Regulatory and functional interactions between the somatic sex regulatory gene *transformer* and the germline genes *ovo* and *ovarian tumor*

Shannon Hinson and Rod N. Nagoshi

Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242-1234, USA

Author for correspondence (e-mail: rodney-nagoshi@uiowa.edu)

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SUMMARY

In *Drosophila*, compatibility between the sexually differentiated state of the soma and the sex chromosome constitution of the germline is required for normal gametogenesis. In this study, we defined important aspects of the soma-germline interactions controlling early oogenesis. In particular, the sex-specific germline activity of the *ovarian tumor* promoter was found to be dependent upon somatic factors controlled by the somatic sex differentiation gene *transformer*. This regulation defines whether there is sufficient *ovarian tumor* expression in adult XX germ cells to support oogenesis. In addition, the *ovarian tumor* function required for female germline differentiation is dependent on the activity of another germline gene, *ovo*, whose regulation is *transformer*-independent. These and other data indicate that *ovarian tumor* plays a central role in coordinating regulatory inputs from the soma (as regulated by *transformer*) with those from the germline (involving *ovo*). We also demonstrate that *transformer*-dependent interactions influence whether XX germ cells require *ovarian tumor* or *ovo* functions to undergo early gametogenic differentiation. These results are incorporated into a model hypothesizing that the functions of *ovarian tumor* and *ovo* are dependent on an early sex determination decision in the XX germline that is at least partially controlled by somatic *transformer* activity.

Key words: Oogenesis, Germline, *ovarian tumor*, *transformer*, *ovo*, *Drosophila*

INTRODUCTION

In *Drosophila melanogaster*, the sexual differentiation of the germline requires a complex interplay between cell autonomous factors controlled by the X:A ratio of the germ cells and sex-specific somatic functions (Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1994a,b; Nagoshi et al., 1995). For example, certain allele combinations of *transformer*, *transformer-2* and *doublesex* can cause chromosomally female (XX) flies to develop with most of their somatic tissues having a male identity, i.e., 'XX pseudomales' (Sturtevant, 1945; Watanabe, 1975; Nöthiger et al., 1980). In these flies, oogenesis is aborted and there is even occasionally what appears to be early spermatogenic development (Sturtevant, 1945; Seidel, 1963; Watanabe, 1975; Nöthiger et al., 1980, 1987, 1989). Since the germline expressions of these sex regulatory genes are not required for early stages of gametogenesis (Marsh and Wieschaus, 1978; Schüpbach, 1982), the aberrant germline phenotypes must result from the male transformation of the soma.

The interpretation of these studies is complicated by the difficulty in diagnosing the differentiated state of abnormal germ cells when using only morphological criteria. Therefore, we took advantage of molecular probes that facilitated the identification of sex- and stage-specific germline structures. These characteristics are described as follows. In the most apical portion of the adult germarium (region 1), germline stem cells divide asymmetrically to produce daughter stem cells and cystoblasts. These two cell types are identified by the presence of a globular spectrosome which can be detected with antibodies and reagents specific for spectrin, a product from the *hu-li tai shao* (*hts*) gene (an adducin-like protein), and f-actin (Lin et al., 1994; Rodesch et al., 1997). The cystoblast undergoes a set of four mitotic divisions noted by incomplete cytokinesis, to produce a 16-cell cyst connected by cytoplasmic bridges (ring canals) through which passes a branched, proteinaceous structure called the fusome (Lin et al., 1994; Lin and Spradling, 1995). The fusome results from the elongation and branching of the spectrosome during the cystocyte divisions, a transition associated with decreased levels of fusome f-actin (Rodesch et al., 1997). Shortly after the completion of the cystocyte divisions, the somatic follicle cells begin to associate with the 16-cell syncytium. This marks the beginning of germarial region 2, during which the fusome disappears and f-actin and another *hts* product (HTS-RC) integrates into the ring canals (Lin et al., 1994; Robinson et al., 1994). In region 3, the germline cyst is surrounded by follicle cells to form a stage 1 egg chamber. This period is noted by the localization of a product from the *kelch* gene to the ring canals (Xue and Cooley, 1993; Robinson et al., 1994). These events differ from that seen in spermatogenesis in two aspects relevant to our study. First, the multibranched spermatogenic fusome contains substantial levels of f-actin and
persists past the 16-cell stage into the meiotic divisions (Hime et al., 1996). Second, male germline ring canals do not incorporate f-actin, HTS-RC or KELCH proteins, indicating a different maturation process (Hime et al., 1996). Therefore, these components of spectrosomes, fusomes and ring canals provide important tools to identify the differentiated state of germ cells under different mutant conditions.

It is not clear which germ line genes are influenced by the proposed somatic interactions. Three possible candidates based on their early and sex-specific roles in female germ line differentiation are ovarian tumor (otu), ovo and Sex- lethal (Sxl). The expression of otu is required in the germ line at several stages, if not continually, in oogenesis (reviewed in King and Storto, 1988). The null mutant phenotype is characterized by the absence of egg chambers in an otherwise normal ovary, denoted as the quiescent phenotype (King and Riley, 1982), although substantial numbers of germ cells are still present in the germarium (Rodesch et al., 1995). Null and severe loss-of-function mutations can also produce ‘ovarian tumors,’ a phenotype characterized by egg chambers containing hundreds of seemingly undifferentiated germ cells. Both the quiescent and tumorous cells are aborted at early oogenic stages, during the cystocyte divisions prior to cyst formation (Rodesch et al., 1997). Mutations in otu have no significant effect on spermatogenesis, although some aberrations in male courtship behavior have been reported (Tirronen et al., 1993).

The ovo gene has been implicated in regulating sex determination and dosage compensation in the germ line (Oliver et al., 1990, 1993, 1994; Wei et al., 1994). This is based primarily on observations that ovo null XX germ cells are typically not found in the adult ovary, presumably because of reduced cell viability (Oliver et al., 1987, 1990; Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). In addition, certain ovo allele combinations produce tumorous germ cells which morphologically resemble primary spermatocytes (Oliver et al., 1987, 1990). These phenotypes make ovo a candidate target for a somatic signal regulating early oogenesis, although the expression of ovo in adult germ cells does not appear to be responsive to somatic influences (Oliver et al., 1994). Interestingly, there is recent evidence that ovo might directly regulate otu (Hager and Cline, 1997; Lu et al., 1997). The OVO protein can bind to sites in the otu promoter, which displays sensitivity to changes in the dosage of ovo* function. It is not known when this putative regulation of otu occurs nor what role it plays in oogenesis.

Besides its essential role in the female germ line, the Sxl gene controls sex determination and dosage compensation in somatic cells (reviewed in Cline and Meyer, 1996). Female-sterile alleles represent hypomorphic mutations that specifically disrupt the germ line Sxl function but allow normal somatic development (Perrimon et al., 1986; Salz et al., 1987). These result in ovarian tumors superficially similar to those produced by otu and ovo (reviewed in Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992). Pole cell transplantation studies have led to the suggestion that the soma might influence aspects of germ line sex determination through the regulation of Sxl (Steinmann-Zwicky, 1994b).

With respect to interactions with the germ line, transformer (tra) is the most extensively studied of the somatic sex regulatory genes. The masculinization of XX soma due to loss-of-function tra mutations cause germ cell aberrations during first instar larval stages (Seidel, 1963; Steinmann-Zwicky, 1994a) and misregulated sex-specific germ line gene expression in the embryo (Staab et al., 1996). Furthermore, when XY soma is feminized by ectopic tra expression (to form ‘pseudofemales,’ McKeown et al., 1988), the somatic components of the ovaries are sufficiently ‘female’ that they can support the maturation of transplanted XX germ cells (Steinmann-Zwicky, 1994a). The pseudofemale soma also appears to partially feminize the XY germ line, as these cells now required the normally female-specific otu function for optimal proliferation (Nagoshi et al., 1995). These observations indicate that tra controls a substantial portion of the somatic-germline interactions affecting early gametogenic differentiation.

In this paper, we tested for regulatory and functional interactions between the somatically required tra gene and the germ line functions of otu and ovo. We found that tra function determines whether sufficient otu is expressed in adult XX germ cells to support oogenesis. This regulation occurs at the level of the otu promoter, and imposes stage- and sex-specific expression of oto that differs significantly from the pattern of ovo promoter activity. We show that oto has two functions, one regulating oogenic differentiation that is dependent on ovo, and one influencing germ cell proliferation that appears to be ovo-independent. These and other observations indicate oto plays a critical role in linking the somatic sex regulatory pathway with the germ line genes controlling early oogenesis. Finally, we present evidence that the germ line requirement for ovo and oto are influenced by tra-dependent somatic interactions. These data give rise to a new hypothesis for the regulation of early oogenesis that differs significantly from current models.

MATERIALS AND METHODS

Fly strains and crosses
Flies were raised on standard cornmeal, molasses, yeast, agar media containing propionic acid as a mold inhibitor and supplemented with live yeast. Unless otherwise noted, alleles and chromosomes used are described in Lindsay and Zimm (1992). The hs-oto strain was obtained from the laboratory of P. Geyer. The construct consists of a 4 kb HpaI oto genomic fragment starting 64 bp downstream of the transcription start site and ending 139 bp downstream of the translation stop codon, under the regulation of the Drosophila hsp70 promoter and the polyadenylation sequences of the α-tubulin gene. The transgene is marked with ry and is inserted into an unmarked second chromosome. The otu-lacZ strain contains the w+ marked transgene inserted on the second chromosome (Rodesch et al., 1995). To construct pOtul04, a 0.4 kb HpaI-PstI fragment containing the oto polyadenylation site was inserted into the equivalent sites in the w+ Drosophila transformation vector Casper-3 (Pirrotta, 1988), to form Casper-3-otu. This was followed by the insertion of a 736 bp EcoRV (artificial site)-HpaI oto promoter fragment, containing 672 bp of sequence upstream of the primary transcription start site, into the HpaI site of the Casper-3-otto subclone. Csp-3-8 represents the orientation in which the oto promoter and polyadenylation sequences are now separated by a single HpaI site. Finally, a cDNA specific for the 104 kDa oto isoform was inserted in the appropriate orientation into the Csp-3-8 HpaI site. Germine transformation of pOtul04 gene was carried out by standard methods (Rubin and Spradling, 1982).

Construction of XX pseudomales
XX flies that carry mutations in transformer develop as somatic males.
of the germline-specific *vasa* promoter and was a gift from F. Laski (Rodesch et al., 1995; Heller and Steinmann-Zwicky, 1998). *ovo-lacZ* places β-galactosidase under the control of the *ovo* promoter (Mével-Ninio et al., 1995).

Gonads were dissected in PBS, then incubated in 50% fixative: 50% heptane in a covered depression slide with agitation for 3 minutes. The tissue was rinsed 3× in PBS + 0.1% Triton X-100. The tissue was incubated in staining solution overnight at room temperature in the dark. After staining, the preparation was washed 5× for 20 minutes with PBS. The tissue was mounted in 50% glycerol in PBS. Stock solutions: solution A, 6.75 g/l NaCl, 6.63 g/l KCl, 0.66 g/l MgSO$_4$·7H$_2$O, 0.54 g/l MgCl$_2$·6H$_2$O; 0.33 g/l CaCl$_2$·2H$_2$O; solution B, 1.4 g/l Na$_2$HPO$_4$, 0.1 g/l KH$_2$PO$_4$, taken to pH 7 with NaOH; and solution C, the same as solution A but with 3.7% formaldehyde. Fixative: 9 parts solution C and 10 parts solution B. Staining solution: 0.75 ml's of a mixture of 9 parts solution A and 10 parts solution B, 0.1 ml 50 mM potassium ferricyanate, 0.1 ml 50 mM potassium ferrocyanate, 50 μl 100 μg/ml 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside (X-gal) in N, M'-dimethylformamide, to a total volume of 1.0 ml in water.

**Microscopy**

Confocal images were obtained on a Nikon Optiphot using a Bio-Rad MRC 1024 confocal laser apparatus. Sections were manipulated using Bio-Rad Lasersharp image analysis software and transferred to Adobe Photoshop for figures. Other microscopy was performed on an Olympus Vanox AHHFT3 microscope using an Optronics LX450A camera for image capture.

**RESULTS**

**Fusome and ring canal phenotypes of XX pseudomale germ cells**

XX pseudomales produced by *tra* mutations contain three types of gonads that differ in the viability and sexual phenotype of their germ cells (Brown and King, 1961; Seidel et al., 1963; Nöthiger et al., 1989). We repeated this analysis using molecular probes that provided more definitive criteria for categorizing the germline phenotypes. Gonads from ‘normal’ XX pseudomales (i.e., carrying wild-type alleles of *ovo*, *otu* and *Sxl*) were simultaneously labeled with antibodies for spectrin and HTS-RC to identify spectrosomes, fusomes and oogenic ring canals. Three categories of gonads were defined.

The most common class were the ‘atrophic’ gonads, found in about half of the pseudomales examined (39/80; Fig. 1A). These contained no differentiating germ cells, as spectrosomes, fusomes, or ring canals were not observed. Confirmation of this interpretation came from a separate experiment in which gonads of this type were double-labeled with antibodies for the germline-specific VASA protein (Hay et al., 1988) and spectrin. In every case examined (n=10), gonads lacking cells with spectrosomes and fusomes also failed to label with VASA (Fig. 2A).

The next two categories were composed of gonads with germ cells initiating gametogenic differentiation, as demonstrated by the presence of spectrosomes and fusomes (Fig. 2B). These were subdivided depending on whether they expressed the HTS-RC protein, an oogenic characteristic. Gonads without HTS-RC-positive cells were designated as ‘non-oogenic’ and were found in 19% (15/80) of pseudomales (Fig. 1A). These could derive from aborted oogenesis before HTS-RC expression or if the germ cells underwent male
Fig. 1. Distribution of XX pseudomale gonad types produced by mutations in ovo, otu and Sxl. tra mutant XX gonads were labeled with antibodies specific for VASA and HTS-RC. Atrophic gonads are VASA-negative. Non-oogenic gonads have VASA-positive germ cells that do not express HTS-RC in their ring canals. Oogenic gonads are VASA-positive and HTS-RC-positive. Number at the top of each column indicates the number of gonads examined for that genotype. All genotypes are XX; tra1 kar2 ry3 red/tra1. Other mutations in each genotype: (A) y w ovoD1r5/FM6. (B) y w ovoD1r5/y w ovoD1r5. (C) otoPs1 v f/otoPs1 v f. (D) y SxdP3/ y SxdP3. (E) y w ovoD1r5/FM6; hs-otuCyO. (F) y w ovoD1r5/y w ovoD1r5; hs-otuCyO. (G) y SxdP3/ y SxdP3; hs-otuCyO.

**Differentiation** It appears that the latter make up the majority of non-oogenic gonads in normal pseudomales, as germ cells of this class were typically connected by multibranched fusomes containing f-actin, a spermatogenic characteristic (Fig. 2C). The remaining ‘oogenic’ gonads were found in 33% (26/80) of XX pseudomales. These were characterized by one or more cell clusters expressing HTS-RC (Fig. 2D,E). In these gonads, the ‘feminized’ clusters were surrounded by cells without HTS-RC expression, indicating female germline differentiation was infrequent and sporadic (Fig. 2E).

**ovo, otu and Sxl are only required in XX germ cells undergoing female differentiation**

We next compared the effects of an ovo null mutation, ovoD1r5, on XX germ cells developing in pseudomale testes and female ovaries. In females, ovoD1r5 mutant XX germ cells typically arrest beginning at larval gonial stages (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995, although see Oliver et al., 1990 for evidence of earlier arrest). Occasionally, mutant germ cells survived to the adult stage, where they were sufficiently viable to express the germline-specific VASA protein (Fig. 2F1; Rodesch et al., 1995). However, these cells generally failed to undergo gametic differentiation as seen by the absence of spectrosomes, fusomes or ring canals (Fig. 2F2; R. N. Nagoshi and S. H., unpublished data). We reasoned that, if the requirement for ovo is solely dependent on the X:A ratio, then the phenotype of ovo mutant germ cells in pseudomales should be at least as severe. In this case, the ovoD1r5 mutant XX pseudomale gonads should be either atrophic or contain a few clusters of mostly undifferentiated germ cells. Such a result had previously been reported, though without supporting data (Steinmann-Zwicky, 1989).

Our studies showed a more complicated phenotype. Although there was an increase in the frequency of atrophic gonads (82%) compared to normal pseudomales (48%), we also found many of the non-oogenic type (11/60, Fig. 1B). The non-oogenic gonads contained VASA-positive germ cells (Fig. 2G), with spectrosomes and multibranched fusomes (Fig. 2H.I). This indicates that not only were a substantial fraction of the mutant germ cells viable in adults, but gametogenic differentiation was occurring as well. The frequency of the non-oogenic gonads in ovo mutant pseudomales was essentially unchanged from that observed in normal pseudomales (18% versus 19%). This suggests the observed increase in the atrophic category was due primarily to the loss of the oogenic class (Fig. 1B).

Mutations in otu gave results similar to that described for ovo (Fig. 1C). Again there was a reduction in oogenic gonads occurring concomitantly with a compensating increase in atrophic testes. As with both ovo+ and ovo mutant pseudomales, approximately 20% of gonads were non-oogenic and these frequently contained multibranched fusomes. This suggests that otu and ovo mutations specifically disrupt only those germ cells attempting female differentiation, rather than the indiscriminate elimination of the entire XX germline. This possibility was supported by more detailed analysis of a sampling of the ovo+ non-oogenic pseudomales. In all gonads examined (8/8), multibranched fusomes were present containing f-actin, a characteristic of early spermatogenesis (the same phenotype as that seen in Fig. 2C for non-oogenic ovo+ pseudomales).

A different phenotype was observed in XX pseudomales mutant for the germline function of Sxl. As with ovo and otu, Sxl female-sterile mutations blocked oogenic differentiation in XX pseudomales (Fig. 1D). However, this did not lead to more atrophic gonads. Instead there was an increase in the non-oogenic category such that the frequency of non-atrophic gonads was equal to Sxl+ pseudomales (compare Fig. 1A and D). These observations indicate a fundamental difference in the germline phenotype caused by this Sxl allele compared to ovo and otu mutations, and demonstrate that aborted oogenesis does not inevitably lead to reduced germ cell viability in pseudomales.

**Increased otu expression can feminize XX pseudomale germ cells**

The hs-otu construct places the otu gene under the control of the Drosophila heat-shock hsp70 promoter. This construct can suppress all otu mutations to fertility at room temperature (Rodesch et al., 1995; data not shown). We previously showed that hs-otu could alter the XX pseudomale gonadal phenotype;
however, the nature of this change was not determined (Nagoshi et al., 1995). To examine whether and to what degree oto expression could induce oogenic development in pseudomales, immunohistochemical studies were performed. When continually cultured at 20-25°C, hs-oto pseudomale gonads were as much as two to three times longer than normal (Fig. 3A,B). In addition, 88% of the hs-oto gonads examined (36/41) showed extensive HTS-RC-labeling of ring canals (Figs 1E, 3C). These ‘feminized’ gonads displayed a developmental progression of gametogenic stages, which could be subdivided into three sections corresponding to germarial regions 1-3 in normal oogenesis (I-III, Fig. 3C).

Section I includes the most apical portion of the hs-oto XX pseudomale testis and was found to contain gametogenic stages characteristic of germarial region 1 in ovaries (Fig. 3D). Localized at the distal tip was a group of cells containing spectrosomes, a characteristic of germline stem cells and cystoblasts. Immediately adjacent were clusters of cells connected by branched fusomes containing spectrin.

In section II, the fusomes have disappeared and the differentiation of the ring canals continues with the deposition of HTS-RC and f-actin (Fig. 3E). The localization of these two components to the rings does not occur simultaneously. Clusters of germ cells were detected in which all the ring canals contained HTS-RC, but only a subset were labeled by phalloidin. In comparison, by the end of section II all the ring canals were found to contain both factors. This suggests sequential deposition in which HTS-RC is localized first to the ring canal, followed by f-actin. A similar sequence of events is thought to occur during normal oogenesis in germarial region 2 (Yue and Spradling, 1992).

In section III, the pseudomale germ cells have differentiated to postgerminal stages as defined by the expression of kelch. The KELCH protein is localized to female ring canals after the deposition of HTS-RC and f-actin (Robinson et al., 1994). It first becomes detected in female ring canals in stage 1 egg chambers, but is not seen in all ring canals until stage 4 (Robinson and Cooley, 1997). In hs-oto XX pseudomales, the germ cell clusters in section III contained thick ring canals with virtually all showing KELCH deposition along the inner surface of the f-actin layer (Fig. 3F). In comparison, no KELCH-labeled ring canals were observed in XX pseudomales without hs-oto (n=15), indicating that oogenesis was not only less frequent, but also more limited. Consistent with the more mature ring canal phenotype were the large germ cell nuclei that stained extensively with DAPI, characteristics suggestive of polyploidy (Fig. 3G). Frequently, clusters of large cells were associated with a smaller cell containing at least four ring canals. This phenotype suggests oocyte differentiation and nurse cell polyploidy, consistent with oogenic development to as late as stage 4.

Taken together, these results indicate the masculinizing effect of male soma (or the absence of female soma) on XX germ cells can be partially, but consistently, overridden by the expression of oto from a heterologous promoter. The resulting fusome and ring canal development follows the same sequence of events as occurs in normal oogenesis. Therefore, pseudomale germ cells are competent to both initiate and undergo substantial oogenesis if provided with adequate levels of oto.

ovo and Sxl are required for oto-induced oogenic differentiation in XX pseudomales

We next determined whether the oto-induced feminization of

Fig. 2. Germ cell and gonadal phenotypes of ovo and ovo- XX pseudomales. tro mutant XX pseudomale gonads were examined by immunohistochemical analysis for the presence of germ cells, fusomes, and ring canals. Nuclei were stained by DAPI. (A) Atrophic XX pseudomale testis (germarial region at top) labeled with anti-VASA. Gonads of this class are devoid of VASA-positive germ cells and do not have spectrosomes or fusomes (data not shown). (B) XX pseudomale non-oogenic gonad labeled with anti-spectrin. Spectrosomes (s) are found in stem cells and cystoblasts. Spectrosomes give rise to branched fusomes (f) that connect cystocyte clusters. (C) Typical fusome from non-oogenic XX pseudomale germ cells labeled with phalloidin. Presence of f-actin is characteristic of spermatogenic fusome. (D) HTS-RC-labeled ring canals from oogenic class XX pseudomale gonad (arrow). (E) Oogenic XX pseudomale testis labeled with anti-HTS-RC. Arrow points to one of five germ cell clusters with HTS-RC-positive ring canals. (F1) ovo mutant XX female ovariole labeled with anti-VASA antibodies. Large clusters of VASA-positive germ cells are present in some germaria. (F2) Same ovariole as (F1) labeled with anti-spectrin. Cell periphery is labeled but there are no spectrosomes or fusomes. (G) ovo mutant XX pseudomale testis of the non-oogenic class labeled with anti-VASA antibodies. Note clusters of VASA-positive germ cells in anterior half of testis. These do not express the female-specific HTS-RC protein. (H) Germarium of ovo mutant XX pseudomale testis labeled with anti-spectrin antibodies. Spectrosomes (s) and fusomes (f) are present. (I) A higher magnification of a highly branched fusome from an ovo mutant XX pseudomale gonad labeled with anti-spectrin. All plates except D are confocal images.
the XX pseudomale germline was dependent on ovo and Sxl function. XX pseudomales were constructed carrying one copy of hs-otu and either homozygous mutant for ovo^{B1S1} or Sxl^{B3}. The mutant gonads were labeled for the germline-specific product, VASA, and the female ring canal protein, HTS-RC. In neither genotype was there an increased frequency of oogenic gonads due to hs-otu, indicating that ovo and Sxl mutant germlines cannot be feminized (Fig. 1F,G). However, the hs-otu construct did cause an increase in the number of gonads possessing VASA-positive cells (compare the non-oogenic and atrophic classes in Fig. 1B,F). The proportion of non-oogenic ovo mutant pseudomale gonads increased from 18% without hs-otu to 64% with the construct, in effect equaling the frequency of non-atrophic gonads in ovo^{+} pseudomales (Fig. 1A). A more modest improvement was also obtained in Sxl mutant XX pseudomales, where the frequency of VASA-positive gonads increased from 52% to 74% (Fig. 1D,G). These results indicate an additional role for oto in some process affecting germline viability and/or proliferation that is separable from oogenic differentiation and independent of ovo and, possibly, Sxl functions.

### The oto and ovo promoters are differentially regulated

The finding that hs-otu can feminize XX pseudomale germ cells suggests oogenesis is blocked because of insufficient oto levels. We therefore examined whether tra-induced sexual transformation affects the level of oto gene expression. In addition, we compared the regulation of the ovo and oto promoters to confirm our previous suggestion that these two genes are differentially regulated (Nagoshi et al., 1995) and to investigate the possibility that ovo may control oto promoter activity (Lu et al., 1997).

Two constructs were utilized in which the oto (oto-lacZ, Rodesch et al., 1995) or ovo (ovo-lacZ, Mèvel-Ninio et al., 1995) promoters were fused to the structural lacZ gene. We first compared their developmental expression patterns in wild-type gonads. Both were expressed specifically in the adult female germline, but differed in their activity during preadult stages. oto-lacZ was expressed in most, if not all, larval and pupal germ cells in both female (Rodesch et al., 1995) and male gonads (Fig. 4A,B). Sex-specific regulation only became apparent in the adult testis where male germline expression became restricted to a few cells at the apical tip (Fig. 4C; see also Hager and Cline, 1997 for analogous results with a similar construct). As with oto, the ovo promoter is initially active in both male and female larval gonads (Mèvel-Ninio et al., 1995). However, ovo-lacZ becomes sex-specific at an earlier stage, showing restricted expression in male gonads during the third instar larval and pupal periods (Mèvel-Ninio et al., 1995; Figs. 4D,E). The expression pattern in the adult testis is identical to that seen with oto-lacZ and has been published elsewhere (Oliver et al., 1994). These results demonstrate that the oto and ovo promoters are under different regulatory control in the preadult germline.

**oto, but not ovo, promoter activity is influenced by tra-induced sexual transformation**

We next examined how changes in the somatic sex influenced oto-lacZ and ovo-lacZ activities. oto-lacZ expression was greatly reduced in XX pseudomales (data not shown); however, the interpretation of this result was complicated by the high frequency of degenerating germ cells in these gonads. To compensate for this problem, oto-lacZ was assayed in hs-otu XX pseudomale gonads which typically contained viable, oogenic germ cells (Fig. 1E). We found that even under these optimized conditions, oto-lacZ activity was...
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reduced and sporadic, with β-galactosidase staining in a few isolated cell clusters in only a minority (3/13) of gonads (Fig. 5A,B). Even germ cells with clear oogenic phenotypes (such as large, nurse-like nuclei) typically showed little otu-lacZ expression. In contrast, ovo-lacZ was extensively expressed in all (10/10) hs-otu XX pseudomale gonads (Fig. 5C), consistent with previous studies demonstrating ovo promoter activity in XX pseudomales using a similar construct (Oliver et al., 1994). The same result was seen with a germline-specific enhancer-trap line, BC69, in which lacZ is controlled by the vasa promoter (Rodesch et al., 1995). All (14/14) hs-otu XX pseudomale gonads carrying BC69 showed substantial expression of β-galactosidase (Fig. 5D). These data demonstrate that the tra-induced sexual transformation specifically inhibits otu promoter activity.

We also performed the reciprocal experiment in which otu-lacZ activity was examined in XY germ cells developing in a female somatic background. XY pseudofemales produced by the ectopic expression of tra result in ovaries containing tumorous egg chambers (McKeown et al., 1988). Because XY pseudofemale germ cells become sufficiently ‘feminized’ that they acquire a need for otu function for optimal proliferation (Nagoshi et al., 1995), we anticipated they would also be permissive for otu promoter activity. This was in fact the case. Even in the absence of ovo function, XY pseudofemale germ cells consistently expressed otu-lacZ (Fig. 5E). This indicates that the feminizing effects of tra, but not ovo, are necessary for otu transcription in this genotype. In comparison, the ovo promoter is not detectably active in XY

Fig. 4. otu and ovo promoter expression in wild-type animals. Animals carrying one copy of otu-lacZ or ovo-lacZ were stained for β-galactosidase expression (green stain). otu-lacZ expression in (A) a third instar larval testis, (B) a pupal testis and (C) an adult testis (expression is restricted to a few apical cells, arrow). ovo-lacZ expression in (D) a third instar larval testis (expression is restricted to only a few cells at most anterior region, arrow), and (E) a pupal testis (expression is still restricted to only a few apical cells, arrow). Size bar equals 100 μm.

Fig. 5. otu promoter expression in sexually transformed animals. Adult XX pseudomale testes stained for β-galactosidase (green stain) and DAPI (β-galactosidase staining tends to suppress DAPI fluorescence). (A,B) XX pseudomales whose germlines were feminized by one copy of hs-otu. (A1) No β-galactosidase expression is observed although DAPI staining (A2) detects many germ cell clusters. (B1) Same genotype as A showing a subset of germ cells expressing β-galactosidase. Most cells do not express β-galactosidase, even those with large, nurse-like nuclei (B2, arrow). (C) ovo-lacZ expression in an XX pseudomale gonad. Unlike otu-lacZ, ovo is expressed in the majority of germ cells. (D) XX pseudomale carrying the BC69 enhancer trap. Most germ cell clusters express β-galactosidase. (E) otu-lacZ expression (one copy) in ovoD1sr1/Y pseudofemale somatically feminized by hs-tra. Even in the absence of ovo, the otu promoter is highly active. Size bar equals 100 μm.
Fig. 6. Frequency of HTS-RC-expressing gonads in differentially aged XX pseudomales. tra mutant XX gonads were labeled with antibodies specific for HTS-RC. Genotypes from A-C are derived from the cross otuP51 v fFM6; tra4 kar2 ry3 red/TM3 X pOtu104/pOtu104; tra4/TM6. (A, B) +/FM6; pOtu104/+; tra4/tra4. (C) otuP51/+; pOtu104/+; tra4/tra4. (D, E) hs-otu/+; tra4/tra4 from the cross: hs-otu/his-otu; tra4 kar ry red/TM6 X +/BFY; tra4/TM6. 1-3d, aged 1-3 days posteclosion before dissection; 7-10d, aged 7-10 days posteclosion; n, number of gonads examined for that genotype.

XX tra- pseudomales

We previously demonstrated that female somatic signals can cause XY germ cells to require otu for proliferation (Nagoshi et al., 1995). This was the first indication that the germline function of otu was influenced by the sexual identity of the soma. We now extend these findings to show that a separable otu function controlling oogenic differentiation is also affected by somatic interactions. The heterologous expression of otu feminizes the XX pseudomale gonad at two levels. First, hs-otu expression dramatically increases the number of viable, differentiating germ cells as seen by the absence of atrophic gonads (Fig. 1E). Second, the germline can differentiate to more mature oogenic stages than typically seen in XX pseudomales, following the same progression of fusome and ring canal maturation events as occurs in normal oogenesis. In fact, the hs-otu XX pseudomale gonad becomes organized much like a normal ovarian gerarium.

These results demonstrate a critical and rate-limiting role for otu in the somatic regulation of oogenesis. In effect, the developmental program for oogenic differentiation to about stage 4 remains intact in tra mutant XX pseudomale germ cells, but is usually inactive specifically because of inadequate levels of otu function. This is illustrated by our observation that otu-induced oogenesis cannot occur in the absence ovo or Sxl.

**DISCUSSION**

**Soma-dependent maintenance of otu activity in the XX germline is necessary for oogenesis**

We previously demonstrated that female somatic signals can cause XY germ cells to require otu for proliferation (Nagoshi et al., 1995). We now extend these findings to show that a separable otu function controlling oogenic differentiation is also affected by somatic interactions. The heterologous expression of otu feminizes the XX pseudomale gonad at two levels. First, hs-otu expression dramatically increases the number of viable, differentiating germ cells as seen by the absence of atrophic gonads (Fig. 1E). Second, the germline can differentiate to more mature oogenic stages than typically seen in XX pseudomales, following the same progression of fusome and ring canal maturation events as occurs in normal oogenesis. In fact, the hs-otu XX pseudomale gonad becomes organized much like a normal ovarian gerarium.

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**Fig. 7.** Model for how tra influences the regulation and function of ovo and otu. XX germ cells initiate female development (A) in a process influenced by sex-specific somatic signals controlled by tra. Female differentiation of XX germ cells results in a requirement for ovo function during larval gonial stages (B). At about this stage, ovo promoter activity becomes restricted to XX germ cells. The function of ovo is required for germ cells to become responsive to the oogenic differentiation function of otu (C), beginning in pupal stages. At this time, sex-specific somatic signals controlled by tra induce female-specific otu promoter activity.
functions (Fig. 1C,D). Therefore, both these and other essential gene products must either be present in pseudomales or induced by additional otu activity. We conclude that the tra-dependent somatic interaction required for early stages of oogenesis acts primarily by modulating otu expression.

**tra is required to maintain otu, but not ovo, promoter activity in the XX germline**

The mechanism by which the soma influences otu function is through the regulation of the otu promoter. The male transformation of XX soma inhibits otu activity in XX germ cells, while the presence of pseudofemale soma allows otu expression in XY germ cells. This pattern of soma-dependent regulation is specific to otu, as two other germline promoters (vasa and ovo) were not affected by the same somatic sex transformations.

Further comparisons between the otu and ovo promoters are instructive. Both the otu-lacZ and ovo-lacZ promoter constructs initially have non-sex-specific expression patterns that subsequently become very restricted in the male germline (Mèvel-Ninio et al., 1995; Rodesch et al., 1995; Fig. 4). Although the potential perdurance of the β-galactosidase products preclude the precise determination of when sex-specific regulation begins, it is clear that it occurs earlier for ovo than for otu. Sex-specificity in the ovo-lacZ pattern is seen in 2nd and 3rd instar larval gonads (Mèvel-Ninio et al., 1995), compared to adult stages for otu-lacZ (Fig. 4). It is notable that these time periods approximately correspond to when the ovo and otu mutant germline phenotypes first become apparent (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995).

These observations demonstrate that sex-specific promoter activity for both genes is not initially determined by the germline X:A ratio. Instead, there is some type of activity for both genes is not initially determined by the xenomic constitution. This ‘femaleness’ is defined by the presence of ovo-lacZ activity in female germ cells, while the presence of pseudofemale soma allows otu expression in XY germ cells. This pattern of soma-dependent regulation is specific to otu, as two other germline promoters (vasa and ovo) were not affected by the same somatic sex transformations.

**ovo-lacZ**

In XX ovaries, mutations in otu affect germ cell numbers as well as their differentiation, suggesting a possible proliferation function. Support for this came from our earlier study using sexually transformed XY animals, which allowed us to separately examine the proliferation and differentiation phenotypes (Nagoshi et al., 1995). In this case, XY germ cells developing in a somatic ovary (XY pseudofemales) required otu to maintain germ cell and egg chamber numbers. We now demonstrate in pseudomales that a similar effect occurs in XX germ cells. The ovo null mutation prevents oogenic differentiation in XX pseudomales, even with hs-ottu (Fig. 1B). However, the increased otu expression substantially improved the recovery of non-oogenic gonads (compare Fig. 1B with F). Therefore, otu appears to improve either the viability or proliferation of ovo mutant germ cells independent of further oogenic differentiation.

These observations have an additional ramification. In contrast to the otu proliferation function, the capacity for otu to induce oogenesis clearly requires ovo product (compare Fig. 1E with 1F). This dichotomy in the responsiveness of ovo mutant germ cells to otu activity indicates differences in how these two genes interact in regard to maintaining XX germ cell numbers and oogenic differentiation.

**The genetic basis of the XX pseudomale germline phenotypes**

From the data in Fig. 1, some inferences can be made concerning the genetic derivation of the pseudomale phenotypes. In every genotype examined, at least 10-20% of the pseudomales were of the non-oogenic class, even in those gonads containing hs-ottu (Fig. 1). We believe this represents an early commitment of the germ cells in this subset to a male fate, as suggested by their fusome composition and operationally defined by their insensitivity to changes in the level of otu and mutations in ovo and Sxl. In this regard, they are similar to XY germ cells developing in testes which also do not require the functions of these oogenic genes. These data are consistent with previous studies based on morphological criteria that also identified a subset of ‘spermatogenetic’ tra mutant pseudomales (Nöthiger et al., 1989). The implication is that a tra-dependent somatic process can sexually transform XX germ cells, albeit inefficiently, such that they no longer require ovo, otu or Sxl for at least some aspects of gametogenesis.

In the remaining oogenic and atrophic gonads, we believe the germ cells maintain a female identity, reflecting their chromosomal constitution. This ‘femaleness’ is defined by the continued sensitivity of these cells to the oogenic effects of heterologous otu expression (compare Fig. 1A and E). The degree of female development, and thereby the distinction between atrophic and oogenic gonads, depends upon the level of otu expressed, as demonstrated in Fig. 6.

**tra-dependent somatic influences on ovo and otu germine functions**

Mutations in the ovo gene typically result in the absence of germ cells in adult ovaries and aborted oogenic differentiation. In fact, ovo null XX germ cells were reported to die beginning in embryonic stages (Oliver et al., 1987, 1990). This effect on both the differentiation and early viability of the XX germine has led to the proposal that ovo is a primary regulator of germline sex determination, perhaps having a role in germline dosage compensation or in determining the germ cell X:A ratio (Oliver et al., 1990, 1993, 1994).

One assumption of these hypotheses is that the reduction in the number of ovo mutant germ cells is due to increased lethality. Evidence in support of this supposition comes from electron microscopic examination of presumptive ovo mutant embryos, where a subset of pole cells appeared to be dead or dying (Oliver et al., 1990). However, two separate studies demonstrate that consistent and significant abnormalities in ovo mutant gonads were not evident until the second instar
larval stage, bringing into question the significance of the reported embryonic phenotype (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). In neither of these later reports was there evidence that the larval gonadal abnormalities stemmed from germ cell lethality, as opposed to, for example, reduced cell proliferation. Therefore, it is not established that ovo is required for germ cell viability during oogonial development.

A second assumption is that the requirement for ovo is determined primarily, if not solely, by the germ line X:A ratio. Support for this comes from two complementary observations: XY germ cells do not require ovo when made to develop in a female soma (Steinmann-Zwicky et al., 1989; Oliver et al., 1994), and the presence of male-transformed soma did not eliminate the requirement for ovo in XX germ cells (Steinmann-Zwicky et al., 1989; Nagoshi et al., 1995). However our use of molecular germline markers defined a more complex pseudomale phenotype. While we confirmed that an ovo null allele did prevent oogenic differentiation, no significant alterations in the frequency of the non-oogenic pseudomale class were observed. Therefore, the pseudomale transformation allows a substantial fraction of ovo null XX germ cells to perdure to the adult stage and produce spectrosomes and fusomes. This contrasts with the absence of similar differentiation typically observed in the infrequent ovo null germ cells found in adult ovaries (Fig. 2), although it should be noted that a small subset of these mutant cells can occasionally produce fusomes (R. N. N. and S. H., unpublished data). A similar phenomenon is seen with otu. In females, null mutations in otu block oogenesis at the first cystocyte division, producing small, linear fusomes (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). In contrast, non-oogenic pseudomales lacking otu produce large, multibranched fusomes (data not shown). We therefore conclude that the requirements of XX germ cells for ovo and otu functions, at least with regard to early gameticogenesis, can be substantially influenced by tra-dependent changes in the sexual state of the soma.

One explanation for these results follows from our genetic rationalization of the pseudomale phenotypes. We hypothesize that, despite their female chromosomal constitution, XX germ cells committed to a male fate do not need ovo or otu to initiate gametogenesis. In one simple permutation of this model, the sexual identity of the germline is defined prior to ovo and otu functions by a process involving tra-dependent somatic interactions. In this case, XX pseudomale germ cells committed to a male fate initiate spermatogenesis independent of ovo and otu. In contrast, those that retain a female identity still require these genes and so become atrophic in their absence.

This early placement of a somatic interaction contributing to germline sex determination is consistent with studies indicating that tra influences the development of the XX germline during embryonic and early larval stages (Seidel, 1963; Steinmann-Zwicky, 1994a; Staab et al., 1996), while ovo and otu appear to be required later in development (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). However, in this regard, it should be noted that there is a significant maternal contribution of ovo product, which could mask possible effects of ovo mutations during early stages of germline differentiation (Mèvel-Ninio et al., 1995).

To summarize our results, we suggest a model describing the regulatory relationships between ovo, otu and tra. We propose that a somatic signal controlled by tra contributes to an early regulatory commitment to female development (Fig. 7A). This creates a requirement for ovo activity during the larval gonial stages, at which time the ovo promoter becomes more sex specific in its expression (Fig. 7B). We believe that during the pupal and adult stages, two critical events occur in the female germarium (Fig. 7C). First, ovo activity allows XX germ cells to become receptive to the otu function controlling oogenic differentiation. Second, tra-dependent somatic signals allow continued expression of otu in the female germline by maintaining otu promoter activity. By this mechanism, we believe that the otu gene serves to link the somatic sex differentiation pathway controlled by tra with a female germline developmental pathway controlled by ovo.

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