

***Drosophila* OVO regulates ovarian tumor transcription by binding unusually near the transcription start site**

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SUMMARY

Evolutionarily conserved *ovo* loci encode developmentally regulated, sequence-specific, DNA-binding, C₂H₂-zinc-finger proteins required in the germline and epidermal cells of flies and mice. The direct targets of OVO activity are not known. Genetic experiments suggest that *ovo* acts in the same regulatory network as *ovarian tumor* (*otu*), but the relative position of these genes in the pathway is controversial. Three OVO-binding sites exist in a compact regulatory region that controls germline expression of the *otu* gene. Interestingly, the strongest OVO-binding site is very near the *otu* transcription start, where basal transcriptional complexes must function. Loss-of-function,

gain-of-function and promoter swapping constructs demonstrate that OVO binding near the transcription start site is required for OVO-dependent *otu* transcription in vivo. These data unambiguously identify *otu* as a direct OVO target gene and raise the tantalizing possibility that an OVO site, at the location normally occupied by basal components, functions as part of a specialized core promoter.

Key words: Initiator, *ovo*, Germ cell, *ovarian tumor*, Transcription, Sex determination, *Drosophila*

INTRODUCTION

Genes encoding a family of OVO C₂H₂-zinc-finger proteins have been identified in insects, nematodes and mammals (Mével-Ninio et al., 1991; Chidambaram et al., 1997; Dai et al., 1998; Lü et al., 1998; Masu et al., 1998). The *Drosophila* zinc-finger domain shows sequence-specific DNA binding in vitro (Lü et al., 1998; Lee and Garfinkel, 2000). This binding, in conjunction with the strong conservation of both structural and predicted DNA contact residues among OVO proteins, suggests that OVO proteins bind similar sequences in a broad range of metazoans. OVO proteins also exhibit remarkably conserved biological function. Mutations in *Drosophila* or mouse *ovo* genes result defective 'hair' formation, even though the denticles of *Drosophila* are exoskeleton structures secreted by individual cells, while hairs in mammals are multicellular structures composed of the keratinized corpses of epidermal cells (Dai et al., 1998; Payre et al., 1999). Additionally, *ovo* mutations result in defective germline development in mice and flies. In *Drosophila*, only germ cells with an XX karyotype require *ovo*⁺ for viability (XX flies are normally female, but *ovo*⁺ is required in XX germ cells regardless of sexual identity), while in mice the male germline requires *Ovo*⁺ (Oliver et al., 1987; Oliver et al., 1994; Dai et al., 1998). *Drosophila* germ cells express two isoforms of OVO, OVO-A and OVO-B, with common DNA-binding domains, but opposite transcriptional activities (Andrews et al., 2000b). OVO-B is an activator, while OVO-A is a repressor. The OVO-B isoform predominates during the most of female germline

development and is sufficient for female germline development. We are interested in the direct targets of OVO activity in the germline. The *otu* locus is a good candidate.

A number of observations suggest that *ovo* and *otu* act in the same genetic pathway. Both *ovo* and *otu* show similar phenotypes. Females homozygous for strong *ovo* or *otu* alleles show extensive loss of germ cells and surviving germ cells overproliferate. Mutations in *ovo* result in more extreme germline death defects as would be expected if *otu* where one of several OVO targets (Oliver et al., 1987; Oliver et al., 1993; Storto and King 1988; Pauli et al., 1993; Rodesch et al., 1995; Stabb and Steinmann-Zwicky, 1996; Oliver and Pauli 1998; Hinson et al., 1999). The *ovo* and *otu* loci both control a common downstream gene. Transplantation and genetic studies suggest that both germline autonomous karyotypic signals (XX for female and X for male) and signals from the soma act to regulate germline *Sex lethal* (*Sxl*) activity in the female germline (reviewed by Cline and Meyer, 1996). The presence of both female-specific and male-specific *Sxl* mRNAs in *ovo* and *otu* mutants (Bopp et al., 1993; Oliver et al., 1993; Pauli et al., 1993) indicate that *ovo*⁺ and *otu*⁺ function to promote female-specific *Sxl* splicing. Furthermore, genetics indicates that this regulation of *Sxl* is crucial, as mutations in both *ovo* and *otu* are partially suppressed by gain-of-function *Sxl* alleles (Oliver et al., 1990; Oliver et al., 1993; Pauli et al., 1993; Oliver and Pauli, 1998). Neither *ovo* nor *otu* are likely splicing regulators suggesting that both *ovo* and *otu* are indirect regulators of germline *Sxl* expression.

Genetic interactions between *ovo* and *otu* alleles also suggest

that both genes function in a common pathway. The *otu* locus is a dose-dependent modifier of *ovo^D* (Pauli et al., 1993). The *ovo^D* alleles inappropriately encode OVO-A isoforms instead of OVO-B isoforms in the ovary (Mével-Ninio et al., 1996; Andrews et al., 1998; Andrews et al., 2000b). Because OVO-A and OVO-B have opposite effects on transcription in a wide range of settings (female germline, epidermis and in yeast), the *ovo^D* alleles are likely to downregulate genes normally upregulated by OVO-B (Andrews et al., 2000b). Heterozygosity for *otu* greatly exacerbates germline development in *ovo^{D/+}* females, while adding additional copies of *otu⁺* ameliorates the mutant phenotype (Pauli et al., 1993; Pauli et al., 1995). Thus, high *otu⁺* copy number counters the negative effect of *ovo^D* on transcription. Similarly, partial rescue of the *ovo* phenotype by forcing expression of wild-type OTU protein also indicates that high *otu* activity counteracts the negative effect of loss of *ovo* function (Hinson et al., 1999). Genetic models where *ovo* and *otu* act to regulate *Sxl* in parallel (Nagoshi et al., 1995) or in series (Oliver et al., 1994) have been proposed.

The relationship between *ovo* and *otu* has been explored at the molecular level, but there is no consensus on whether *ovo* regulates *otu* and if so, how important this regulation might be. The *ovo* and *otu* genes are expressed in, and function in, the germline (Oliver et al., 1987; Steinhauer et al., 1989; Comer et al., 1992; Garfinkel et al., 1994; Mével-Ninio et al., 1995). Some evidence supports the idea that *ovo* regulates *otu*. Flies mutant for *ovo* show downregulated expression of *otu* reporters (Lü et al., 1998; Hinson et al., 1999; Andrews et al., 2000b). Importantly, OVO-binding sites are present at the *otu* locus within a segment of DNA sufficient to drive reporter genes in the *otu* pattern, raising the possibility that OVO is a direct regulator of *otu* transcription (Lü et al., 1998). Both of these results are consistent with the hierarchical order *ovo*→*otu*.

Other results favor the parallel model. In particular, one study has shown that *otu* reporters are expressed normally in *ovo* mutants, suggesting that *ovo* does not regulate *otu* (Rodesch et al., 1995). Finally, it is possible that *ovo* controls only minor aspects of *otu* function and that other genes in the pathway are the prominent regulators of *otu* expression. Clearly, somatic signals and *stand still* regulate *otu* transcription (Hinson and Nagoshi, 1999; Sahut-Barnola and Pauli, 1999). It has been suggested that somatic signals play the major, or even the sole role, in *otu* expression (Nagoshi et al., 1995; Hinson and Nagoshi, 1999). This is supported by evidence that *ovo* mutations moderately influence only late expression of *otu* (in terminal stages of oogenesis), and have no effect on the crucial early stages of *otu* expression (Hinson et al., 1999). In contrast, it has also been shown that *otu* reporters respond in a dramatic fashion to loss-of-function and dominant-negative *ovo* alleles in all adult ovarian germ cells, suggesting that *ovo* does indeed control early *otu* expression (Andrews et al., 2000b). Thus, the question of whether *ovo* regulates *otu* expression remains unanswered.

Using an exhaustive set of transgenic animals, we show that OVO regulates the activity of the *otu* promoter, by binding to an OVO site surprisingly near the transcription start site. These data unambiguously indicate that OVO is direct regulator of *otu* transcription. This is the first direct interaction demonstrated in the cell-autonomous portion of the regulatory hierarchy controlling *Drosophila* germline sex determination.

MATERIALS AND METHODS

Alleles

See Table 1 for descriptions of alleles. Flies were grown at 25±0.2°C. See FlyBase for Balancer chromosomes, visible markers and a compendium of references (<http://flybase.bio.indiana.edu>). Reporter gene constructs were introduced into flies by P-element mediated transformation as previously described (Andrews et al., 1998).

General molecular biology

We used standard molecular biology techniques throughout (Sambrook et al., 1989; Dieffenback and Dveksler, 1995). All deletions and point mutations were introduced by PCR. Insertions were introduced by either PCR or by opening engineered restriction sites (Table 1). We verified constructs by sequencing using fluorescent dye terminators (ABI-PRISM, dRhodamine Terminator Cycle Sequencing and an ABI-377 sequencer, Perkin-Elmer). Radioactivity was detected and measured with a phosphorimager and the Imagequant program (Molecular Dynamics).

RACE

Total ovarian RNA from flies bearing reporter genes was extracted using TRIZOL (GIBCO/BRL). We used a RACE kit (Rapid Amplification of cDNA ends v2.0) according to manufacturer's instruction (GIBCO/BRL). 5 µg of total RNA was reverse transcribed, lyophilized, resuspended, tailed and PCR amplified. 0.05% of the original PCR reaction product was subjected to an additional round of PCR. Amplicons were resolved by agarose-gel electrophoresis. Resolved bands were excised and cloned into PCR vector 2.1. The gene-specific primer used for reverse transcription was within the *lacZ*-encoding region (5'-CGGGCCTCTTCGCTATTA-3'). Nested primers were used for the PCR reactions. The first and second gene-specific PCR primers were within the *lacZ*-encoding region (5'-GGGATGTGCTGCAAGGCGATTA-3', and 5'-CCCACTCACGAC-GTTGTAAAA-3'). The abridged anchor primer was used for the first round of PCR, and the universal amplification primer was used for the second round of PCR. The primer used for sequencing cloned RACE products was from the *Adh* region of pCaSpeR-AUG-β-gal (5'-AGCCTCCCAGACCGCAACGA-3').

OVO binding

We performed gel shifts and in-gel DNaseI protection as previously described (Lü et al., 1998). For each experiment, lane 1 had 200 ng of protein from sham-induced *Escherichia coli*, while lanes 2 to 5 had 8, 40, 200, 1000 and 5000 ng of protein with OVO DNA-binding polypeptide from induced *E. coli*. The concentrations of OVO DNA-binding polypeptide in these reactions were about 0, 1.6, 8, 40, 200 and 1000 nM. We used a single preparation of recombinant OVO for all shown experiments. In the gel-retardation experiments, we observed sequence-specific OVO-dependent shifted bands with as little as 1.6 nM recombinant OVO and non-sequence-specific OVO-dependent shifted bands with greater than 200 nM OVO (Lü et al., 1998; additional data not shown). Bound versus unbound was determined. For each series of sites, we arbitrarily set the binding affinity of fragments with a wild-type OVO-binding site at 100%.

Blotting

For RNA blotting experiments, we dissected ovaries from 2- to 5-day-old flies in PBS and flash froze them on dry ice. RNA was extracted with TRIZOL (GIBCO/BRL). 20 µg of total RNA was loaded in each lane, subjected to formaldehyde agarose gel electrophoresis, and transferred to Nytran N⁺ membranes (Amersham). Probes from the *ovo* (2201-2385 bp; numbering convention of Mével-Ninio et al., 1991), *otu* (1325-1525 bp; numbering convention of Steinhauer et al., 1989) and *ribosomal protein 49* (*rp49* (now known as *RpL32* – Flybase) 551-750 bp; numbering convention of Ramos-Onsins et al., 1998) loci were labeled using Rediprime-II (Amersham) and ³²P-

dCTP (NEN). The same RNA samples were used to test for expression of the three genes. We performed hybridization in Quickhyb (Stratagene) according to the manufacturer instructions. Counts in each band were background subtracted. Then, we divided the counts from bands loaded with ovary RNA from females with three copies of *ovo*⁺ by the counts from bands loaded with ovary RNA from females with one copy of *ovo*⁺.

Histology

We tested all transgenic flies bearing reporters by in situ detection of β -galactosidase activity. For each experiment, we assayed ovaries of flies with one copy of the promoter reporter gene, and with one, two or three copies of *ovo*⁺. Genotypes with respect to *ovo* copy number throughout this paper are one copy, *y cho ovo^{D1rv23} v²⁴/+*; two copies *FM7a/+* (siblings of the *y cho ovo^{D1rv23} v²⁴/+* females); three copies, *y w; ovo^{D1r+/+}*. We performed chromogenic staining using master mixes and positive or negative controls, for 24 hours at 37°C (Andrews et al., 2000b). Stained tissues were mounted in DPX (Sigma) and examined under bright field or DIC optics (Zeiss). We always assayed germline and somatic components of the internal

female reproductive system to look for OVO-dependent expression in the germline and to assay for non-OVO-dependent activity in somatic cells. In some cases, whole adults were stained and then dissected to look for adult tissues expressing reporters in a non-OVO-dependent fashion.

RESULTS

OVO binds to a well-defined consensus DNA sequence in vitro (TTACMGTTACA, Lü et al., 1998; ACNGTTACA, Lee and Garfinkel, 2000). Several of these OVO-binding sites are present at the *otu* locus. These sites may be functional in vivo, as *otu* reporter genes respond positively to OVO-B expression in the female germline (Andrews et al., 2000b). While these data are consistent with direct regulation of *otu* by OVO, the function of the OVO-binding sites has not been assayed. We first confirmed that *otu* reporter activity and endogenous *otu* mRNA responded to the dose of *ovo*⁺ in trans. We then assayed

Table 1. Alleles and transgenes used

Alleles	Notes*
<i>ovo^{D1rv23}</i>	Germline amorph. Insertion of HMS beagle (~4.0-4.6 kb) in <i>ovo^{D1}</i> . ‡
<i>P{ovo^{D1r+}}</i>	Wild-type allele. Genomic <i>ovo</i> DNA +1 to about +10.5 kb, in pCaSpeR2. ‡
<i>P{ovo::lacZ^{L1}}</i>	Genomic <i>ovo</i> DNA 0 to 1082 bp (convention of Mével Ninio et al., 1991). Linker at insertion site with initiation codon. ‡
<i>P{otu::lacZ}</i>	Genomic <i>otu</i> DNA -423 to +119 bp (this, and all other <i>otu</i> positions, are according to the convention of Comer et al., 1992) in pCaSpeR-AUG- β gal. Note that this is a new construct.
<i>P{otu::lacZ^{Δ1}}</i>	potu::lacZ with deletion of -294 to -276 bp, concomitantly introducing a <i>SpeI</i> site by an A to C substitution at -311 bp and an A to T substitution at -312 bp.
<i>P{otu::lacZ^{Δ2}}</i>	potu::lacZ with deletion of -261 to -242 bp.
<i>P{otu::lacZ^{Δ3}}</i>	potu::lacZ with deletion of -294 to -242 bp, concomitantly introducing a <i>SacII</i> site by an A to C substitution at -302 bp and an A to G substitution at -300 bp, and a <i>SpeI</i> site by an A to C substitution at -311 bp and an A to T substitution at -312 bp.
<i>P{otu::lacZ^{Δm1}}</i>	potu::lacZ with GCGATA to AAACCC substitution at -260 to -255 bp and CGCTAT to AAACCC substitution at -289 to -279 bp.
<i>P{otu::lacZ^{Δs1}}</i>	potu::lacZ ^{Δ3} with one copy of <i>ovo</i> DNA +606 to +629 bp (from between the <i>ovo-A</i> and <i>ovo-B</i> promoters, numbering convention of Mével Ninio et al., 1991) inserted between the <i>SacII</i> and <i>SpeI</i> sites.
<i>P{otu::lacZ^{Δs2}}</i>	potu::lacZ ^{Δ3} with two copies of <i>otu</i> DNA -263 to -242 bp between the <i>SacII</i> and <i>SpeI</i> sites.
<i>P{otu::lacZ^{Δs3}}</i>	potu::lacZ ^{Δ3} with four copies of <i>otu</i> DNA +12 to +34 bp between the <i>SacII</i> and <i>SpeI</i> sites.
<i>P{otu::lacZ^{Δs3m}}</i>	potu::lacZ ^{Δ3} with four copies of <i>otu</i> DNA +12 to +34 bp with G to C substitution at +24 inserted between the <i>SacII</i> and <i>SpeI</i> sites.
<i>P{otu::lacZ^{Δspm}}</i>	potu::lacZ with deletion of -3 to +42 bp.
<i>P{otu::lacZ^{Δspm}g}</i>	potu::lacZ ^{Δpm} with TCGCG to GTAAC substitution at -31 to -27 bp and T to G substitution at -23 bp.
<i>P{ovo-B::lacZ}</i>	<i>ovo-B</i> core promoter (-71 to +59 bp, where the '+1' transcription start is at +852 bp in the numbering convention of Mével-Ninio et al., 1995) in pCaSpeR-AUG- β gal.
<i>P{otu::lacZ^{swa}}</i>	The <i>otu</i> UAS region (-423 to -94 bp) and <i>ovo-A</i> core promoter (-64 to +50 bp, where the '+1' transcription start is at +361 bp in the numbering convention of Mével-Ninio et al., 1995).
<i>P{otu::lacZ^{swat}}</i>	potu::lacZ ^{swa} with CAAGACG to TATAAAA substitution at -25 to -32 bp, where the <i>ovo-A</i> start site is +1 bp.
<i>P{otu::lacZ^{swad}}</i>	potu::lacZ ^{swa} with GCAAT to AGTCG substitution at +30 to +34 bp, where the <i>ovo-A</i> start site is +1 bp.
<i>P{otu::lacZ^{swai}}</i>	Replacing <i>ovo-A</i> sequence (-6 to +5 bp, where the <i>ovo-A</i> start site is +1 bp) of potu::lacZ ^{swa} with the <i>otu</i> Inr (-44 to -31 bp).
<i>P{otu::lacZ^{swb}}</i>	The <i>otu</i> UAS region (-423 to -94 bp) and <i>ovo-B</i> core promoter (-71 to +59 bp, where the '+1' transcription start is at +852 bp in the numbering convention of Mével-Ninio et al., 1995).
<i>P{otu::lacZ^{swb2}}</i>	The <i>otu</i> UAS region (-423 to -94 bp) and <i>ovo-B</i> core promoter (-35 to +35 bp, where the '+1' transcription start is at +852 bp in the numbering convention of Mével-Ninio et al., 1995). A <i>BglII</i> site was engineered in potu::lacZ by a TT to GA at -95/96 bp and a G to T substitution at -92 bp to create potu::lacZb. <i>ovo-B</i> amplicons with <i>BglII</i> and <i>BamHI</i> sites were introduced into potu::lacZb.
<i>P{otu::lacZ^{swba}}</i>	potu::lacZ ^{swb} with the <i>ovo-B</i> Inr (-5 to +9 bp, where the '+1' transcription start is at +852 bp in the numbering convention of Mével-Ninio et al., 1995) changed to the sequence around <i>ovo-A</i> start site (-5 to +9 bp, where the '+1' transcription start is at +361 bp in the numbering convention of Mével-Ninio et al., 1995).
<i>P{otu::lacZ^{swbi}}</i>	potu::lacZ ^{swb} with the <i>ovo-B</i> Inr (-5 to +9 bp, where the '+1' transcription start is at +852 bp in the numbering convention of Mével-Ninio et al., 1995) changed to the <i>otu</i> Inr sequence (-44 to -31 bp).
<i>P{otu::lacZ^{snf}}}</i>	The <i>snf</i> core promoter (-35 to +36 bp, where '+1' is the transcription start; Flickinger and Salz, 1994) introduced into potu::LacZb between the <i>BglII</i> and <i>BamHI</i> sites.
<i>P{otu::lacZ^{E75A}}</i>	The <i>E75A</i> core promoter (-35 to +35 bp, where '+1' is the transcription start; Segreaves and Hogness, 1990) introduced into potu::LacZb between the <i>BglII</i> and <i>BamHI</i> sites.
<i>P{otu::lacZ^{act}}}</i>	The <i>actin</i> core promoter (-35 to +38, where '+1' is the transcription start; Manseau et al., 1988) introduced into potu::LacZb between the <i>BglII</i> and <i>BamHI</i> sites.
<i>P{otu::lacZ^{lam}}}</i>	The <i>lamin</i> core promoter (-46 to +35 bp, where '+1' is the transcription start; Osman et al., 1990) introduced into potu::LacZb between the <i>BglII</i> and <i>BamHI</i> sites.
<i>P{otu::lacZ^{stil}}}</i>	The <i>stand still</i> core promoter (-35 to +38 bp, where '+1' is the transcription start; Pennetta and Pauli, 1997) introduced into potu::LacZb between the <i>BglII</i> and <i>BamHI</i> sites.

*This study, unless indicated.

‡<http://flybase.bio.indiana.edu>

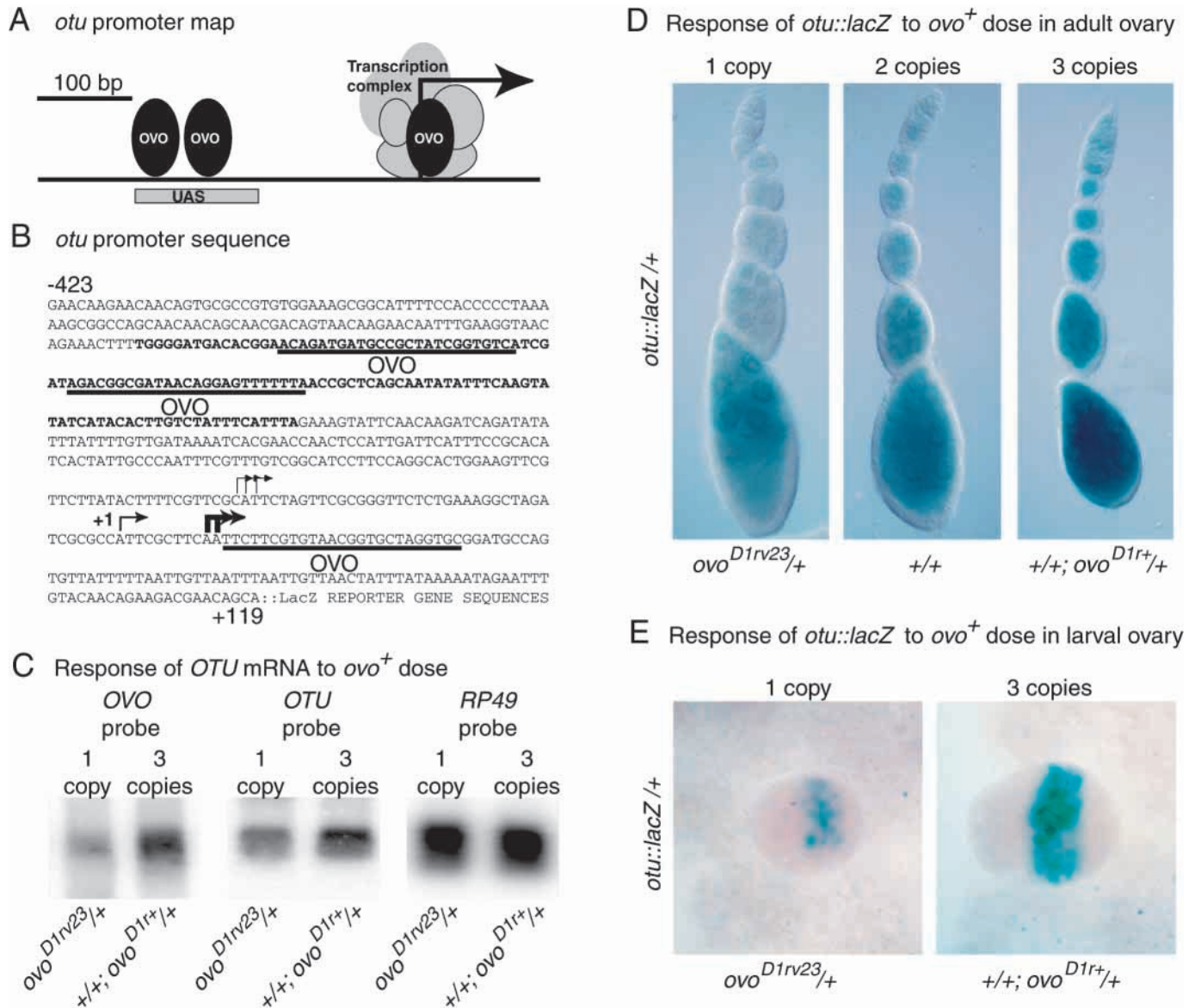


Fig. 1. OVO regulates *otu* transcription in vivo. (A) The *otu* promoter. An upstream region required of *otu* expression (UAS) is indicated as are OVO binding sites (dark ovals) and the basal transcription apparatus (gray) flanking the start site (arrow). (B) *otu* promoter sequence (convention of Comer et al., 1992) in the reporter gene. OVO footprints (underlined); weak (thin arrows) and strong (thick arrows) transcription start sites; UAS (bold type). One transcription start site is defined as +1 by convention, but the strongest start sites are at +10 and +11 bp respectively. Three minor start sites are located at -38, -39 and -40 bp (Comer et al., 1992). There are consensus *Drosophila* Inr elements around each transcription start site (Arkhipova, 1995). (C) Northern blots of *ovo*, *otu* and *ribosomal protein 49* (*RP49*) mRNA levels in ovaries bearing one or three copies of *ovo*⁺ (copy number above each panel, genotype below). (D) X-Gal staining of ovarioles from flies bearing one copy of *otu* reporter gene (left) and with one, two or three copies of *ovo*⁺ (copy number above each panel, genotypes below each panel). (E) X-Gal staining of third larval instar ovaries bearing one copy of the *otu* reporter gene with one or three copies of *ovo*⁺ (copy number above each panel, genotypes below each panel).

for the function of OVO-binding sites in a series of transgenic flies in which OVO-binding sites were deleted or added in *cis*. We then determined the response of these constructs to the dose of *ovo*⁺ in *trans*.

Three OVO-binding sites are present in the 542 bp region that is sufficient for correct expression of *otu* in the germline (Fig. 1A,B). In-gel DNaseI protection experiments indicated that recombinant OVO bound to the consensus site at the major core promoter with highest affinity and protected a region just two bp downstream of the major transcription start site. This

site is certainly within the region from about -20 to +40 where transcription complexes would be expected to assemble on the major +1, +10, and +11 start sites (Burke and Kadonaga, 1996). Thus, if this OVO site is occupied in vivo, it would be expected to influence transcription initiation at the major *otu* start site. Additionally, two closely spaced OVO-binding sites are found within a previously mapped upstream regulatory region between -310 and -190 (Comer et al., 1992). These OVO-binding sites are much weaker and diverge considerably from the consensus sequence (Lü et al., 1998; Lee and

Garfinkel 2000). Previous work also suggests that regions between the UAS and just upstream of the transcription start site are not important for *otu* expression in vivo (Sass et al., 1993).

Endogenous *otu* transcripts and *otu* reporter genes respond to *ovo*⁺ copy number in *trans*

Our previous work suggests that *otu* functions downstream of *ovo* (Pauli et al., 1993; Lü et al., 1998; Andrews et al., 2000b). If OVO directly controls the expression of *otu*, then the absence of *ovo* should have an effect on the *otu* mRNA levels in the female germline. As *ovo*⁺ is required for female germline viability (Oliver et al., 1987), the expression of *otu* in flies that lack OVO cannot be easily analyzed (see Discussion). Instead, we asked if altered *ovo*⁺ gene dose influenced *otu* mRNA levels.

We performed northern blotting experiments to examine endogenous *otu* mRNA levels in flies are altered by differing doses of *ovo* (Fig. 1C). As a first control, we measured steady-state *ovo* mRNA levels to determine if increased *ovo*⁺ copy number resulted in increased *ovo* transcripts. There was a clear relationship between *ovo*⁺ copy number and *ovo* transcription, as a threefold increase in *ovo*⁺ genetic dose resulted in greater than a fourfold increase in *ovo* mRNA levels. Thus, this control indicates that the increase in *ovo* genetic dose results in the expected increase in steady-state *ovo* mRNA. We controlled for loading by probing for *Ribosomal protein 49* (also known as *RpL32*). Lanes of RNA from ovaries with one or three copies of *ovo*⁺ showed equal *Ribosomal protein 49* mRNA levels. For the experimental result, we found that increasing the copy number of the *ovo*⁺ gene from one to three resulted in greater than a twofold increase in the steady state level of *otu* mRNA in the ovary. The increased *otu* expression was likely to be due to increased OVO protein levels in flies with more copies of *ovo*⁺. Although we do not have antibodies that we can use to detect OVO protein, genetic tests indicate that increased *ovo*⁺ copy number results in increased OVO activity. For example, dominant-negative OVO proteins can be genetically titrated by increasing the dose of *ovo*⁺ alleles, but not by increasing the dose of mutated *ovo* genes (Andrews et al., 1998). These data indicate that increased *ovo* transcription results in increased OVO protein accumulation and increased *otu* transcription.

We constructed a new *otu* reporter gene that served as a base for all other constructs in the study. We tested this construct for appropriate expression patterns and response to *ovo*⁺ dose in *trans*. The base construct had 542 bp of *otu* sequence (−423 to +119), including the three OVO-binding sites, fused to the *lacZ* gene (Fig. 1B). As expected, we observed expression of the wild-type *otu* reporter gene in all germ cells of the adult ovary (Fig. 1D). β-galactosidase activity (X-Gal staining) was clearly observed in the germarium, where germline stem cells and dividing cystocytes are located. Activity was moderate in early egg chambers beginning the differentiation process, while more mature egg chambers showed markedly increased activity. Like the endogenous transcripts, the *otu* reporter responded to the dose of *ovo*⁺ in *trans*. Females with one copy of *ovo*⁺ showed weak X-Gal staining, while female with two copies of *ovo*⁺ showed moderate X-Gal staining, and females with three copies of *ovo*⁺ showed very robust X-Gal staining. Thus, the reporter faithfully replicated the response of *otu* mRNA expression to *ovo*⁺ dose in *trans*. In contrast to some

previous reports, our results show that *otu::lacZ* activity increases with increased *ovo*⁺ copy number in the stem cells and cystocytes of the germarium (Fig. 1D; Lü et al., 1998; Andrews et al., 2000b) and in the germ cells of the larval ovary (Fig. 1E), suggesting that *ovo* regulates *otu* expression in early and late stages of germline development. Additionally, we find that both *ovo* and *otu* reporters are expressed in the larval germ cells of both females and males (Fig. 1E; additional data not shown). These data are consistent with the presence of OVO protein in the larval germ cells that express *otu*. Briefly, the arrangement of OVO-binding sites at *otu*; the expression patterns of *ovo* and *otu*; and the response of endogenous *otu* and reporters to *ovo* copy number in *trans*; all suggest that *ovo* is a regulator of *otu*.

Promoter proximal OVO binding

The effect of *ovo*⁺ dose on *otu* mRNA levels suggest that at least some of the OVO-binding sites at the *otu* promoter are functional in vivo. To rigorously test this hypothesis and to determine which OVO sites are important, we constructed a series of new reporter genes.

The proximal OVO footprint abuts the major transcription start site of the *otu* promoter. This proximity is intriguing, but also makes the analysis of the function of the OVO-binding site problematic. Deletion of a large region flanking the OVO-binding site might destroy the sequences bound by the basal transcriptional machinery. Even if we made more subtle mutations that abolished promoter activity, it would be difficult to say with any certainty that this loss of activity was due specifically to reduced OVO-binding. Therefore, we tried two related approaches. First, we asked if we could ameliorate OVO responsiveness by introduction of an OVO-binding site at a core promoter. Second, we swapped the *otu* core promoter for short fragments (usually 60 bp) of other core promoters, some with an OVO binding site and some without. All of the core promoter constructs minimize the potential damage to essential core promoter sequences. Additionally, some of these constructs rely on the higher standard of conferring responsiveness, rather than simply abolishing function. We assayed OVO binding to core promoters by gel retardation assays. We performed in-gel DNaseI protection assays to determine the precise location of OVO binding relative to the start sites. We introduced the constructs into flies by P-element-mediated transformation to assay promoter activity in vivo. The start sites used in the constructs themselves were mapped by RACE. We describe some of the more crucial constructs in detail below.

Gel retardation analysis of the region including the wild-type transcription start sites revealed very strong OVO-binding activity to the wild-type *otu::lacZ* reporter (Fig. 2A). There is a strong shift at low OVO concentration and a further shift at higher OVO concentrations. In-gel DNase protection revealed a single predominant protected region beginning 2 bp downstream of the +11 start site of the wild-type reporter (Fig. 2A). The same footprint is developed from DNA extracted from either shifted band, suggesting that the first shift is due to specific binding to the OVO site and that the second shift is due to non-sequence-specific binding distributed along the length of the DNA fragment in addition to the sequence-specific binding (also see Materials and Methods). In *otu::lacZ^{Δpm}*, we introduced a deletion of 45 bp of DNA (from

-3 to +42) that contained the core promoter OVO-binding site and major transcription start sites. This deletion eliminated sequence-specific OVO binding, as assayed by both gel retardation and DNase protection experiments (Fig. 2B). The *otu::lacZ^{Δpm}* deletion leaves only the weak *otu* start sites, which we then attempted to strengthen by adding an OVO-binding site. We reconstituted the ability of the core promoter region to bind OVO in *otu::lacZ^{Δpmg}* (Fig. 2C). This construct was modified from *otu::lacZ^{Δpm}* by converting 6 of 13 bases, between 7 bp to 19 bp downstream of the -40 start site, to the same sequence that appeared 7 bp to 19 bp downstream of the major +11 start site in the wild-type *otu* core promoter. This reconstructed *otu::lacZ^{Δpmg}* core promoter DNA fragment

bound recombinant OVO as well as the wild-type major core promoter. In-gel footprint analysis of the retarded bands revealed the predicted protected region immediately downstream of the minor transcription start (Fig. 2C). These three constructs measure the activities of the wild-type major and minor promoter, the minor promoter only, and the minor promoter with a new OVO-binding site.

We also employed native core promoters from the *ovo* locus. Transcripts driven from the tightly linked *ovo-B* and *ovo-A* core promoters are differentially expressed and encode distinct OVO proteins with opposite activities (Andrews et al., 2000b). The *ovo-B* core promoter has an OVO-binding site. The *ovo-A* core promoter does not (Lü et al., 1998). We replaced the entire

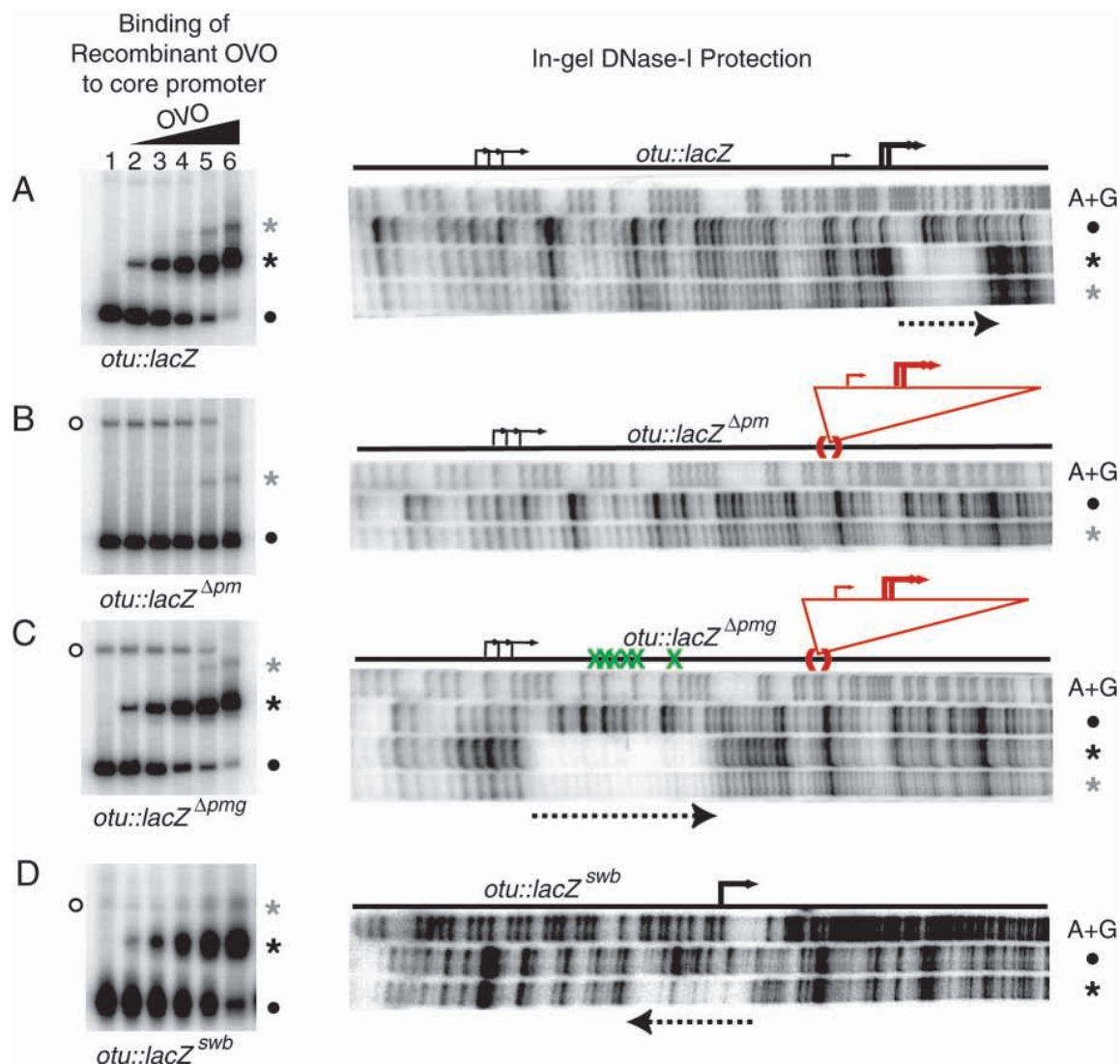
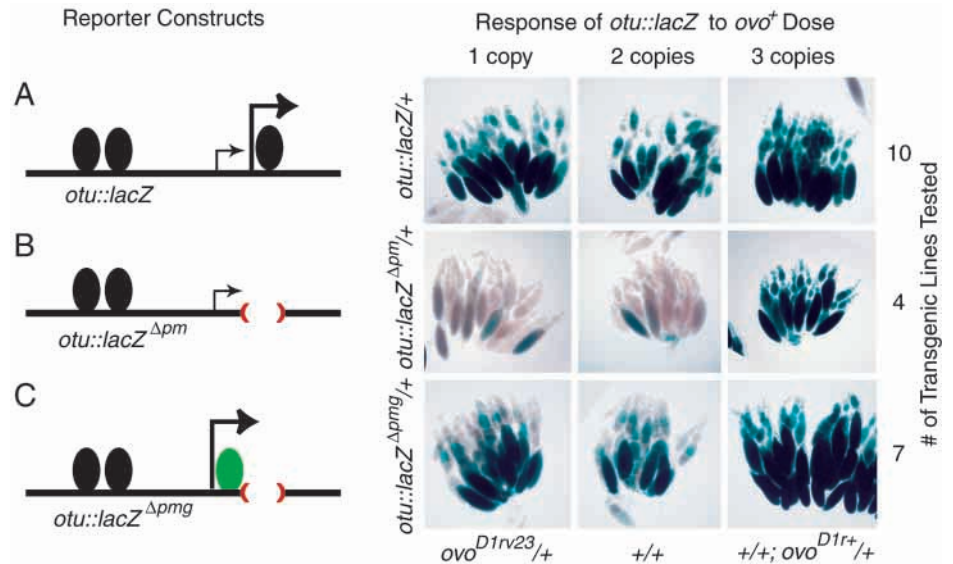


Fig. 2. OVO-binding sites at native or mutated core promoters. In the first column, which shows gel shifts, unshifted (black circle), sequence-specific shifted (black asterisk) and nonspecific shifted bands (gray asterisk), owing to the OVO DNA-binding polypeptide (see Materials and Methods), as well as nonspecific shifts caused by bacterial proteins (white circles) are indicated. Lanes 1 to 6 have approximately 0, 1.6, 8, 40, 200 and 1000 nM of recombinant OVO DNA binding domain, respectively. In the second column, showing footprints, orientation of OVO binding sites (broken arrows), transcription start sites (thin and thick arrows), deletions (brackets) and point mutations (Xs) are indicated. Loss-of-function mutations are in red and gain-of-function mutations in green. The DNase-I protection experiments were developed from the indicated bands (circles, asterisks to the right). The chemical sequencing DNA reference lane (A+G) is shown. (A) *otu::lacZ*: Wild-type core promoter DNA fragment from *otu::lacZ*. (B) *otu::lacZ^{Δpm}*: Core promoter DNA fragment with the OVO-binding site deleted from *otu::lacZ*. (C) *otu::lacZ^{Δpmg}*: Core promoter DNA fragment from *otu::lacZ^{Δpm}* with a reconstituted core promoter OVO binding site. (D) *otu::lacZ^{swb}*: *ovo-B* core promoter DNA fragment from *otu::lacZ^{swb}*.

Fig. 3. Function of OVO-binding sites at the *otu* core promoter. The promoter reporter constructs (left) and the X-Gal staining in ovaries (right) are shown. The number of transgenic lines tested is shown (right). (A) *otu::lacZ*. (B) *otu::lacZ^{Δpm}*. (C) *otu::lacZ^{Δpmg}*. The new OVO binding site is shown (green oval), and weak (thin arrows) and strong (thick arrows) transcription start sites are indicated. X-Gal staining in ovaries (right) is shown. *ovo*⁺ copy number (top), *ovo* genotype (bottom), reporter genotype (left) and the number of transgenic lines tested (right) are also indicated.



otu core promoter region (211bp, -93 to +119) with the *ovo-B* or with the *ovo-A* core promoter. The *otu::lacZ^{swb}* reporter binds OVO in vitro and the OVO footprint overlaps the transcription start site (Fig. 2D). There is no core promoter footprint for *otu::lacZ^{swa}* (not shown). These two reporters allow us to test for the effect of an OVO-binding site at the core promoter. Additionally, the OVO-binding site at the *ovo-B* core promoter is inverted relative to the OVO site at the *otu* core promoter. Thus, this construct allows us to test both for an overall effect of a core promoter OVO-binding site and for orientation.

Reconstitution of a promoter proximal OVO-binding site confers *ovo* responsiveness

We examined expression of the deleted and reconstituted *otu* promoters first. The wild-type reporter showed characteristic graded response to the dose of *ovo*⁺ in *trans* (Fig. 3A). In contrast, the *otu::lacZ^{Δpm}* reporter with the deletion of the OVO-binding site and major transcription start sites showed strikingly reduced activity in vivo (Fig. 3B). Females bearing *otu::lacZ^{Δpm}* and one or two copies of *ovo*⁺ showed no detectable staining in the early egg chambers and only feeble staining in late chambers. There was residual *ovo*-responsiveness, perhaps owing to upstream OVO-binding sites, as females with three copies of *ovo*⁺ exhibited strong staining activity. Females bearing the crucial reconstituted core promoter of *otu::lacZ^{Δpmg}* showed dramatically increased staining in females bearing one or two copies of *ovo*⁺ (Fig. 3C). Indeed, females bearing *otu::lacZ^{Δpmg}* show a graded reporter response to *ovo*⁺ copy number like females bearing the wild-type reporter. This experiment strongly supports the idea that OVO binding to the promoter proximal site substantially contributes to *otu* promoter activity.

Alterations in the core promoter structure could cause the use of different transcription start sites. We were therefore quite interested in determining which site in the *otu::lacZ^{Δpmg}* reporter served as the transcriptional start site. Start sites within about 30 bp of the OVO footprint would suggest that OVO protein can function in some aspect of core promoter function, while start sites further away would suggest a more traditional

transcription factor function. We extracted RNA from ovaries of *otu::lacZ^{Δpmg}* females for RACE analysis. The expected amplification product for the minor start site was found by agarose gel electrophoresis (data not shown). We sequenced two RACE products from the excised band, and those products terminated 14 and 17 bp upstream of the OVO footprint, suggesting that the transcription start site in *otu::lacZ^{Δpmg}* is also promoter proximal. Thus, as with the wild-type *otu* promoter, the OVO-binding site in the transcriptional active *otu::lacZ^{Δpmg}* construct is within the region where the basal transcriptional machinery should be arrayed at the start site.

Promoter swapping reveals a striking requirement for promoter proximal OVO-binding sites

In the remaining promoter constructs, no native *otu* transcription start sites were retained. We swapped the *otu* transcription start sites, for bone fide *Drosophila* core promoters from a variety of genes. The endogenous *ovo-A* promoter is expressed most strongly in late stages of oogenesis (Andrews et al., 2000b). Thus, all the requisite core promoter sequences for expression in the ovary are present. The gel-retardation assays showed that the *ovo-A* core promoter region did not bind OVO in vitro, other than non-sequence-specific binding at high OVO concentration (Fig. 4B). The *otu* upstream region and *ovo-A* core promoter construct, *otu::lacZ^{swa}*, did not show detectable activity in vivo, even in the presence of three copies of *ovo*⁺ in *trans* (Fig. 4B). Thus, the two upstream OVO-binding sites have no discernable effect on transcription from the *ovo-A* promoter. In dramatic contrast, the *ovo-B* core promoter bound OVO strongly in vitro (Figs 2D, 4C) and *otu::lacZ^{swb}* was as active as the wild-type *otu* reporter (Fig. 4C). Females heterozygous for *otu::lacZ^{swb}* with one copy of *ovo*⁺ showed weaker staining, while females with two copies of *ovo*⁺ showed moderate staining, and females with three copies of *ovo*⁺ showed strong staining activity. The core OVO-binding site in the robustly expressed *otu::lacZ^{swb}* reporter gene is inverted, and offset, relative to the OVO-binding site at the *otu* core promoter, suggesting that functional binding of OVO at the core promoter is not strictly orientation or position dependent. The *ovo-B* core promoter alone (in the absence of

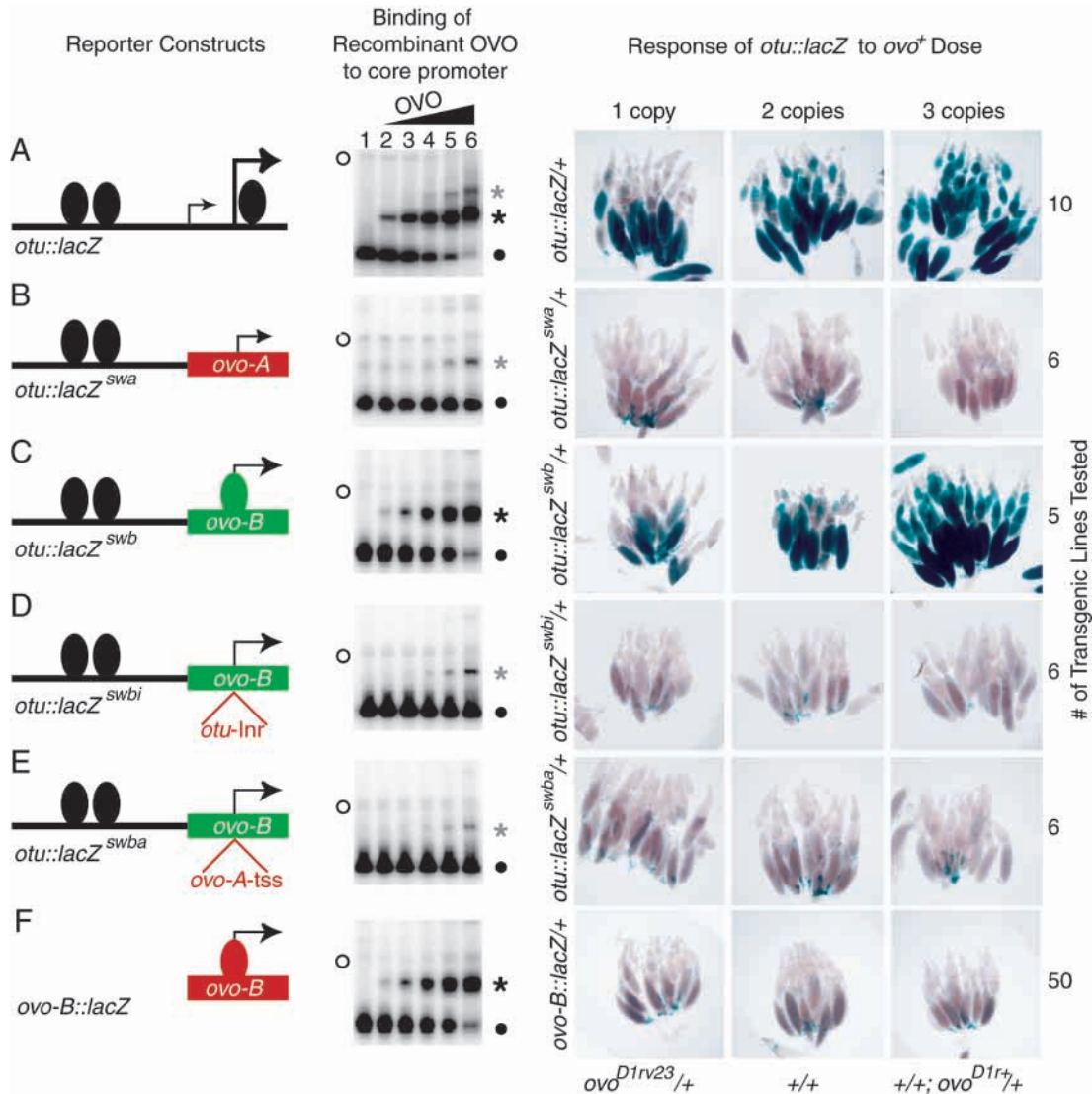


Fig. 4. The *ovo-A*, *ovo-B*, and derivative core promoters. Replaced core promoter sequences are indicated by rectangles. Sequences common to the reporters are indicated by black lines. Non-functional (red) and functional (green) derivations are indicated. (A) *otu::lacZ*. (B) *otu::lacZ^{swa}*. (C) *otu::lacZ^{swb}*. (D) *otu::lacZ^{swbi}* (same as C, except that the *ovo-B* Inr was replaced with an *otu* Inr). (E) *otu::lacZ^{swba}* (same as C, except that the *ovo-B* Inr was replaced with the sequence around the *ovo-A* transcription start site (tss)). (F) *ovo-B::lacZ*. OVO binding sites are shown (ovals), and weak (thin arrows) and strong (thick arrows) transcription start sites are indicated. In the second column, which shows gel shifts, unshifted (black circle), sequence-specific shifted (black asterisk) and nonspecific shifted bands (gray asterisk), owing to the OVO DNA-binding polypeptide (see Materials and Methods), as well as nonspecific shifts caused by bacterial proteins (white circles) are indicated. X-Gal staining in ovaries (right) is shown. *ovo*⁺ copy number (top), *ovo* genotype (bottom), reporter genotype (left) and number of transgenic lines tested (right) are also indicated.

otu sequences) was not expressed (Fig. 4F) suggesting that upstream regions are also required for a response to OVO.

Again, because a slight change in the position of the transcription start site would place bound OVO outside the domain of the core promoter, we performed RACE experiments on RNA from *otu::lacZ^{swb}* ovaries. Two transcription starts, within and 10 bp downstream of the OVO footprint region, were identified. The site within the footprint region was exactly the same site mapped in previous studies (Garfinkel et al., 1994; Mével-Ninio et al., 1995). Thus, as was the case with *otu::lacZ^{Δpmg}* these data suggest that OVO binds and functions usually close to the transcription start site of *otu::lacZ^{swb}*. Indeed, the OVO footprint in *otu::lacZ^{swb}* occludes the

transcription start site, but appears to serve a positive role in transcription.

While the presence of an OVO-binding site is an obvious difference between the *ovo-A* and *ovo-B* core promoters, none of the flanking sequences is strikingly homologous. Thus, the promoter elements responsible for *ovo-B* activity, or for failed *ovo-A* activity, need not be OVO-binding sites. To determine if the OVO-binding sites at the *ovo-B* core promoter are required, we replaced the OVO-binding sites at the core promoter with the same number of residues from other native promoters that lack OVO-binding sites. We replaced the start site of *ovo-B* (−5 to +9) with the corresponding region from the *otu* −39 transcription start site to create the *otu::lacZ^{swbi}* reporter (Fig.

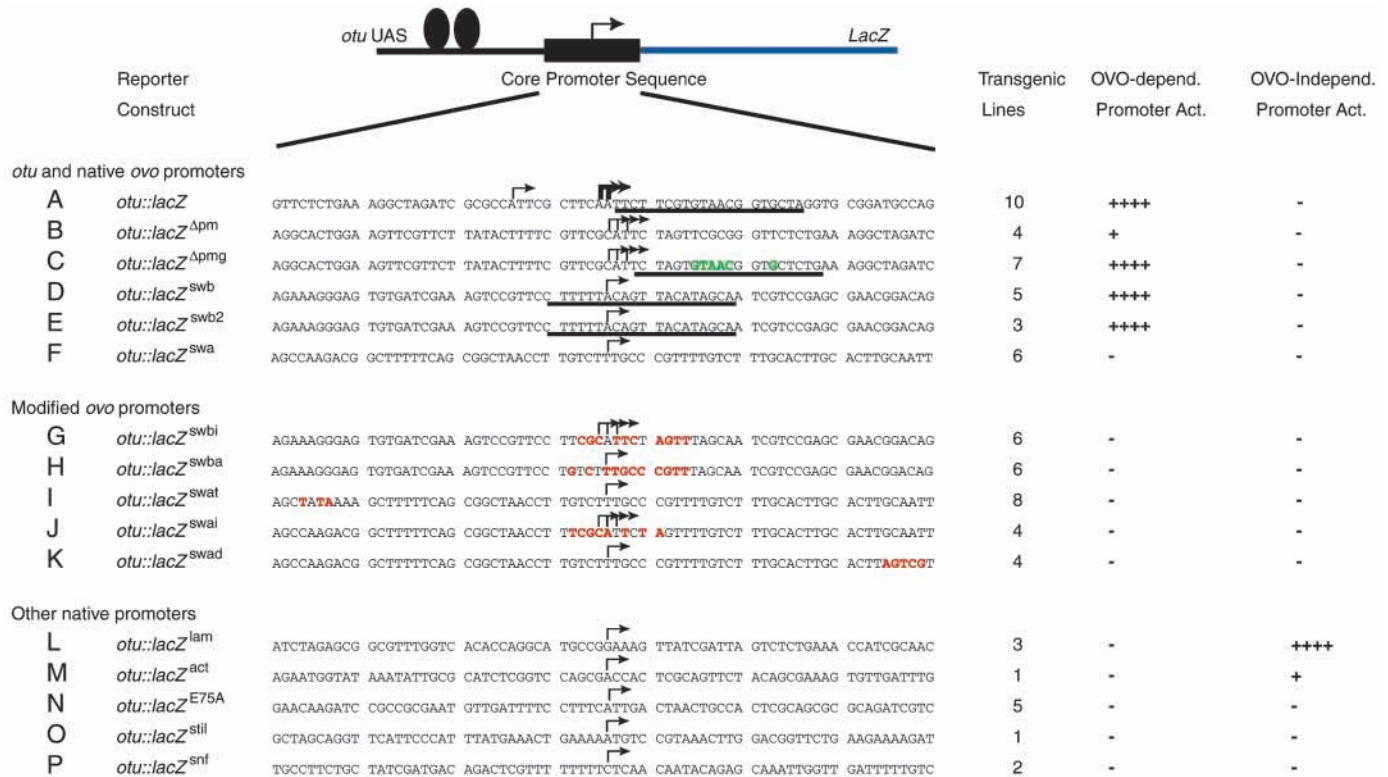


Fig. 5. Testing other core promoters. All these reporters have the same *otu* region, but different core promoter sequences. The base reporter is cartooned above and the core promoter sequence for each reporter is shown below. Some core promoter constructs differ outside the region shown (see Table 1). Minor alterations to core promoters are in bold. Functional changes (green) and nonfunctional changes (red) are shown. Promoter reporter activities were very strong (++++), weak (+), or not detectable (-). There was little intermediate expression. OVO-dependent activity was assayed in the germ cells of adult ovaries. OVO-independent activity was assayed in various adult tissues.

4D), or with the sequence around the *ovo-A* transcription start site to create the *otu::lacZ^{swba}* reporter (Fig. 4E). Both these replacements abolished OVO-binding activity at the *ovo-B* core promoter. We failed to detect reporter activity in the ovaries of females bearing either *otu::lacZ^{swbi}* or *otu::lacZ^{swba}*, even in the presence of three copies of *ovo⁺*. These results suggest that the OVO-binding site at the *ovo-B* initiator is important for promoter function.

The *otu* upstream region does not activate a variety of native core promoters that lack OVO-binding sites

We found that the minor start sites of *otu* responded more robustly to *ovo⁺* when provided with a core OVO binding site, and that the *ovo-A* core promoter failed to respond (Fig. 5A-F). Substitution of even a few bp at the OVO-binding site abolishes activity (Fig. 5G,H), but these mutations might weaken the basal strength of the promoter. Similarly, given that the minor *otu* and *ovo-A* promoters are weakly active in native contexts (Comer et al., 1992; Andrews et al., 2000b), we were interested in asking if core OVO binding is required simply to make weak promoters stronger. If this were the case, then providing known core promoter elements, such as TATA, might circumvent the requirement for proximal OVO binding sites. We introduced a TATA, Inr or DPE element into the *ovo-A* core promoter in an attempt to increase promoter strength (Fig. 5I-K). We generated multiple lines of these reporters with modified *ovo-A* core promoters, but none showed a response to *ovo⁺* dose. These data suggest a potentially stronger *ovo-A*

core promoter is still unresponsive. Because we do not fully understand what makes a core promoter strong, it was also possible that the changes we made in *ovo-A* were insufficient.

Unlike our designer *ovo-A* promoters, strong endogenous promoters are more likely to contain the requisite information for high promoter activity. Thus, if core promoter OVO sites are important to strengthen an otherwise weak core promoter, then some endogenous promoters should substitute for the *otu* and *ovo-B* promoters in the reporter system. We therefore replaced the *otu* core promoter region with core promoters from the *Actin 87E*, *E75A* (also known as *Eip75B*) *sans fille*, *stand still* and *Lamin* genes (Fig. 5L-M). We selected these promoters based on expression patterns and promoter element configuration. Like *otu* and *ovo*, the *sans fille* and *stand still* loci function in the female germline (Oliver et al., 1988; Pennetta and Pauli, 1997; Cline et al., 1999). The *Actin 87E* and *Lamin* genes are highly, and widely, expressed genes (Tobin et al., 1990; Ulitzur et al., 1992). Additionally, both the *Actin 87E* and *Lamin* genes have potential TATA elements (Manseau et al., 1988; Osman et al., 1990). The *E75A* and *sans fille* genes have potential DPE elements (Segraves and Hogness, 1990; Flickinger and Salz, 1994; Burke and Kadonaga, 1996). We introduced all these promoters in exchange for the *otu* promoters in the *otu::lacZ* reporter. None of these reporters responded to *ovo⁺* copy number in *trans*.

The reporters made from the *Actin 87E*, *E75A*, *sans fille* and *stand still* core promoters never showed detectable activity in ovaries (Fig. 5N-P). It is highly unlikely that all these

promoters failed to respond due to trivial defects in construction. Indeed, we can rule out trivial explanations for failed expression for two of the reporters.

The reporter made from the *Lamin* core promoter, *otu::lacZ^{lam}*, was expressed throughout the ovary in a non-*ovo* dependent fashion (Fig. 5L) and thus clearly functions, but does not respond to *ovo*⁺ dose in trans. The *Actin 87E* reporter, *otu::lacZ^{act}*, showed weak expression visible through the adult eye, but also failed to respond to *ovo*⁺ dose in trans (Fig. 5M). Thus, even with a strong and functional basal promoter, we were unable to reconstitute the correct *otu* expression pattern. This indicates that the role of the OVO-binding site at the core promoter does more than simply increase promoter strength. These data support the idea that OVO bound at the core promoter is informational.

Upstream regions are required for *otu* reporter function

Several core promoter sequences bear remarkable tissue-specific regulatory information. For example, very short regions at the *Drosophila decapentaplegic* (Schwyter et al., 1995) or *bicoid* core promoters (Ruez et al., 1998) are able to

direct specific expression patterns during development. In contrast, isolated *ovo-B* core promoters were not active in our reporter system (Fig. 4F). These data suggest that upstream sequences are also required. Indeed the two weak OVO sites at *otu* map to a region known to be important for *otu* function (Comer et al., 1992). The failed expression of multiple core promoter swaps indicates that upstream OVO sites can not be sufficient for *ovo* responsiveness (Fig. 5), but they might play a role in conjunction with the promoter proximal OVO-binding sites. Indeed, the weak response of *otu::lacZ^{Δpm}* is consistent with a minor role for upstream sites (Fig. 3). We made another series of reporters to directly assay for upstream OVO-binding site function.

Deletion of a 19 bp DNA segment including the distal upstream OVO-binding site in the *otu::lacZ^{Δ1}* construct reduced OVO binding affinity in the upstream region to 64% of the wild-type fragment (Fig. 6A,B). This deletion had a minimal effect on reporter activity in the female germline (Fig. 6B). The ovaries of females bearing a single copy of *otu::lacZ^{Δ1}* showed strong staining activity and the reporter responded positively to increased *ovo*⁺ dose in vivo. Deletion of a 20 bp DNA fragment that included the second upstream

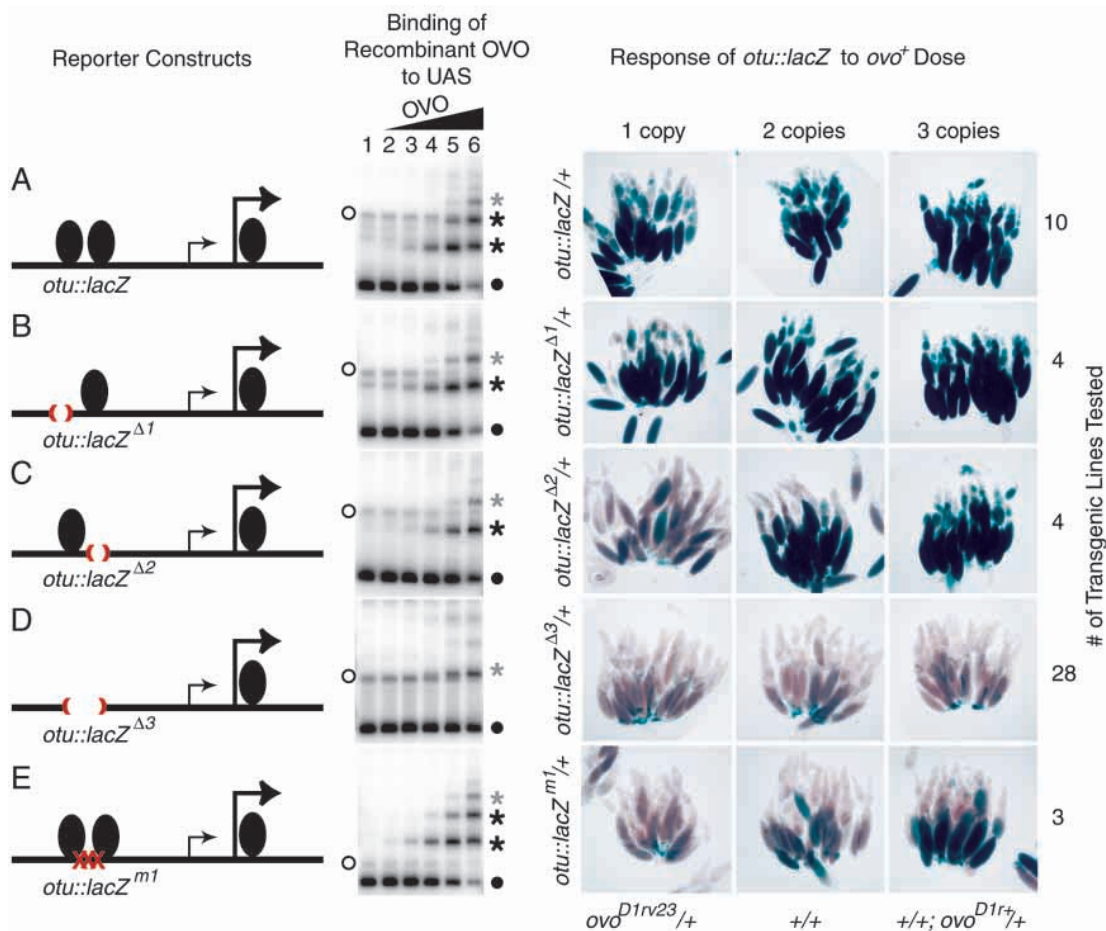


Fig. 6. Deletions of upstream OVO-binding sites. (A) *otu::lacZ*. (B) *otu::lacZ^{Δ1}*. (C) *otu::lacZ^{Δ2}*. (D) *otu::lacZ^{Δ3}*. (E) *otu::lacZ^{m1}* (with clustered point mutations between the OVO binding sites). Weak (thin arrows) and strong (thick arrows) transcription start sites are indicated. In the second column, which shows gel shifts, unshifted (black circle), sequence-specific shifted (black asterisk) and nonspecific shifted bands (gray asterisk), owing to the OVO DNA-binding polypeptide (see Materials and Methods), as well as nonspecific shifts caused by bacterial proteins (white circles) are indicated. On the right, X-Gal staining in ovaries (right) is shown. *ovo*⁺ copy number (top), *ovo* genotype (bottom), reporter genotype (left) and number of transgenic lines tested (right) are also indicated.

OVO-binding site reduced binding affinity of the upstream region to 36% of wild type (Fig. 6C). Similarly, the deletion of the second binding site resulted in more significantly reduced reporter activity in vivo (Fig. 6C). In females with one copy of *ovo*⁺, *otu::lacZ*^{Δ2} reporter activity was minimal in late egg chambers and absent from earlier stages. There was a similar relative reduction in reporter activity in the ovaries of females with two or three copies of *ovo*⁺. Thus, while the deletion of the second site was clearly consequential, the reporter still responded to the dose of *ovo*⁺ in vivo. Deletion of a 53 bp DNA fragment that included both the upstream OVO-binding sites abolished sequence-specific OVO binding in vitro (Fig. 6D). The effect on reporter activity in flies was equally dramatic. We did not detect *otu::lacZ*^{Δ3} reporter activity in the ovaries of females with one, two or three copies of *ovo*⁺ (Fig. 6D). These results indicate that a crucial control region for *otu* expression lies in the 53 bp region between -294 and -242. The two upstream OVO-binding sites lie within this interval. Either half of this control region, each bearing an OVO-binding site (in opposite orientations), allows for

response to *ovo*⁺ dose. These data indicate that the *otu* core promoter is nonfunctional without the upstream control region.

Upstream control regions are typically composites of binding sites for multiple proteins (Carey, 1998), so failed expression from the reporter could be due to the simultaneous deletion of binding sites unrelated to OVO. Indeed, the *otu* locus is transcriptionally regulated by somatic signals and *stand still* activity (Hinson and Nagoshi, 1999; Sahut-Barnola and Pauli, 1999), so we expect that a number of transcription factors will bind in the compact *otu* regulatory region. Indeed, when we mutated the DNA segment between the upstream OVO-binding sites, we found that this region is required for *otu* reporter activity, but not for OVO binding (Fig. 6E). These data suggest that either the upstream OVO sites have no function, or that the upstream OVO-binding sites contribute to the activity of a complex control region. The later seems more likely, given the weak response of *otu::lacZ*^{Δpm} to *ovo*⁺ dose and the moderate effect of clean deletion of the first or second upstream OVO sites.

If upstream OVO sites are contributory, it might be possible

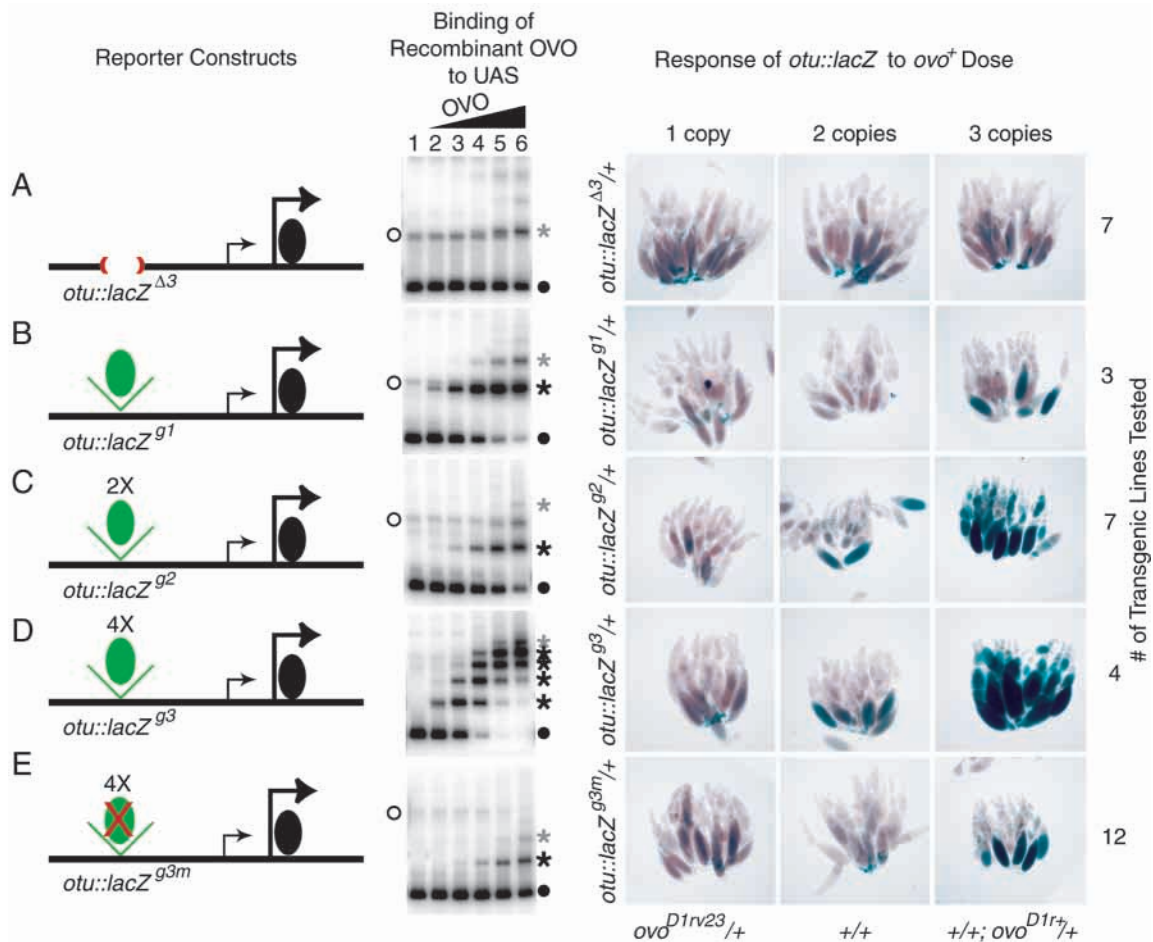


Fig. 7. Reconstitution of upstream OVO binding sites. (A) *otu::lacZ*^{Δ3}. (B) *otu::lacZ*^{g1} (with one OVO-binding site from the *ovo* locus introduced into *otu::lacZ*^{Δ3}). (C) *otu::lacZ*^{g2}: with two copies of the second *otu* proximal upstream OVO-binding site introduced into *otu::lacZ*^{Δ3}. (D) *otu::lacZ*^{g3} with four copies of the *otu* core promoter OVO-binding site introduced into *otu::lacZ*^{Δ3}. (E) *otu::lacZ*^{g3m}: same as (D) but with one base pair mutation in each binding site. The new OVO binding site is shown (green oval), and weak (thin arrows) and strong (thick arrows) transcription start sites are indicated. In the second column, which shows gel shifts, unshifted (black circle), sequence-specific shifted (black asterisk) and nonspecific shifted bands (gray asterisk), owing to the OVO DNA-binding polypeptide (see Materials and Methods), as well as nonspecific shifts caused by bacterial proteins (white circles) are indicated. On the right, X-Gal staining in ovaries (right) is shown. *ovo*⁺ copy number (top), *ovo* genotype (bottom), reporter genotype (left) and number of transgenic lines tested (right) are also indicated.

to restore at least some *otu::lacZ^{Δ3}* reporter activity by reintroducing OVO binding sites with different flanking sequences (Fig. 7). Furthermore, this reporter activity should respond to the dose of *ovo*⁺ in *trans*. To test this, we introduced a 24 bp fragment containing a strong, non-core promoter, OVO-binding site from the *ovo* locus into *otu::lacZ^{Δ3}* to create *otu::lacZ^{Δ3}1*. Gel retardation assays showed that this binding site was stronger (117%) than the two combined binding sites in the wild-type reporter. When this construct was introduced into flies there was clear, albeit weak, restoration of promoter activity. Females with one or two copies of *ovo*⁺ showed no reporter activity, but females with three copies showed moderate staining in late egg chambers and faint staining in early egg chambers (Fig. 7B). In the second reconstitution construct, we introduced two copies of a 22 bp DNA fragment containing the proximal OVO-binding site from the *otu* UAS region to create *otu::lacZ^{Δ3}2*. These two sites restored in vitro OVO binding to 60% of wild type and resulted in more impressive rescue of reporter activity in vivo (Fig. 7C). Females with one copy of *ovo*⁺ showed no reporter activity, but female with two copies of *ovo*⁺ showed moderate staining in the late egg chambers and faint staining in early egg chambers. Females with three copies of *ovo*⁺ showed much more robust staining in mature egg chambers and also showed moderate staining in early egg chambers. In the third reconstitution construct, we introduced four copies of the strong OVO-binding site from the *otu* core promoter (this 23 bp DNA fragment unit does not include the transcription start site) to create *otu::lacZ^{Δ3}3*. The reconstructed upstream region bound OVO twice as well as the wild-type construct, and resulted in multiple shifted bands, owing to the reiterated OVO sites in the upstream region. When introduced into flies, we observed reporter expression in females with two or three copies of *ovo*⁺ (Fig. 7D). Staining was moderate in the advanced egg chambers of females with two copies of *ovo*⁺, but females with three copies showed very strong staining in late stages and moderate staining in early egg chambers. The reintroduced upstream OVO sites only modestly restored function. To assure that this modest activity was due to the introduced OVO sites, and not an unintended reconstruction of flanking non-OVO sites, we created *otu::lacZ^{Δ3}3m*, which differs from the *otu::lacZ^{Δ3}3* construct at a single critical base in each of the four OVO-binding sites (Fig. 7E). DNA fragments bearing these four mutated sites showed feeble OVO binding in vitro, when compared with the otherwise identical construct (Fig. 7D,E). When introduced into flies, the *otu::lacZ^{Δ3}3m* reporter was much less active than the corresponding *otu::lacZ^{Δ3}3* reporter with wild-type binding sites (Fig. 7D,E). Females with one or two copies of *ovo*⁺ showed no detectable X-Gal staining and, even in the presence of three copies of *ovo*⁺, showed only moderate staining in late stage egg chambers and faint staining in early egg chambers (Fig. 7E). Thus, weaker OVO binding to an upstream region at *otu* is also functional, but appears to be less important than the strong promoter proximal binding site.

DISCUSSION

OVO directly regulates *otu*

Dominant genetic interactions and bypass suppression suggest that *ovo* and *otu* operate in the same genetic pathway (Pauli et

al., 1993; Oliver and Pauli, 1998; Hinson et al., 1999). However, reports on whether *ovo* is required for *otu* transcription have been neither consistent nor conclusive.

First, several reports indicate that *ovo* is not absolutely required for *otu* expression, but that *ovo* might well be required for high level expression. It has been suggested that somatic sex determination signals control *otu* transcription, leaving little role for other regulatory inputs (Nagoshi et al., 1995; Hinson and Nagoshi, 1999). Rodesch et al. (Rodesch et al., 1995) reported *otu::lacZ* expression in female germ cells in the absence of *ovo*. However, they did not measure the relative expression of this reporter in *ovo*⁻ versus *ovo*⁺. Additionally, because nearly all *ovo*⁻ germ cells in XX females die, one can not be sure if the rare surviving *ovo*⁻ female germ cells express *otu* at the same level as in those that succumbed.

Five studies suggest that high *otu* promoter activity requires *ovo* in at least some stages of germline development (Hager and Cline, 1997; Lü et al., 1998; Hinson et al., 1999; Andrews et al., 2000b; this study). Two of these reports examined the expression of *otu* reporters in an *ovo*⁻ background in males (Hager and Cline, 1997; Lü et al., 1998). Because XX germ cells require *ovo* for viability, examining the effect of *ovo* on *otu* expression in the XY male germline overcomes the inherent problem of assaying promoter activity in dead cells. From a practical point of view, even though *ovo*, *otu* and the *otu* target, *Sxl*, have no clear role in male germline development, at least some of the regulation that occurs in the female germline can also be seen in male germ cells (Hager and Cline, 1997; Lü et al., 1998). In fully viable *ovo*⁻ male germ cells, only feeble *otu* promoter activity was detected, while activity was robust in *ovo*⁺ males (Hager and Cline, 1997; Lü et al., 1998). Further, supernumerary copies of *ovo*⁺ increase *otu* reporter activity in males (Lü et al., 1998). It is also clear that *otu* expression is altered in the ovaries of females bearing combinations of *ovo* alleles that result in defective oogenesis, but not obligatory germ cell lethality. Importantly, the more extreme the *ovo* allele, the stronger the reduction in *otu::lacZ* activity (Hinson et al., 1999; Andrews et al., 2000b).

Even assuming that *ovo* regulates *otu* transcription, there are differences on when *ovo* regulation is relevant. It has been reported that *ovo* mutations only affected *otu* expression in differentiating egg chambers (Hinson et al., 1999). This idea was supported by the finding that *ovo* reporters are expressed in only a subset of the larval gonadal cells that express *otu* reporters (Hinson and Nagoshi, 1999). A different conclusion was reached in other studies. In those studies, *otu* reporters were downregulated in all germ cell stages in the adult ovary when the dose of *ovo*⁺ was lowered (Lü et al., 1998), or when *ovo^D* alleles were employed (Andrews et al., 2000b). The later results suggest that *ovo* functions to control *otu* expression in non-differentiating cells as well as cells undergoing oogenic differentiation.

In this study, we provide conclusive evidence that *Drosophila* OVO, a developmentally regulated C₂H₂-zinc-finger transcription factor, directly regulates the *otu* promoter. We showed that increased *ovo*⁺ dose results in increased *ovo* mRNA and genetic activity. This important control means that an increased *ovo*⁺ copy number translates into increased functional OVO protein. In those flies with increased OVO activity, endogenous *otu* transcripts were present in greater quantity. We also show that transgenes driven by the *otu*

promoter respond positively to increased OVO activity. This response is not limited to late stages. We showed that *otu* reporters respond to increased OVO activity in larval gonads and in the stem cells and cystocytes of the adult ovary. We also showed that cells expressing *otu* reporters also express *ovo* reporters, suggesting that OVO is at the scene of *otu* promoter activity. These data suggest that OVO controls *otu* expression in early stages of oogenesis.

More importantly, we made an extensive set of transgenes with deleted and reconstituted OVO-binding sites, which we tested in females with differing doses of *ovo*⁺. Removal of OVO-binding sites reduces or eliminates the response to *ovo*⁺ activity in *trans*, while reconstituting OVO-binding sites conferred activity. These data in conjunction with previous genetic and molecular studies indicate that OVO protein directly regulates *otu* transcription.

OVO at the core promoter

Surprisingly, OVO functions very close to the transcription start site of *otu*. OVO footprints within 20 bp of the transcription start sites of all but one of the reporter genes responding to *ovo* dose in *trans*. Indeed, in the case of the *ovo-B* promoter, the transcription start site is in the middle of the region protected by OVO. It is a reasonable assumption that RNA Polymerase II and basal transcription complex components also bind this region (see reviews by Burley and Roeder, 1996; Verrijzer and Tjian, 1996). For example, the TFIID complex protects about 60 bp centered on the core promoter. Certainly, RNA Polymerase II must contact the +1 position in the *ovo-B* promoter that is covered by OVO protein *in vitro*.

A standard model for transcriptional regulation holds that the binding of regulatory factors at control regions modulates the transcriptional activity of a variety of core promoters. In this model, core promoters (where the start site is +1 and the core promoter is from -35 to +35) can have different basal strengths, but they have little regulatory information. While in many cases different core promoters respond similarly to a given enhancer, there is some evidence supporting the idea that core promoters can bear important regulatory information. Our data suggest that the *ovo-B* and *otu* core promoters have a regulatory function. We explore several possible mechanistic explanations for the promoter proximal binding of OVO to these core promoters.

Binding of a regulatory protein to the transcription start site is unusual. There are only a few core-promoter binding proteins, such as AEF1 and YY1, that function in tissue or promoter-specific transcriptional control (Ren and Maniatis, 1998; Shi et al., 1997). Binding a short distance away from the transcription start is more common. Start sites are not often mapped to the base. Thus, a trivial explanation of the effect of promoter proximal binding of OVO is that it binds near the start sites, but not at them. This is unlikely. For example, two groups (Garfinkel et al., 1994; Mével-Ninio et al., 1995) have mapped the *ovo-B* transcription start site to the same location. Full-length cDNAs, showing evidence of 5' caps, also end at this site (Mével-Ninio et al., 1995). The sequenced RACE product from the *otu::lacZ^{svb}* transgene ends precisely at the same site (this study). Similarly, the *otu* transcription start sites have been mapped by primer extension (Comer et al., 1992) and by RACE (this study). The *otu* start sites in reporter genes are

within 20 bp upstream of the OVO footprints, well within the region expected to bind basal factors. Thus, OVO and basal transcription factors occupy the same region of the *otu* core promoter, concurrently or in series.

The concurrent occupancy model for OVO function at the core promoter places OVO in the basal transcriptional apparatus. Core promoters typically have binding sites for basal factors at characteristic locations (Buratowski 1997; Burke et al., 1998; Smale et al., 1998). The best-studied site is the TATA element at about -30 to -25, but about half of *Drosophila* genes are TATA-less (Arkhipova 1995). In addition, Initiator elements (Inr) at the transcription start site, and downstream promoter elements (DPE) at about +28 to +34 have been described. The proteins that bind core promoter sites are components of the enormous pre-initiation complex, TFIID, which protects the entire 60 bp core promoter region (see reviews by Burley and Roeder, 1996; Verrijzer and Tjian, 1996). The combinatorial binding of TFIID components to characteristically spaced sequence elements provides enhanced specificity and binding strength. OVO could function as a tissue-specific core element to augment TFIID binding, but this seems unlikely for three reasons. First, the OVO-binding site is slightly downstream of the *otu* start site, but overlaps the *ovo-B* initiation site. We would expect a more constrained position relative to the start site. Second, the promoter proximal OVO binding sites at *otu* and *ovo-B* are in opposite orientation. Transcription is certainly directional. If OVO serves to orient the complex at the transcription start site in a manner analogous to TATA, Inr and DPE elements, then directionality would be expected. To account for function in each orientation, OVO would need a flexible domain between the DNA binding and complex contact domains, or a highly symmetrical structure outside the DNA-binding domain. Third, we have tested for dose-dependent genetic interactions between *ovo^D* and mutations in the *Drosophila* TBP associated factors (TAFs) that are components of TFIID (Wassarman et al., 2000). Mutations in any of several TAFs fail to interact with *ovo^D* (B. O., unpublished). This is a circumstantial argument against an intimate relationship between OVO and TFIID.

If OVO and basal factors occupy the *otu* core promoter serially, orientation and spacing issues are less important. OVO binding might alter the structure of the core promoter to make it more accessible to transcription initiation complexes. There is precedent for preconditioning a core promoter. For example, a bent configuration can enhance the binding of TBP to the TATA element (Parvin et al., 1995). Similarly, RNA Polymerase II can initiate from a melted or negatively supercoiled core promoter in the absence of the normal stable of transcription factors (Parvin and Sharp, 1993; Usheva and Shenk, 1994; LeBlanc et al., 2000). Thus, OVO could precondition the core promoter to allow stronger and/or more precise subsequent binding by the transcriptional apparatus, by generating or stabilizing bends or single stranded regions. Indeed, retrotransposon targeting suggests that the *ovo-B* promoter has an unusual structure. The *ovo-B* promoter region, and the OVO-binding sites in particular, are preferred targets for de-novo *gypsy*-transposon insertion (Mével-Ninio et al., 1989; Dej et al., 1998). Transposable element targeting is believed to be sensitive to chromatin structure in many systems (reviewed by Sandmeyer et al., 1990). It is thus possible that OVO binding makes the chromatin especially available for

gypsy insertion. Such accessibility could also promote the entry of transcriptional machinery. Finally, the presence of bound OVO might even circumvent the need for TFIID. The YY-1 protein, also a C₂H₂-zinc-finger protein that binds core promoters (Shi et al., 1997), binds double-stranded DNA and a single-stranded bubble in the direction of transcription (Houbaviy et al., 1996; Usheva and Shenk, 1996). YY-1 binding and RNA Polymerase II, but not TFIID, are sufficient for transcription from those core promoters in vitro (Usheva and Shenk, 1994). In summary, while we do not have a mechanistic understanding of OVO function at the core promoter, it seems likely that OVO and components of the machinery performing the work of transcription bind to the same sequence, but not at the same time.

Core promoter diversity and enhancer/promoter compatibility

Classical assays for *cis*-regulatory sites involve the introduction of a segment of DNA in front of an exogenous promoter and reporter gene. While in many cases different core promoters respond similarly to a given enhancer, there is some evidence supporting the idea that core promoters can bear important regulatory information. For example, it has been shown that some core promoter/control region pairs are selectively active, suggesting that core promoter diversity may play an important role in gene regulation (Li and Noll, 1994; Schwyter et al., 1995; Merli et al., 1996; Ohtsuki et al., 1998). We do not know what *cis*-sequences mediate this promoter specificity, nor do we know what proteins are involved. However, there is intriguing evidence that different core promoters bind different arrays of proteins. Both promoter sequence analysis and the isolation of TATA binding protein-related factors (TRF) suggest that there is greater core promoter diversity than previously thought (Parvin et al., 1992; Arkhipova, 1995; Buratowski et al., 1997; Hori and Carey, 1998; Dantonel et al., 1999; Rabenstein et al., 1999). Why have divergent core promoters? It could be simply that different core promoter strengths are required for different genes. Strong promoters might be useful for ubiquitous high-level expression, while weaker promoters, more susceptible to the action of other auxiliary transcription factors, might provide better regulatory control during development or in response to stimuli. Alternatively, divergent core promoters might be required to communicate with a subset of nearby regulatory proteins, while ignoring others. This could provide specificity in a crowded nuclear environment.

Interestingly, most of the core promoters we tested in swapping experiments fail to respond to the UAS sequences required for the *otu* expression pattern. Most of these core promoters show no activity in the *otu* context, even if they have typical core promoter sequence (such as an assortment of TATA, Inr and DPE elements). Furthermore, those promoters that are active in the *otu* milieu, show aberrant expression patterns that do not depend on OVO (ubiquitous or in the eye). These data suggest that the core promoters with OVO-binding sites communicate differently with transcription factors bound to the UAS. It will be interesting to determine if promoter/UAS communication might be established by OVO before assembly of the transcription initiation complex. In this scenario, OVO orchestrates basal transcription machinery binding to the core promoter and the positioning of the factors bound upstream.

Thus, even in the presence of identical basal transcriptional complexes at the *Actin 87E* and *ovo-B* core promoters for example, *ovo-B* communicates with the *otu* upstream region, while *Actin 87E* does not. While we do not know if a crowded nuclear environment make core promoter choice important for *otu* expression, it is clearly important at the *ovo* locus. Expression from the *ovo-B* promoter is required for female germline development, while inappropriate expression from the nearby *ovo-A* core promoter is highly detrimental to the female germline (Andrews et al., 2000b). While the details remain to be worked out, there is evidence that *ovo* is autoregulatory (Oliver et al., 1994; Lü et al., 1998; Andrews et al., 2000b), raising the possibility that OVO-B and OVO-A proteins act in *ovo-B* versus *ovo-A* promoter selection.

OVO at the upstream sites

OVO also binds more weakly to sites about 200 bp upstream of the *otu* transcription start site, but these sites appear to have limited function. Certainly, the upstream OVO-binding sites are insufficient to drive correct expression from a host of wild-type core promoters that lack promoter proximal OVO sites. Additionally, while the upstream region is important for *otu* expression, much of this activity seems to be due to the binding of factors other than OVO. Point mutations between the two upstream OVO-binding sites have no effect on OVO binding in vitro, but eliminate expression in vivo.

The best data supporting a role for upstream OVO-binding sites comes from two experiments. First, when we deleted the major *otu* transcription start site and associated OVO-binding site, there was residual OVO responsiveness. As the upstream sites remain, this implies a function. Alternatively, it is also true that the minor *otu* promoter has a reasonable-looking OVO-binding sequence that might bind OVO in vivo, even though we were unable to detect binding in vitro. Second, there was restoration of expression of an upstream deletion construct by adding OVO-binding sites. However, the rescue of transcriptional activity was moderate. It remains to be seen if there is an important role for upstream OVO binding, perhaps in promoting homophilic interaction with the core promoter.

More targets

OVO is likely to act to control the expression of more than one gene. Distinct phenotypes associated with *ovo* mutations can be suppressed by different genes, indicating that *ovo* acts at a branch in the germline sex determination hierarchy (Oliver et al., 1993; Oliver and Pauli, 1998). Similarly, the partial suppression of *ovo* by constitutive *otu* expression suggests that OVO targets more than *otu* expression in the germline (Hinson et al., 1999). Additionally, OVO protein is required in the epidermis, while *otu* is not required or expressed in this tissue (Payre et al., 1999). Thus, there are likely to be OVO target genes expressed in the epidermis. We are developing a gonad microarray (Andrews et al., 2000a) in part to determine what changes in gene expression profiles occur in germ cells bearing loss-of-function or gain-of-function *ovo* alleles. Additionally with the availability of somatic cDNA clones for microarray manufacture (Rubin et al., 2000), we can begin to look for OVO target genes in the soma as well. Finally, as the OVO-binding site consensus is well established (Lü et al., 1998; Lee and Garfinkel, 2000), and as the *Drosophila* genome is sequenced (Adams et al., 2000), we should be able to readily

identify genes with OVO-binding sites. By careful examination of genes with OVO-binding sites and which respond to OVO in expression profiling experiments, we should be able to home in on additional direct target genes.

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