

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

Jining Lü and Brian Oliver*

Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892-2715, USA

*Author for correspondence (e-mail: oliver@helix.nih.gov)

Accepted 6 February; published on WWW 5 April 2001

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 germ line 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 oto locus is a dose-dependent modifier of ovoD (Pauli et al., 1993). The ovoD alleles inappropriately encode OVO-A isofoms instead of OVO-B isofoms 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 ovoD alleles are likely to downregulate genes normally upregulated by OVO-B (Andrews et al., 2000b). Heterozygosity for ovo greatly exacerbates germline development in ovoD/+ females, while adding additional copies of oto* ameliorates the mutant phenotype (Pauli et al., 1993; Pauli et al., 1995). Thus, high oto* copy number counters the negative effect of ovoD on transcription. Similarly, partial rescue of the ovo phenotype by forcing expression of wild-type OTU protein also indicates that high oto activity counteracts the negative effect of loss of ovo function (Hinson and Nagoshi, 1999). Genetic models where ovo and oto 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 oto has been explored at the molecular level, but there is no consensus on whether ovo regulates oto and if so, how important this regulation might be. The ovo and oto genes are expressed in, and function in, the germline (Oliver et al., 1987; Steinhauser et al., 1989; Comer et al., 1992; Garfinkel et al., 1994; Mével-Ninio et al., 1995). Some evidence supports the idea that ovo regulates oto. Flies mutant for ovo show downregulated expression of oto reporters (Lü et al., 1998; Hinson et al., 1999; Andrews et al., 2000b). Importantly, OVO-binding sites are present at the oto locus within a segment of DNA sufficient to drive reporter genes in the oto pattern, raising the possibility that OVO is a direct regulator of oto transcription (Lü et al., 1998). Both of these results are consistent with the hierarchical order ovo→oto.

Other results favor the parallel model. In particular, one study has shown that oto reporters are expressed normally in ovo mutants, suggesting that ovo does not regulate oto (Rodesch et al., 1995). Finally, it is possible that ovo controls only minor aspects of oto function and that other genes in the pathway are the prominent regulators of oto expression. Clearly, somatic signals are and stand still regulate oto 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 oto expression (Nagoshi et al., 1995; Hinson and Nagoshi, 1999). This is supported by evidence that ovo mutations moderately influence only late expression of oto (in terminal stages of oogenesis), and have no effect on the crucial early stages of oto expression (Hinson et al., 1999). In contrast, it has also been shown that oto 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 oto expression (Andrews et al., 2000b). Thus, the question of whether ovo regulates oto expression remains unanswered.

Using an exhaustive set of transgenic animals, we show that OVO regulates the activity of the oto promoter, by binding to an OVO site surprisingly near the transcription start site. These data unambiguously indicate that OVO is direct regulator of oto 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; Diefenback 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 phosphoimager 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, ligylized, 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 electrophoreses. 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’-CGGGGCTTCTTGCATATTA-3’). Nested primers were used for the PCR reactions. The first and second gene-specific PCR primers were within the lacZ-encoding region (5’-GGGATGTGCTCAAGCCGATT-3’ and 5’-CCCCACTCACGC- GTTGAAA-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’-AGGCTCCCAGGCCGAACGA-3’).

**OVO binding**

We performed gel shifts and in-gel DNasel 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 using Trizol (GIBCO/BRL). 20 μg of total RNA was loaded in each lane, subjected to formaldehyde agarose gel electrophoreses, and transferred to Nytran N+ membranes (Amersham). Probes from the ovo (2201-2385 bp; numbering convention of Mével-Ninio et al., 1991), oto (1325-1525 bp; numbering convention of Steinhauser 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 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 where 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; ACNGTACA, 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>
<thead>
<tr>
<th>Alleles</th>
<th>Notes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ovo⁺</td>
<td>Germline amorph. Insertion of HMS beagle (−4.0−4.6 kb) in ovo⁺.‡</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>Wild-type allele. Genomic ovo DNA +1 to about +10.5 kb, in pCaSpeR2.‡</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>Genomic ovo DNA 0 to 1082 bp (convention of Mével Ninio et al., 1991). Linker at insertion site with initiation codon.‡</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>Genomic oto DNA −423 to +119 bp (this, and all other oto positions, are according to the convention of Comer et al., 1992) in pCaSpeR-AUG-fggl. Note that this is a new construct.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −242 bp, concomitantly introducing a SacI site by an A to C substitution at −302 bp and an A to G substitution at −303 bp, and a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with GCGATA to AAACC substitution at −260 to −255 bp and CCATAT to AAACC substitution at −289 to −279 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with one copy of ovo DNA +606 to +629 bp (from between the ovo-A and ovo-B promoters, numbering convention of Mével Ninio et al., 1991) inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with two copies of oto DNA −263 to −242 bp between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with four copies of oto DNA +12 to +34 bp between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with four copies of oto DNA +12 +34 bp with G to C substitution at +24 inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −3 to +42 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with TCGCG to GTAAC substitution at −31 to −27 bp and T to G substitution at −23 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>ovo-B core promoter (−71 to −59 bp, where the ‘+1’ transcription start is at +852 bp in the numbering convention of Mével-Ninio et al., 1991) in pCaSpeR-AUG-fggl.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>The oto UAS region (−423 to −94 bp) and ovo-A 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).</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with two copies of oto DNA −263 to −242 bp between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with GCGATA to AAACC substitution at −260 to −255 bp and CCATAT to AAACC substitution at −289 to −279 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with one copy of ovo DNA +606 to +629 bp (from between the ovo-A and ovo-B promoters, numbering convention of Mével Ninio et al., 1991) inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −242 bp, concomitantly introducing a SacI site by an A to C substitution at −302 bp and an A to G substitution at −303 bp, and a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with GCGATA to AAACC substitution at −260 to −255 bp and CCATAT to AAACC substitution at −289 to −279 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with one copy of ovo DNA +606 to +629 bp (from between the ovo-A and ovo-B promoters, numbering convention of Mével Ninio et al., 1991) inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with GCGATA to AAACC substitution at −260 to −255 bp and CCATAT to AAACC substitution at −289 to −279 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with one copy of ovo DNA +606 to +629 bp (from between the ovo-A and ovo-B promoters, numbering convention of Mével Ninio et al., 1991) inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with GCGATA to AAACC substitution at −260 to −255 bp and CCATAT to AAACC substitution at −289 to −279 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with one copy of ovo DNA +606 to +629 bp (from between the ovo-A and ovo-B promoters, numbering convention of Mével Ninio et al., 1991) inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −294 to −276 bp, concomitantly introducing a SpeI site by an A to C substitution at −311 bp and an A to T substitution at −312 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with GCGATA to AAACC substitution at −260 to −255 bp and CCATAT to AAACC substitution at −289 to −279 bp.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with one copy of ovo DNA +606 to +629 bp (from between the ovo-A and ovo-B promoters, numbering convention of Mével Ninio et al., 1991) inserted between the SacI and SpeI sites.</td>
</tr>
<tr>
<td>ovo⁺</td>
<td>potu::lacZ with deletion of −261 to −242 bp.</td>
</tr>
</tbody>
</table>

†http://flybase.bio.indiana.edu

*This study, unless indicated.
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 (−243 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). \(\beta\)-galactosidase activity (X-Gal staining) was clearly observed in the germarium, where germ line 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 stems 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 DNase1 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 DNase1 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\(^{wm}\), 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 \textit{otu::lacZ} \textsubscript{pm} deletion leaves only the weak \textit{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 \textit{otu::lacZ} \textsubscript{pmg} (Fig. 2C). This construct was modified from \textit{otu::lacZ} \textsubscript{pm} 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 \textit{otu} core promoter. This reconstructed \textit{otu::lacZ} \textsubscript{pmg} 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 \textit{ovo} locus. Transcripts driven from the tightly linked \textit{ovo-B} and \textit{ovo-A} core promoters are differentially expressed and encode distinct OVO proteins with opposite activities (Andrews et al., 2000b). The \textit{ovo-B} core promoter has an OVO-binding site. The \textit{ovo-A} core promoter does not (Lü et al., 1998). We replaced the entire

\textbf{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) \textit{otu::lacZ}: Wild-type core promoter DNA fragment from \textit{otu::lacZ}. (B) \textit{otu::lacZ} \textsubscript{pm}: Core promoter DNA fragment with the OVO-binding site deleted from \textit{otu::lacZ}. (C) \textit{otu::lacZ} \textsubscript{pmg}: Core promoter DNA fragment from \textit{otu::lacZ} \textsubscript{pm} with a reconstituted core promoter OVO binding site. (D) \textit{otu::lacZ} \textsubscript{swb}: \textit{ovo-B} core promoter DNA fragment from \textit{otu::lacZ} \textsubscript{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<sup>swb</sup>*. (C) *otu::lacZ<sup>swm</sup>*. 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<sup>+</sup>* 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<sup>swb</sup>* 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<sup>swa</sup>* (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<sup>+</sup>* in *trans* (Fig. 3A). In contrast, the *otu::lacZ<sup>swm</sup>* 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<sup>swm</sup>* and one or two copies of *ovo<sup>+</sup>* showed no detectable staining in the early egg chambers and only feeble staining in late chambers. There were residual *ovo* responsiveness, perhaps owing to upstream OVO-binding sites, as females with three copies of *ovo<sup>+</sup>* exhibited strong staining activity. Females bearing the crucial reconstituted core promoter of *otu::lacZ<sup>swm</sup>* showed dramatically increased staining in females bearing one or two copies of *ovo<sup>+</sup>* (Fig. 3C). Indeed, females bearing *otu::lacZ<sup>swm</sup>* show a graded reporter response to *ovo<sup>+</sup>* 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 starts sites. We were therefore quite interested in determining which site in the *otu::lacZ<sup>swm</sup>* 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<sup>swm</sup>* 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<sup>swm</sup>* is also promoter proximal. Thus, as with the wild-type *otu* promoter, the OVO-binding site in the transcriptional active *otu::lacZ<sup>swm</sup>* 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 (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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 (Andrews et al., 2000). 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 concentra...
1678  J. Lü and B. Oliver

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::lacZswa. (C) otu::lacZswb. (D) otu::lacZswbi (same as C, except that the ovo-B Inr was replaced with an otu Inr). (E) otu::lacZswba (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 (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 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::lacZswb, these data suggest that OVO binds and functions usually close to the transcription start site of otu::lacZswb. Indeed, the OVO footprint in otu::lacZswb 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::lacZswbi reporter (Fig.
OVO and enhancer/promoter compatibility

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 TA TA 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\textsuperscript{lam}, was expressed throughout the ovary in a non-ovo dependent fashion (Fig. 5L) and thus clearly functions, but does not respond to ovo\textsuperscript{+} dose in trans. The Actin 87E reporter, otu::lacZ\textsuperscript{act}, showed weak expression visible through the adult eye, but also failed to respond to ovo\textsuperscript{+} 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\textsuperscript{lam} 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\textsuperscript{A} 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\textsuperscript{A} showed strong staining activity and the reporter responded positively to increased ovo\textsuperscript{+} dose in vivo. Deletion of a 20 bp DNA fragment that included the second upstream sequence decreased OVO-binding affinity in the upstream region to 64% of the wild-type fragment (Fig. 6A,C).

**Fig. 6.** Deletions of upstream OVO-binding sites. (A) otu::lacZ. (B) otu::lacZ\textsuperscript{A}. (C) otu::lacZ\textsuperscript{A} 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\textsuperscript{+} 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\(\Delta^2\) 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\(\Delta^3\) reporter activity in the ovaries of females with one, two or three copies of ovo+. 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\(\Delta^3\) 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...
to restore at least some \(otu::lacZ3\) 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 \textit{trans}. To test this, we introduced a 24 bp fragment containing a strong, non-core promoter, OVO-binding site from the \(ovo\) locus into \(otu::lacZ3\) to create \(otu::lacZ3\). 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 \(otu::lacZ6\). 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 \(otu::lacZ3\). The reconstituted 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 \(otu::lacZ5\), which differs from the \(otu::lacZ3\) 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 \(otu::lacZ3\) reporter was much less active than the corresponding \(otu::lacZ5\) 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., 2000; 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., 2000).

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 \(ovo^+\) alleles were employed (Andrews et al., 2000). 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 \textit{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 TFIIID 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 to 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.

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 (Buratowskii 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, TFIIID, which protects the entire 60 bp core promoter region (see reviews by Burley and Roeder, 1996; Verrijzer and Tjian, 1996). The combinatorial binding of TFIIID components to characteristically spaced sequence elements provides enhanced specificity and binding strength. OVO could function as a tissue-specific core element to augment TFIIID 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 ovoD and mutations in the Drosophila TBP associated factors (TAFs) that are components of TFIIID (Wassarman et al., 2000). Mutations in any of several TAFs fail to interact with ovoD (B. O., unpublished). This is a circumstantial argument against an intimate relationship between OVO and TFIIID.

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; Déj 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 factors 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 otu 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 provide some reference materials in this paper.

We thank Justen Andrews, Virginia Boulais and George Poy for technical assistance with transgenics and sequencing; and Justen Andrews, David Clark, Ann Dean, Judy Kassis and Alan Kimmel for comments on the manuscript.

REFERENCES


Oliver, B., Kim, Y. J. and Baker, B. S. (1993). Sex-lethal, master and slave:


