et al.* 2000). We therefore assayed for reporter expression in flies heterozygous for ovo^{D1}, an antimorphic allele

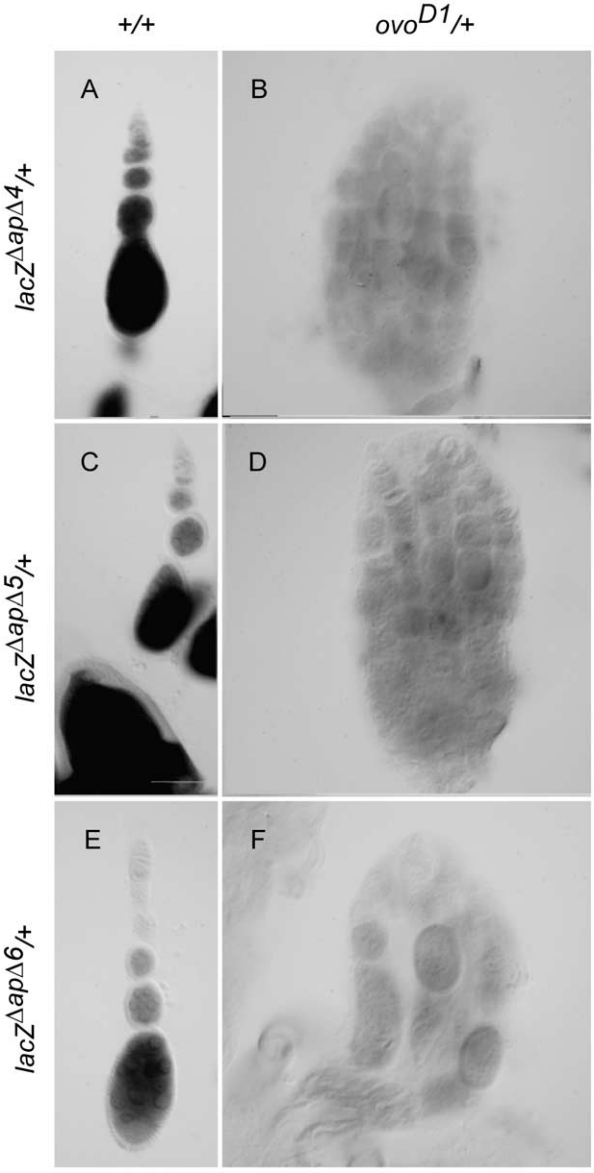

Figure 6.—Examples of *ovo* reporter expression in an ovo^{D1}/+ background. The genotype with respect to *ovo* encoding alleles is shown at the top and the reporter genotype is shown at the left. An ovariole is shown for the +/+ micrographs, and an entire atrophic ovary is shown from the ovo^{D1}/+ flies. (A, C, and E) Ovarioles from females wild type for *ovo*. (B, D, and F) Ovaries from females heterozygous for ovo^{D1}. (A and B) Heterozygous for $ovo^{\Delta ap\Delta 4}$. (C and D) Heterozygous for $ovo^{\Delta ap\Delta 5}$. (E and F) Heterozygous for $ovo^{\Delta ap\Delta 6}$.

encoding an OVO-A isoform from the *ovo-B* promoter, and in flies bearing various copy numbers of $ovo^{\Delta ap}$, an OVO-B encoding transgene.

The ovo^{D1} allele resulted in nearly complete silencing of both the $lacZ^{\Delta ap}$ and the $lacZ^{\Delta bp}$ reporter gene series (Figure 6). The $lacZ^{1.1}$ reporter that bears both the *ovo-B* and the *ovo-A* start sites was repressed, as were deletion reporters. Indeed, any reporter that is expressed has several OVO binding sites (Figure 2) and all such reporters can be repressed by expression of OVO-A. Collec-

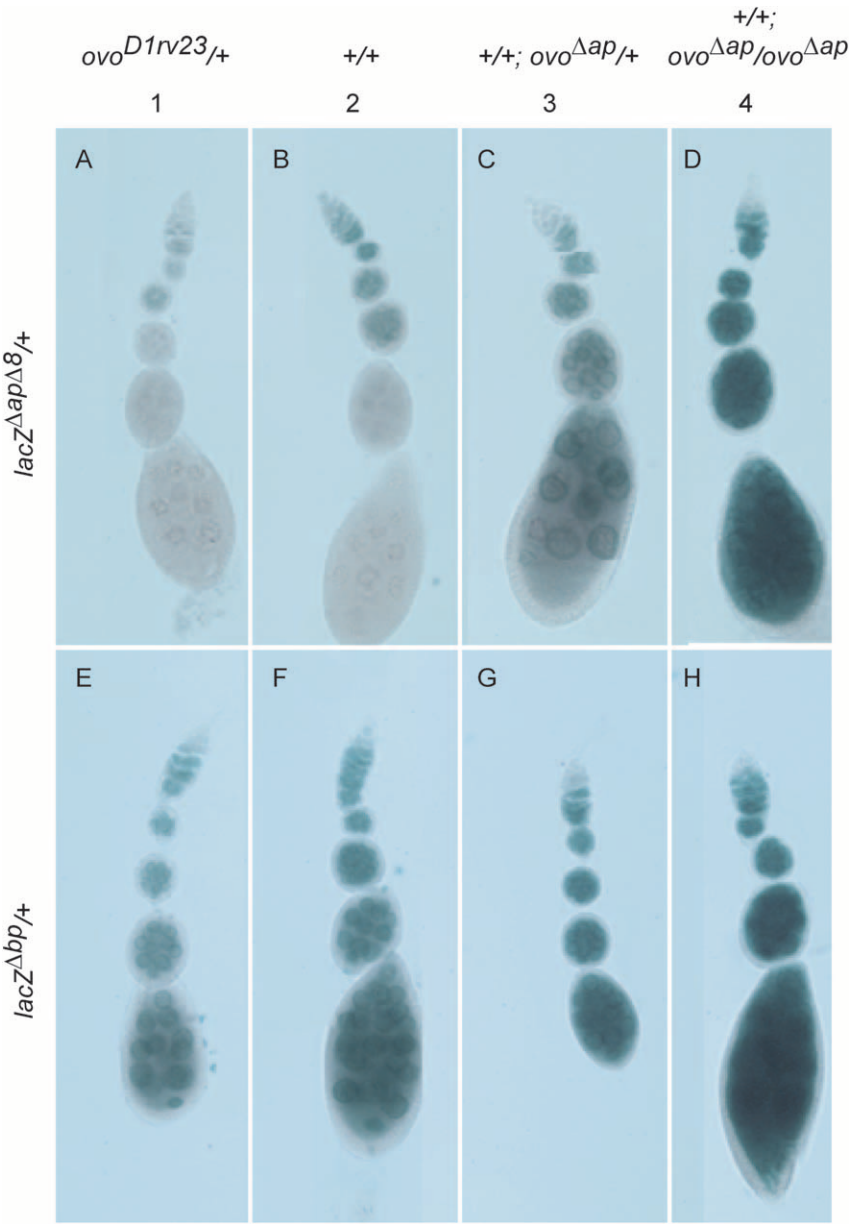

FIGURE 7.—Examples of *ovo-B* and *ovo-A* reporter expression in flies with different OVO-B encoding copy numbers. The genotype with respect to *ovo* encoding alleles is shown at the top and the reporter genotype is shown at the left. Staining of gonads bearing the *ovo-B*-specific reporter (A–D) $lacZ^{\Delta ap\Delta 8}$, and the *ovo-A*-specific reporter (E–H) $lacZ^{\Delta b}$, in flies with (A and E) one, (B and F) two, (C and G) three, or (D and H) four copies of *ovo* alleles encoding *ovo-B* isoforms is shown. The $ovo^{\Delta ap}$ allele encodes only OVO-B, while the wild-type alleles also express OVO-A, albeit at a lower level.

tively, the reporters that are downregulated by ovo^{D1} remove all the strong OVO binding sites at the *ovo* locus and all but one of the weak sites (Figure 6), raising the distinct possibility that any OVO binding site can act as a silencer in conjunction with OVO-A proteins.

We were unable to find convincing evidence for female germline positive *ovo* autoregulation in previous work (ANDREWS *et al.* 2000), even though the *ovo-B* core promoter positively responds to OVO-B when swapped into the *otu* regulatory region (LU and OLIVER 2001). This suggests that there are *cis*-sequences required for positive autoregulation present in *otu*, but not *ovo*, or that there are *cis*-sequences that block positive autoregulation at *ovo*, or both. We therefore asked if any of the deletion reporters are influenced by the dose of OVO-B produced *in trans*. Females bearing the $lacZ^{\Delta ap\Delta 8}$ construct showed a striking increase of reporter expression with increasing copies of the OVO-B encoding transgene $ovo^{\Delta ap}$ (Figure 7). Additionally, reducing the copy number of endogenous *ovo* greatly reduced the expression of the $lacZ^{\Delta ap\Delta 8}$ reporter. The expression of other reporters was only marginally increased with the dose of OVO-B encoding transgenes in 2X females (Figure 7). Thus, OVO binding sites at *ovo* can respond negatively to OVO-A and positively to OVO-B.

DISCUSSION

Transcriptional circuits at *ovo*: We are beginning to have a reasonable understanding of the germline pathway centered on *ovo* (Figure 8). OVO-A and OVO-B functions are in a delicate balance in the female germline. OVO-B is absolutely required for oogenesis and is downregulated by OVO-A. An excess OVO-A results in

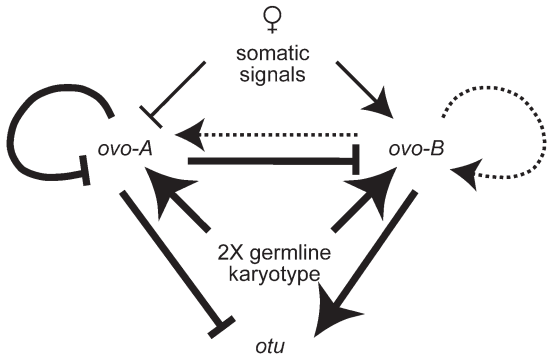

FIGURE 8.—The regulatory circuit centered on the *ovo* locus. Positive effects (arrows) and negative effects (blocked end) are shown. The lines represent strong (thick), moderate (thin), and weak (dashed) effects. See text for details.

defective oogenesis and subsequent embryogenesis, while too little results in defective germline function in progeny (MEVEL-NINIO *et al.* 1996; ANDREWS *et al.* 1998, 2000). Having the female soma repress *ovo-A* function in the germline may prevent damage to developing eggs, while the positive effect of a 2X karyotype may ensure that OVO-A protein is ultimately deposited in those eggs. We show that OVO-B can have a positive effect on the *ovo-B* promoter following the deletion of some promoter-proximal sequences, but negative autoregulation occurs in all reporters. This difference between response to OVO-A *vs.* OVO-B does not appear to be due to different inherent strengths of the two transcription factors, as the *otu* promoter, a direct target of *ovo*, is strongly positively regulated by OVO-B (LU and OLIVER 2001) in addition to being negatively regulated by OVO-A (ANDREWS *et al.* 2000). Further, this difference in response dose not appear to be due to the *ovo-B* core promoter sequence, as in the *otu* sequence milieu, the *ovo-B* promoter is also strongly positively regulated by OVO-B (LU and OLIVER 2001). Thus, the *ovo* context is likely to specifically dampen the *trans* effect of OVO-B, but not OVO-A, on *ovo-B* promoter activity.

The *ovo-B* promoter encodes the OVO-B isoforms required and sufficient for female germline development and is regulated by the number of X chromosomes in the germline cells, and the sex of the surrounding soma positively regulates *ovo-B*, even though neither signal is absolutely required (ANDREWS and OLIVER 2002). For example, only 1X males fail to robustly express *ovo-B* in the germline, suggesting that both the intrinsic 2X signal and the extrinsic female somatic signal can upregulate *ovo-B* independently. Also we know that somatic signaling is not required for *ovo* genetic function, because 2X males have germline cells, while 2X males lacking *ovo* do not (HINSON and NAGOSHI 1999; ANDREWS and OLIVER 2002). This dual regulatory input ensures that *ovo-B* is most highly expressed in the cells that require *ovo* activity—wild-type female germ cells. We show that *ovo-A* expression is more dynamically regulated. The highest *ovo-A* promoter activity is in 2X males, followed by 2X females, 1X males, and 1X females. This pattern suggests that a 2X karyotype activates *ovo-A*, while a female soma inhibits *ovo-A* activity within the germline.

The combination of negative and positive autoregulation adds considerable complexity to the regulatory circuit. For example, the positive effect of a female soma on the expression of *ovo-B* in our working model could be due to repression of *ovo-A* expression by a female soma, followed by derepression of *ovo-B* because of lowered OVO-A levels, or a more direct positive effect of the female soma on *ovo-B*.

The *ovo-B* core promoter: Analysis of promoters active in the germline of Drosophila suggests that they are often more compact than many of the promoters studied in somatic cells (ARNOSTI 2003). This may be the case for *ovo-B*. While the *ovo-B* core promoter alone is insufficient for transcription, transcriptional activity from *ovo-B* is remarkably resistant to deletions from either the 5′ or the 3′ direction. The $lacZ^{\Delta ap\Delta 6}$ reporter has only 268 bp of *ovo* sequence but is expressed in the female germline. The overlap between the $lacZ^{\Delta ap\Delta 6}$ and $lacZ^{\Delta ap\Delta 8}$ reporters, both of which are expressed, is only 73 bp. This is unusually close to the transcription start site. The OVO binding site footprints overlap the transcriptional start sites of both *otu* and *ovo-B* (LU and OLIVER 2001), and we show that there are proteins or complexes in gonad extracts that bind to this core sequence. We therefore suggest that OVO alters the structure of the core promoter and promotes preinitiation complex formation (LU and OLIVER 2001). The highly conserved position of OVO binding sites at *ovo-B* in multiple species of flies supports the idea that OVO functions at the transcription start site. A recent study of human promoters suggests that the binding of transcription factors within 100 bp of the transcription start site may be more common than previously thought (FITZGERALD *et al.* 2004).

The importance of the core promoter raises some interesting questions about how *ovo* interprets the number of X chromosomes in the germline and the sex of the surrounding soma. For example, the *Sex-lethal* gene counts X chromosomes in the soma by binding several transcription factors, encoded on the X chromosome, to a region rich in the corresponding binding sites. The balance toward expression of *Sxl* is thus tipped by a graded occupancy at a complex *cis*-regulatory module (LOUIS *et al.* 2003). There does not appear to be an extended *cis*-regulatory module that is essential for the qualitative expression of *ovo*. Perhaps sex-determination signals indirectly regulate *ovo*. The molecular nature of the karyotype and somatic signals to the germline is a major unresolved problem in germline sex determination.

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LITERATURE CITED

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

Andrews, J., and B. Oliver, 2002 Sex determination signals control *ovo-B* transcription in *Drosophila melanogaster* germ cells. Genetics **160:** 537–545.

Andrews, J., I. Levenson and B. Oliver, 1998 New AUG initiation codons in a long 5′ UTR create four dominant negative alleles of the *Drosophila* C2H2 zinc-finger gene *ovo*. Dev. Genes Evol. **207:** 482–487.

Andrews, J., D. Garcia-Estefania, I. Delon, J. Lu, M. Mevel-Ninio *et al.*, 2000 OVO transcription factors function antagonistically in the *Drosophila* female germline. Development **127:** 881–892.

Arnosti, D. N., 2003 Analysis and function of transcriptional regulatory elements: insights from *Drosophila*. Annu. Rev. Entomol. **48:** 579–602.

Ashburner, M., 1989 *Drosophila. A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell and D. L. Wheeler, 2004 GenBank: update. Nucleic Acids Res. **32:** D23–D26.

Celniker, S. E., D. A. Wheeler, B. Kronmiller, J. W. Carlson, A. Halpern *et al.*, 2002 Finishing a whole-genome shotgun: release 3 of the *Drosophila melanogaster* euchromatic genome sequence. Genome Biol. **3:** R79.

Dai, X., C. Schonbaum, L. Degenstein, W. Bai, A. Mahowald *et al.*, 1998 The *ovo* gene required for cuticle formation and oogenesis in flies is involved in hair formation and spermatogenesis in mice. Genes Dev. **12:** 3452–3463.

FitzGerald, P. C., A. Shlyakhtenko, A. A. Mir and C. Vinson, 2004 Clustering of DNA sequences in human promoters. Genome Res. **14:** 1562–1574.

FlyBase, 2003 The FlyBase database of the *Drosophila* genome projects and community literature. Nucleic Acids Res. **31:** 172–175.

Fuller, M. T., 1993 Spermatogenesis, pp. 71–148 in *The Development of Drosophila*, edited by M. Bate and A. Martinez-Arias. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Garfinkel, M. D., A. R. Lohe and A. P. Mahowald, 1992 Molecular genetics of the *Drosophila melanogaster ovo* locus, a gene required for sex determination of germline cells. Genetics **130:** 791–803.

Hinson, S., and R. N. Nagoshi, 1999 Regulatory and functional interactions between the somatic sex regulatory gene *transformer* and the germline genes *ovo* and *ovarian tumor*. Development **126:** 861–871.

Khila, A., A. El Haidani, A. Vincent, F. Payre and S. I. Souda, 2003 The dual function of *ovo/shavenbaby* in germline and epidermis differentiation is conserved between *Drosophila melanogaster* and the olive fruit fly *Bactrocera oleae*. Insect Biochem. Mol. Biol. **33:** 691–699.

King, O. D., and F. P. Roth, 2003 A non-parametric model for transcription factor binding sites. Nucleic Acids Res. **31:** e116.

Lee, S., and M. D. Garfinkel, 2000 Characterization of *Drosophila* OVO protein DNA binding specificity using random DNA oligomer selection suggests zinc finger degeneration. Nucleic Acids Res. **28:** 826–834.

Lenhard, B., A. Sandelin, L. Mendoza, P. Engstrom, N. Jareborg *et al.*, 2003 Identification of conserved regulatory elements by comparative genome analysis. J. Biol. **2:** 13.

Louis, M., L. Holm, L. Sanchez and M. Kaufman, 2003 A theoretical model for the regulation of *Sex-lethal*, a gene that controls sex determination and dosage compensation in *Drosophila melanogaster*. Genetics **165:** 1355–1384.

Lu, J., and B. Oliver, 2001 *Drosophila* OVO regulates *ovarian tumor* transcription by binding unusually near the transcription start site. Development **128:** 1671–1686.

Lu, J., J. Andrews, D. Pauli and B. Oliver, 1998 *Drosophila* OVO zinc-finger protein regulates *ovo* and *ovarian tumor* target promoters. Dev. Genes Evol. **208:** 213–222.

Ludwig, M. Z., C. Bergman, N. H. Patel and M. Kreitman, 2000 Evidence for stabilizing selection in a eukaryotic enhancer element. Nature **403:** 564–567.

Mayor, C., M. Brudno, J. R. Schwartz, A. Poliakov, E. M. Rubin *et al.*, 2000 VISTA: visualizing global DNA sequence alignments of arbitrary length. Bioinformatics **16:** 1046–1047.

Mevel-Ninio, M., R. Terracol and F. C. Kafatos, 1991 The *ovo* gene of *Drosophila* encodes a zinc finger protein required for female germ line development. EMBO J. **10:** 2259–2266.

Mevel-Ninio, M., E. Fouilloux, I. Guenal and A. Vincent, 1996 The three dominant female-sterile mutations of the *Drosophila ovo* gene are point mutations that create new translation-initiator AUG codons. Development **122:** 4131–4138.

Nöthiger, R., M. Jonglez, M. Leuthold, P. Meier-Gerschwiler and T. Weber, 1989 Sex determination in the germ line of Drosophila depends on genetic signals and inductive somatic factors. Development **107:** 505–518.

Ohler, U., G. C. Liao, H. Niemann and G. M. Rubin, 2002 Computational analysis of core promoters in the Drosophila genome. Genome Biol. **3:** R87.

Oliver, B., 2002 Genetic control of germline sex determination in Drosophila. Int. Rev. Cytol. **219:** 1–60.

Oliver, B., N. Perrimon and A. P. Mahowald, 1987 The *ovo* locus is required for sex-specific germ line maintenance in *Drosophila*. Genes Dev. **1:** 913–923.

Oliver, B., J. Singer, V. Laget, G. Pennetta and D. Pauli, 1994 Function of Drosophila *ovo+* in germ-line sex determination depends on X chromosome number. Development **120:** 3185–3195.

Pauli, D., B. Oliver, and A. P. Mahowald, 1993 The role of the *ovarian tumor* locus in *Drosophila melanogaster* germ line sex determination. Development **119:** 123–134.

Payre, F., A. Vincent and S. Carreno, 1999 *ovo/svb* integrates Wingless and DER pathways to control epidermis differentiation. Nature **400:** 271–275.

Salles, C., M. Mevel-Ninio, A. Vincent and F. Payre, 2002 A germline-specific splicing generates an extended ovo protein isoform required for *Drosophila* oogenesis. Dev. Biol. **246:** 366–376.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandelin, A., and W. W. Wasserman, 2004 Constrained binding site diversity within families of transcription factors enhances pattern discovery bioinformatics. J. Mol. Biol. **338:** 207–215.

Spradling, A. C., 1993 Developmental genetics of oogenesis, pp. 1–70 in *The Development of Drosophila*, edited by M. Bate and A. Martinez-Arias. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Steinmann-Zwicky, M., H. Schmid and R. Nothiger, 1989 Cell-autonomous and inductive signals can determine the sex of the germ line of *Drosophila* by regulating the gene *Sxl*. Cell **57:** 157–166.

Tamura, K., S. Subramanian and S. Kumar, 2004 Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. Mol. Biol. Evol. **21:** 36–44.

Waterbury, J. A., J. I. Horabin, D. Bopp and P. Schedl, 2000 Sex determination in the *Drosophila* germline is dictated by the sexual identity of the surrounding soma. Genetics **155:** 1741–1756.

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