Development 126, 1917-1926 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV7706

The *Drosophila* gene *stand still* encodes a germline chromatin-associated protein that controls the transcription of the *ovarian tumor* gene

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Accepted 12 February; published on WWW 6 April 1999

SUMMARY

The *Drosophila* gene *stand still* (*stil*) encodes a novel protein required for survival, sexual identity and differentiation of female germ cells. Using specific antibodies, we show that the Stil protein accumulates in the nucleus of all female germ cells throughout development, and is transiently expressed during early stages of male germline differentiation. Changes of Stil subnuclear localization during oogenesis suggest an association with chromatin. Several mutant alleles, which are point mutations in the Stil N-terminal domain, encode proteins that no longer co-localized with chromatin. We find that Stil binds to many sites on polytene chromosomes with strong preference for decondensed chromatin. This localization is very similar to that of RNA polymerase II. We show that Stil is required for high levels of transcription of the *ovarian tumor* gene in germ cells. Expression of *ovarian tumor* in somatic cells can be induced by ectopic expression of Stil. Finally, we find that transient ubiquitous somatic expression of Stil results in lethality of the fly at all stages of development.

Key words: Germline, Chromatin, Transcription, *Stand still* (*stil*), *ovarian tumor* (*otu*), *ovo* (*ovo*)

INTRODUCTION

In most multicellular organisms, sexual reproduction requires two important cell fate decisions, the separation between soma and germline on the one hand, the choice of the sexual identity on the other hand. These two decisions are usually made early during embryogenesis.

In *Drosophila melanogaster*, the primordial germ cells form at the posterior tip of the embryo less than 2 hours after fertilization, under the control of maternal factors deposited at this pole during oogenesis (reviewed by Williamson and Lehmann 1996). Sex determination of the somatic cells takes place soon after (reviewed by Cline and Meyer, 1996). It is not known exactly when sex determination of the germline occurs, but around the middle of embryogenesis some sexual dimorphisms have been described (Poirié et al., 1995; Staab et al., 1996).

Germline sex determination is controlled by both cell-autonomous (germ cell intrinsic components) and non-cell-autonomous factors (somatic signals) (Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1994; Horabin et al., 1995; Staab et al., 1996). Candidate genes involved in germline sex determination might be identified on the basis of two mutant phenotypes: the disappearance of germ cells in a sex-specific manner and the differentiation of chromosomally female (2X/2A) germ cells as male germ cells. Genetic analyses have shown that the three genes *ovo*, *ovarian tumor (otu)*, and *stand still (stil)* meet both criteria. They are required only in females for survival of the germ cells, establishment of their sexual identity and oogenesis (Oliver et al., 1987, 1990, 1993, 1994; Pauli et al., 1993; Staab and Steinmann-Zwicky, 1995; Nagoshi et al., 1995; Hager and Cline, 1997; Pennetta and Pauli, 1997). In addition to the similar phenotypes shown by mutations in these three genes, genetic interactions between them have been demonstrated. The phenotype of females bearing dominant female-sterile alleles of *ovo*, ovo^D, is sensitive to the gene dosage of *otu* and *stil*, such that decreased amounts of these genes lead to enhancement and increased gene doses to partial suppression of the phenotype (Pauli et al., 1993, 1995; Pennetta and Pauli, 1997). These genetic interactions suggested that the *otu, ovo* and *stil* genes function in a common pathway, or in closely linked parallel pathways, involved in the survival and differentiation of the female germline. Support for the hypothesis of a common pathway comes from the recent finding that the zinc-finger domain of the Ovo protein can bind in vitro to multiple sites at the *otu* promoter (Lü et al., 1998) and that high levels of expression of *otu* both in male and female germ cells is controlled by *ovo* gene products (Hager and Cline, 1997; Lü et al., 1998). The *otu* gene therefore seems to be a direct downstream target of Ovo.

Mutations in other genes such as *Sex-lethal* (*Sxl*), *sans fille* (*snf*), *female lethal(2)d* (*fl(2)d*) result in masculinization of the female germline, without affecting the viability of the germ

cells (Oliver et al., 1988, 1993; Steinmann-Zwicky, 1988; Steinmann-Zwicky et al., 1989; Granadino et al., 1992; Bopp et al., 1993). Molecular and genetic data indicate that *ovo* and *otu* regulate, directly or indirectly, the expression of *Sxl* in the female germline (Oliver et al., 1990, 1993; Bopp et al., 1993; Pauli et al., 1993) and that *fl(2)d* and *snf* are probably involved in female-specific splicing of *Sxl* pre-mRNAs (Granadino et al., 1992; Hager and Cline, 1997).

In this paper, we have focused our attention on the *stil* gene. Mutations of *stil* show the two phenotypes mentioned above (Pennetta and Pauli, 1997). First, the stronger effect is a very severe reduction of the number of germ cells in female gonads: in certain allelic combinations, up to 90% of adult ovaries contain no germ cells. Second, the few germ cells that survive frequently show a morphology typical of spermatocytes. The existence of additional late oogenesis functions can be inferred from the fact that females bearing hypomorphic *stil* alleles show good germline survival and female sexual identity, but oogenesis is arrested and various defects, such as the misplacement of the oocyte within the egg chamber, occur (Gutzeit and Arendt, 1994; Mulligan et al., 1996; Pennetta and Pauli, 1997). *stil* function appears to be dispensable for fly viability and male germline differentiation. The *stil* gene encodes a novel protein of 321 amino acids (Pennetta and Pauli, 1997).

Here we show that Stil is a nuclear protein, which binds to multiple sites on polytene chromosomes, preferentially to decondensed regions. We analyzed the hierarchy of expression between *stil*, *ovo* and *otu*, and found that *stil* positively regulates the transcription of *otu*. Finally, we show that ubiquitous Stil expression in somatic cells is lethal.

MATERIALS AND METHODS

Drosophila stocks

Flies were raised on standard *Drosophila* medium at 25°C. *stil* alleles and deficiencies are described in Pennetta and Pauli (1997). The following reporter strains were used: P{ry^+ *otu::lacZ*}^{C} (Comer et al., 1992); P{ry^+ *ovo::lacZ*} (Mével-Ninio et al., 1995); P{w^+ *ovo::lacZ*}^{4B8} and P{w^+ *ovo::lacZ*}^{2U21} (Oliver et al., 1994).

Preparation of antibodies

A *Nae*I fragment from a *stil* cDNA clone (position 752-1265, Pennetta and Pauli, 1997) was ligated into the *Sma*I site of pQE-30 (Qiagen). The resulting clone encodes an open reading frame that includes 18 vector-encoded amino acids and the last 102 residues (219-321) of the predicted Stil protein. The bacterially expressed fusion protein carried a 6-residue Histidine tag, which was used for affinity purification on a Ni^{2+} column according to the manufacturer's instructions (Qiagen). The affinity-purified recombinant protein was injected into two rabbits by Elevage Scientifique des Dombes (01400 Chatillon/Chalaronne, France).

Western blots

Twelve to fifteen females were dissected in 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0. Ovaries were sonicated until appearance of foam, centrifuged 10 minutes at 15,000 rpm and 1/10 of the supernatant was loaded on a SDS-PAGE gel. Western blots analysis were performed according to Cléard et al. (1997). Crude anti-Stil antisera were used at a 1/1,000 dilution.

Whole-mount antibody staining

Embryos were treated as described in Pauli et al. (1990). Gonads were dissected in 1× PBS, fixed for 20 minutes in 4% paraformaldehyde, washed 3 times in PBT (1× PBS, 0.1% BSA, 0.1% Triton X-100), blocked in PBT for 2 hours and incubated overnight at 4°C with a 1/10,000 dilution of rabbit anti-Stil antibodies, or with a 1/500 dilution of rabbit anti-Otu antiserum (gift from L. Glenn) or with a 1/500 dilution of rat anti-Vasa antiserum (gift from P. MacDonald). They were washed with PBT, twice quickly and three times for 30 minutes. Incubation with goat anti-rabbit secondary antibodies coupled either to peroxydase or to 5-([4,6-dichlorotriazin-2-yl] amino)-fluorescein (DTAF) (Sigma), or with goat anti-rat secondary antibodies coupled to rhodamine, was for 4 hours at room temperature, followed by washes as above and staining in presence of 0.5 mg/ml 3′3 diaminobenzidine and 0.06% H_2O_2 for peroxidase detection. The tissues were washed in PBS and mounted in PBS/glycerol or vectashield mounting medium for fluorescence (Vector Laboratories).

In situ hybridization

Testes for whole-mount in situ hybridization were dissected in PBS and fixed in 5% formaldehyde, 50 mM EGTA for 40 minutes. They were washed in methanol for 10 minutes and fixed again in 5% formaldehyde, 0.1% Tween 20 for 25 minutes. After three washes in PBST (1× PBS, 0.1% Tween 20), testes were treated with 0.1 mg/ml proteinase K for 6 minutes at room temperature, incubated with 2 mg/ml glycine for 3 minutes, washed three times in PBST and fixed again in 5% formaldehyde, 0.1% Tween 20 for 20 minutes. Gonads were then washed 5 times with PBST, pre-hybridized for 4 hours at 55°C in 50% formamide, 5× SSC, 50 mg/ml heparin, 0.1% Tween 20, 100 mg/ml salmon sperm DNA, 100 mg/ml t-RNA, before addition of 20 ng of DIG-labeled riboprobe for 16 hours. Anti-sense *stil* RNA probe was prepared according to a DIG RNA labeling Kit protocol (Boehringer). Washes were done at 55°C: 3 times 20 minutes in hybridization solution, 3 times 20 minutes in hybridization solution/PBT (1/1), 3 times 20 minutes PBT. Detection was as described by the manufacturer's protocol with anti-DIG antibodies at a 1/2,000 dilution.

Sequencing of *stil* mutations

Genomic DNAs extracted from hemizygous mutant flies were PCR-amplified with the following primers: 5′CAGGTTCATTCCCATTTA and 5′AACCATTCTTTAGTGGTCC. The 1.5 kb bands were purified on an agarose gel and sequenced directly with internal primers.

Germline transformation with a *hsp70::stil* transgene

A cDNA *Eco*RI-*Not*I fragment bearing the full Stil open reading frame was inserted in transformation vector pCasper-hs (Thummel and Pirrotta, 1991). In the progeny of over 100 injected surviving embryos, only 3 transformants were recovered.

Immunodetection on polytene chromosomes

Larvae carrying the P{w^+ *hsp70::stil*} construct and control larvae were heat-shocked at 37°C for 45 minutes and then left at 25°C for 2 hours before dissection. Salivary glands were dissected in PBS, 0.1% Triton X-100 and squashed in a fixative solution containing 3.7% formaldehyde, 1% Triton X-100 in PBS. Chromosomes were then treated according to Cléard et al. (1997). Anti-Stil antibodies were used at a 1/1000 dilution. Polytene chromosomes from nurse cells of otu^{11}/otu^7 ovaries were prepared following the same procedure. Images were treated with the deconvolution program MicroTome from VayTek, Inc.

β-galactosidase detection

β-galactosidase detection was performed according to Sahut-Barnola et al. (1995).

RESULTS

Stil is a nuclear protein present in the germline of both sexes

Previous investigations have shown that *stil* encodes a novel

protein required for survival, sex determination and differentiation of germ cells in females (Pennetta and Pauli, 1997). As a step in the analysis of the function of *stil*, we raised two antibodies against the last 102 C-terminal residues of the predicted Stil protein. One of the antisera detected a single band of approximately 40 kDa on western blots of ovarian extracts of wild-type adult flies (data not shown). This size is in close agreement with the one predicted from the *stil* sequence (35.8 kDa). The band was undetectable in ovaries devoid of germ cells (progeny of *tudor* mutant mothers) or in *stil* mutant ovaries containing only a few germ cells. No Stil protein could be detected on western blots of testis extracts (not shown).

Both antisera were found to give germline-specific staining when tested on whole-mount embryos, larval and adult tissues. During embryogenesis, the Stil protein is first detectable at stage 11, in the nucleus of germ cells soon after their migration through the midgut epithelium, but before their separation into two groups. This is very soon after germ cells becomes competent for RNA polymerase II-dependent transcription (Van Doren et al., 1998). At stage 11, the staining is very faint, but becomes strong by stage 13. Double staining with antibodies against the Vasa and Stil proteins showed that all germ cells express Stil (data not shown). We found no evidence of either sex-specific expression during embryogenesis or expression in somatic tissues.

During the first and second larval instar, Stil protein was found in the nucleus of all germ cells in both sexes (Fig. 1A,B). A difference between males and females first becomes apparent during the third larval stage. All female germ cells, which are located in the middle part of larval ovaries, showed strong staining (Fig. 1C). In adult ovaries, Stil expression is maintained in all germ cells (see below). By contrast, Stil is present only during some stages of male germline differentiation (Fig. 1D). At the testis apex, Stil expression is low in stem cells and dividing spermatogonia. In newly formed 16-cell cysts of primary spermatocytes, Stil staining is as strong as that found in female germ cells, but then quickly vanishes during the spermatocyte growth phase. This pattern of expression was also found in adult testes, which have the same apical-distal organization (Fig. 1E,F).

The pattern of Stil expression in testes was unexpected since in situ hybridization had indicated a very weak uniform *stil* mRNA expression over the entire gonad (with the exception of the apex; Pennetta and Pauli, 1997). We repeated the in situ hybridization experiments and found that different conditions of fixation allowed the detection of an RNA pattern similar to the one found for the Stil protein (Fig. 1G).

Because ovaries from flies bearing strongly hypomorphic mutant alleles of *stil* are mostly devoid of germ cells and the few surviving cells are not very healthy, it was difficult to assess the antibody specificity on the basis of the absence of staining in mutant female germ cells. Thus, we took advantage of the expression in testis. No staining was found in the gonads of males homozygous for the $stil^5$ allele. It should be noted that the male germline appears completely normal in $stil^5$ and all other *stil* alleles. Sequencing of the $stil^5$ DNA revealed a small deletion, resulting in a frame shift and truncation of the protein after amino acid 97 with the addition of 20 unrelated residues. This protein could not be detected by our antibodies which were raised against the C-terminal part of Stil. This experiment confirmed the specificity of our antibodies.

We found no Stil expression in most of the somatic tissues that we tested: whole embryos, third instar larval imaginal discs, brain and salivary glands, as well as the somatic cells of the gonads. The only exception was larval fat bodies, which showed low levels of Stil expression.

Immunolocalization of the Stil protein in adult ovaries

An adult ovary is composed of about 16 egg assembly lines called ovarioles. A special region, the germarium, lies near the anterior end of an ovariole. Two to three germline stem cells are located at the anterior end of a germarium. A germline stem cell divides asymmetrically to produce a new stem cell and a cystoblast, which then undergoes four rounds of synchronous incomplete mitotic divisions forming a cluster of 16 interconnected cystocytes. One of the 16 cells becomes the oocyte and the 15 others differentiate into nurse cells. The 16-cell cyst, surrounded by a layer of somatic follicle cells, leaves the germarium and undergoes final differentiation (reviewed by Spradling, 1993).

In adults, we found that Stil is present in the nucleus of all female germline-derived cells, including the oocyte. Staining of the oocyte nucleus was detected transiently, from stage 1 to 10 of oogenesis, and is weak compared to staining in nurse cell nuclei (Fig. 2A). After stage 10, staining was observed only in nurse cell nuclei and disappears when they degenerate (Fig. 2B,C). The oocyte cytoplasm presents no detectable level of

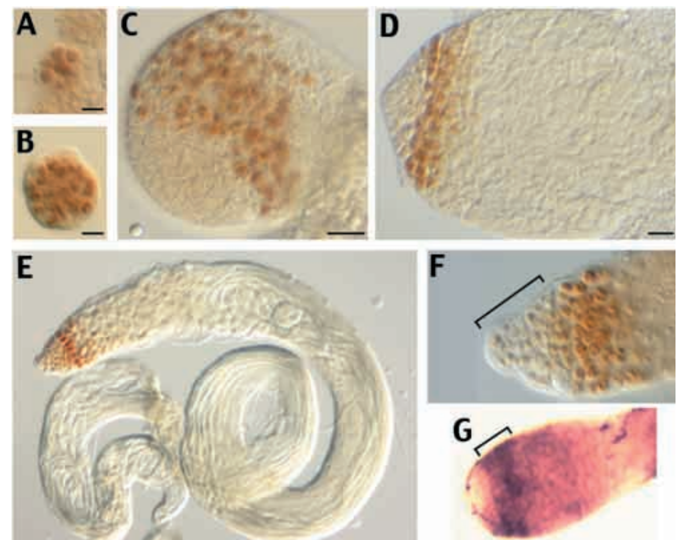

Fig. 1. Expression of Stil in larval gonads and in adult testes. Immunodetection was performed using secondary antibodies coupled to HRP. (A) An ovary and (B) a testis of a first instar larva: all germ cell nuclei contain Stil. (C) An ovary and (D) a testis from a larva at the end of the 3rd instar. All germ cells express Stil in ovaries. In testes, expression is transient: weak in stem cells and dividing spermatogonia at the apex, strong in newly formed cysts of 16 spermatocytes in a sub-apical region, absent in later stages. (E,F) Adult testes show the same transient expression. (F) Higher magnification shows the weak staining in stem cells and dividing spermatogonia (bracket). (G) In situ hybridization on adult testes shows large amounts of *stil* RNAs in the region of newly formed 16-spermatocyte cysts, indicated by the bracket. Bars: 10 μm (A,B), 20 μm (C,D).

Stil protein irrespective of the developmental stage, indicating that most of the Stil protein is not transferred from the nurse cells to the oocyte, and that what is transferred is trapped in the oocyte nucleus.

We were interested in verifying whether Stil was present in all germ cells in the germarium. Thus, ovaries were also stained for the germline-specific cytoplasmic Vasa protein. Fig. 2D-F demonstrates that the Stil protein is expressed in every germ cell, including the germline stem cells.

Stil protein co-localizes with chromatin

We noticed that the nuclear staining was frequently not uniform. This is particularly visible in nurse cell nuclei. During previtellogenic stages, Stil appears to be in a few compact aggregates, while in later stages the staining is more diffuse (Fig. 2G-I). Changes of nuclear morphology of nurse cells during oogenesis have been described (King, 1970). Before stage 4, banded polytene chromosomes can be found. During stages 4 and 5, the banding is lost, chromatin condensation increases, and five large blobs of chromatin (presumably the five major chromosome arms) can be seen. In later stages, chromatin becomes less condensed and nuclei are filled with chromatin of fibrillar appearance. The localization of Stil appears to follow closely the changes of chromatin condensation (Fig. 2G-L), suggesting that Stil is bound to chromatin, possibly at many sites.

Some mutant Stil proteins do not co-localize with chromatin

We next found that our antibodies detected Stil polypeptides in several *stil* mutants, although the intensity of staining is generally reduced (Table 1). Strikingly, the chromatin association appears to be lost (or reduced) in these mutants. Fig. 3 shows an example of a stage 5 egg chamber homozygous for *stil*^{2} (the weakest known hypomorphic allele), in which the pattern of Stil staining in nurse cell nuclei is complementary to the DNA staining. For three other mutations, *stil*^{1}, *stil*^{4} and *stil*^{6}, protein localization could not be assessed in nurse cells because of early arrest in differentiation. However, we found that the staining in spermatocyte nuclei is uniform in *stil* mutants, by contrast to wild-type spermatocytes which show granular staining. These mutations were sequenced and found to be clustered within the first 80 residues of the Stil polypeptide (Table 1). These data identify the N-terminal part of Stil as a possible domain of interaction with chromatin.

Binding of Stil to polytene chromosomes

Polytene chromosomes of larval salivary glands have been intensively used to analyze the binding pattern of chromatin-associated proteins. We therefore wanted to test for the behavior of Stil in this well defined system. To express Stil in salivary glands, we introduced into the *Drosophila* germline, a P-transposon in which a *stil* cDNA was fused downstream of an *hsp70* promoter. A pulse of Stil expression was obtained by heat-shocking transgenic third instar larvae for 45 minutes at 37°C, followed by 2 hours of recovery at 25°C before preparation of the polytene chromosomes. Immunostaining with the anti-Stil antibodies gave a complex pattern (Fig. 4), which was not found in negative controls (chromosomes prepared from non-transgenic flies or chromosomes from heat-shocked transgenic flies stained with pre-immune anti-serum). Very weak staining was found with non-heat-shocked transgenic flies. Many sites were observed along all the chromosome arms, some sites being very strongly stained, others being weakly labeled. Double staining for DNA and for Stil revealed almost complementary patterns. Stil is abundantly present in decondensed regions,

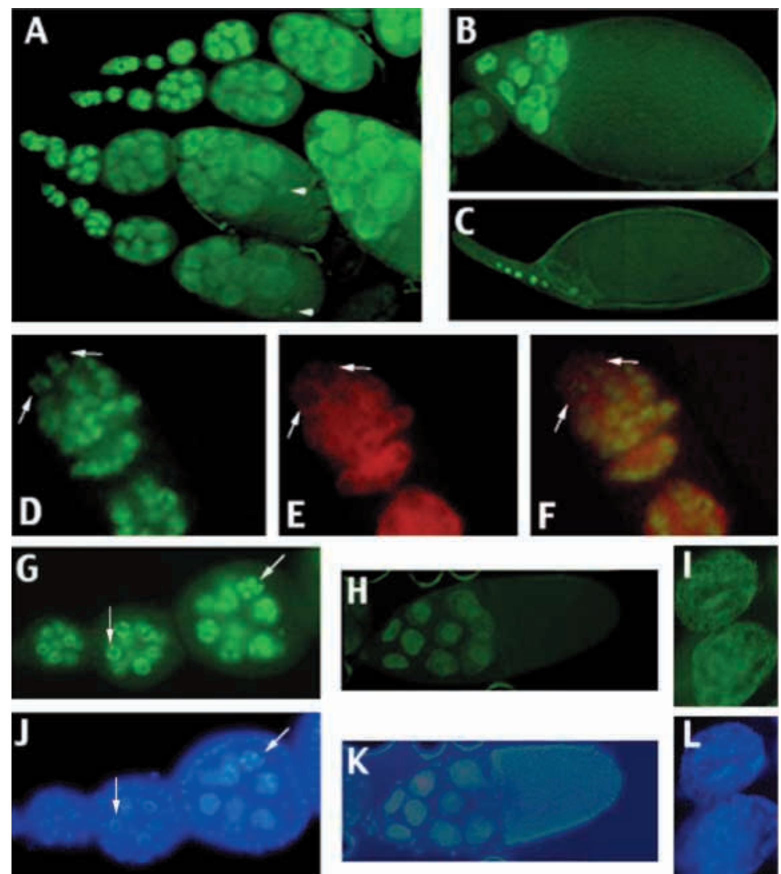

Fig. 2. Stil co-localizes with chromatin in germ cells of adult ovaries. Stil was detected by secondary antibodies coupled to DTAF (green). (A) Four ovarioles showing various early stages of egg chamber maturation. Stil is present in the nucleus of all germ cells: strongly in nurse cells and more weakly in oocyte nuclei (arrowheads). Staining in the oocyte disappears after stage 10. (B) A stage 11 egg chamber in which only nurse cell nuclei are labeled. (C) A stage 13 egg chamber in which weak staining is still detectable in degenerating nurse cells. (D-F) Magnification of a germarium doubly labeled for Stil (D, green) and Vasa (E, red). (F) The merged image of D and E. All germ cells, including the two stem cells at the apex (arrows) express Stil. (G-L) Extensive co-localization of Stil (green) and DNA (DAPI, blue) revealed by similar staining patterns. (G,J) During stages 4 and 5, blobs corresponding to the highly condensed chromosome arms can be seen (arrows point to nurse cell nuclei). Note the absence of Stil in the outer layer of follicular cells whose nuclei are labeled with DAPI. (H,K) Whole egg chamber and (I,L) higher magnification of two nurse cell nuclei, at stage 10. Chromatin is decondensed and shows a fibrillar structure. Stil (I) and DAPI (L) stainings are very similar.

Table 1. Molecular characteristics of *stil* mutations

Allele	Molecular alteration	Antibody staining[a]
stil^{1}	K79M	–/+, delocalized?[b]
stil^{2}	G58R	+++, delocalized
stil^{4}	P19S	+, delocalized
stil^{5}	deletion/insertion[c]: protein truncated after A97	–
stil^{6}	5′ splice site of first intron: consensus GT→AT[d]	++, delocalized

[a]+++ = wild-type level of expression. – = no staining.
[b]The question mark indicates that the protein localization is difficult to estimate due to the very weak staining.
[c]Deletion of 63 bp and insertion of 5 bp. The resulting frame shift leads to a polypeptide containing the first 97 amino acids of Stil followed by 20 unrelated residues.
[d]The first intron is located after amino acid 65. The staining found with antibodies directed against the C terminus of Stil suggests that cryptic 5′ splice site(s) are used.

both puffs and interbands (Fig. 4C, small amounts of DNA, green bands), as well as in regions of intermediate levels of condensation (yellow bands). Stil seems to be completely absent in the most condensed regions (high amounts of DNA, red bands), which correspond to the bands on maps of polytene chromosomes. This data indicates that the Stil protein is able to bind to decondensed chromatin, including sites of active transcription, when expressed in an ectopic tissue.

To give more support to this observation, we analyzed polytene chromosomes of nurse cells, which correspond to the normal tissue expressing *stil*. Polytene chromosomes are present in early stages of oogenesis, but are very difficult to observe. Larger polytene chromosomes have been reported in some *otu* mutations (Storto and King, 1988; Mal'ceva et al., 1997). We analyzed nurse cell polytene chromosomes from *otu*^{7}/*otu*^{11} females and found a pattern consistent with that observed on polytene chromosomes in salivary glands (Fig. 4D). In both normally and ectopically expressing tissues, the binding of Stil appears to inversely correlate with the condensation of chromatin.

Positive control of *otu* expression by Stil

Given the similar phenotypes exhibited by mutations in *stil, ovo* and *otu* as well as the genetic interactions between them, it is likely that these genes function in a common pathway, or in parallel pathways, to determine germline survival and sexual identity (Oliver et al., 1990, 1993; Pauli et al., 1993; Nagoshi et al., 1995; Pennetta and Pauli, 1997). A common pathway is supported by the finding that high levels of *otu* expression requires *ovo* gene activity (Lü et al., 1998). We therefore investigated whether Stil controls the expression of the *ovo* and *otu* genes.

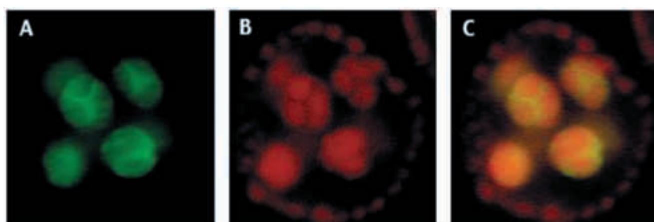

Fig. 3. Delocalization of Stil protein in *stil*^{2} mutant nurse cell nuclei. (A) Stil protein, (B) DNA and (C) the merged image. Note that Stil protein does not appear to co-localize with chromatin.

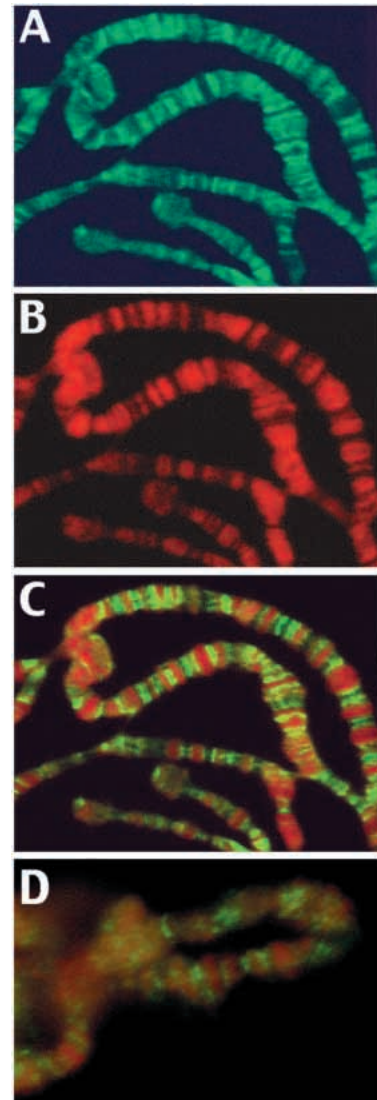

Fig. 4. Stil binds to polytene chromosomes. Stil was detected with secondary antibodies coupled to DTAF (green), and DNA with propidium iodide (PI, red). (A-C) Polytene chromosomes from salivary glands ectopically expressing Stil. Stil is present at many sites along all the chromosome arms. The merged image (C) shows that Stil binding inversely correlates with DNA condensation (red bands). (D) Part of a polytene chromosome from *otu*^{11}/*otu*^{7} nurse cells. Although the quality of these chromosomes is poor compared to those of salivary glands, Stil is clearly localized to the regions of low chromatin condensation.

First, we analyzed the expression of three constructs containing parts of the *ovo* gene fused to a *lacZ* reporter gene (Oliver et al., 1994; Mével-Ninio et al., 1995) in gonads of wild-type, *stil* heterozygous and *stil* homozygous or hemizygous flies. We did not find any effect of *stil* gene dosage on expression of the *ovo::lacZ* reporter genes (not shown).

Second, to determine the effect of *stil* mutations on *otu* expression, we used both antibodies against the Otu proteins (gift from L. Glenn) and an *otu::lacZ* reporter which contains the 5′ region and the first untranslated exon of the *otu* gene (Comer et al., 1992). Using Otu antibodies in wild-type or in *stil* heterozygous backgrounds, we observed a cytoplasmic

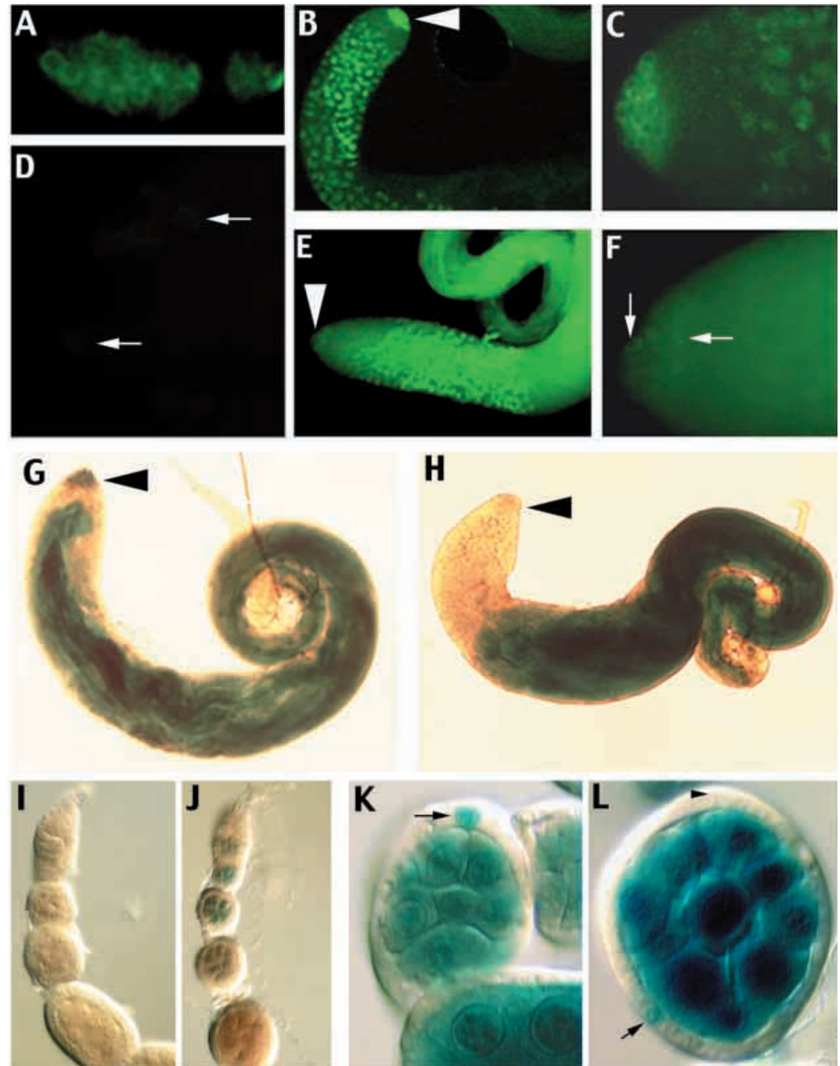

Fig. 5. Stil regulates the expression of *otu*. (A-F) Immunodetection with anti-Otu antibodies. (A) Tip of a single wild-type ovariole. Note the cytoplasmic staining of germ cells. (B,C) Wild-type testis and enlargement of its apex. Anti-Otu antibodies stain the cytoplasm of germ cells at the apex (arrowhead). Nuclear staining was also observed in spermatocytes, but is not due to Otu since it is present in *otu* null mutants (not shown). This staining was used as an internal control. (D) A whole ovary from a $stil^{6}$/*Df(2R)stil-B* female, containing a few germ cells showing a small amount of Otu protein (compare with A). (E) A testis and (F) an enlarged view of its apex from a $stil^{6}$/*Df(2R)stil-B* male. Otu staining in germ cells at the apex is strongly reduced (indicated by an arrowhead in E and arrows in F). Note that the control staining in the spermatocyte nuclei is as strong as in wild type. (G,H) X-gal staining of testes of flies bearing an *otu::lacZ* construct. (G) Testis of a male heterozygous for deletion *Df(2R)stil-B*. Staining at the apex corresponds to *otu::lacZ* expression (arrowhead). Staining in sperm comes from a *lacZ* enhancer trap located on the *Df(2R)stil-B* chromosome and provides a positive internal control. (H) In a $stil^{6}$/*Df(2R)stil-B* testis, *otu::lacZ* staining at the apex (arrowhead) is almost completely absent. (I,J) Three doses of $stil^{+}$ in the ovo^{D2} background increase *otu::lacZ* expression. (I) Anterior part of an ovariole heterozygous for ovo^{D2}. Expression of *otu::lacZ* is almost undetectable. (J) Anterior part of an ovariole heterozygous for ovo^{D2} and bearing an extra copy of a $stil^{+}$ transgene: *otu::lacZ* expression is partially restored. (K,L) Ectopic Stil expression from an *hsp70::stil* transgene can induce the expression of *otu::lacZ* in some follicular cells. Flies were submitted to two 45-minute heat-shocks separated by 4 to 7 hours and dissected 24 hours after the first heat-shock. Occasional staining of follicular cells was observed (arrows). Ectopic *otu::lacZ* expression was most frequently found in polar cells, which are located at the extremities of egg chambers (arrow in K). However, unstained polar cells were also observed (arrowhead in L).

staining in all the germ cells in ovaries and in stem cells and dividing spermatogonia at the apex of testes (Fig. 5A-C). In *stil* mutant ovaries, in which only a few cells are present, we observed a strong reduction in the intensity of Otu antibody staining (Fig. 5D). However, it is difficult to be sure that the reduction of staining is due to decreased expression of *otu* and not to the fact that the mutant cells are unhealthy and dying. In males, *stil* mutant germ cells are completely healthy. We also found a strong reduction of the intensity of Otu antibody staining at the apex of *stil* mutant testes (Fig. 5E-F). Experiments using the *otu::lacZ* transgene gave the same results. In wild-type and in *stil* heterozygotes, expression of *otu::lacZ* is restricted to the apex of testes (Fig. 5G). In *stil* mutant testes, β-galactosidase expression was almost undetectable (Fig. 5H). These results show that transcription of the *otu* gene in male and female germ cells is regulated by Stil.

It has been shown recently that the *ovo* gene products are positive regulators of *otu* expression and that the Ovo zinc-finger domain binds specifically to several sites at the *otu* promoter (Lü et al., 1998). These and our observations raise the possibility that Ovo and Stil work together at the *otu* promoter. One way to get a hint of such a cooperation is to combine *ovo* and *stil* mutations and to test the consequence on *otu* expression. We did observe an effect of $stil^{+}$ gene dosage in a background in which *otu* transcription is strongly impaired by a dominant antimorphic mutation of *ovo*. In ovaries heterozygous for ovo^{D2}, expression of *otu::lacZ* is almost completely abolished (Lü et al., 1998; Fig. 5I). Expression of the *otu::lacZ* transgene was partially restored by the addition of an extra wild-type copy of the *stil* gene (Fig. 5J). Phenotypic analysis had shown that the ovo^{D2} mutation can be partially suppressed by increasing the dosage of $stil^{+}$ (Pennetta and Pauli, 1997). Our result suggests that this phenotypic improvement is in part due to restoration of *otu* expression. We also tested for the effect of decreased amount of both *ovo* and *stil*. In three double transheterozygous $ovo^{-}/ovo^{+};sti^{-}/stil^{+}$ conditions ($ovoD1^{rv22}$/+; $stil^{4}$/+. $ovoD1^{rv23}$/+;$stil^{4}$/+. $ovoD1^{rv23}$/+;$stil^{6}$/+), we have not observed a modification of expression of the *otu::lacZ* reporter gene compared to the level of expression found in heterozygous ovo^{-}/ovo^{+} backgrounds (not shown). This suggests that in these conditions the amount of Stil is not rate-limiting.

To determine if *otu* transcription could be induced in somatic cells, we ectopically expressed Stil using the *hsp70::stil* transgene, and tested for activation of *otu::lacZ* in the follicle

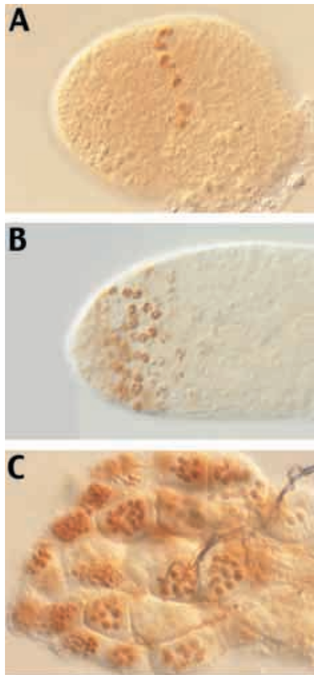

Fig. 6. Expression of Stil is not controlled by *ovo*. Immunodetection of Stil in *ovo* mutants. (A) Ovary from a late third instar larva homozygous for a null allele of *ovo*. A few germ cells are present and express Stil. (B) Testis from an adult male mutant for a null allele of *ovo*. Expression of Stil is similar to wild-type. (C) Ovary from a female heterozygous for the dominant antimorphic allele *ovo*^{D1}. Oogenesis is blocked at stage 4, but germ cells express Stil like in wild-type egg chambers at this stage. Genotypes: *ovo*^{D1rv22} (A,B), *ovo*^{D1} *v*^{24}/+ (C).

cells surrounding egg chambers. Adult flies were subjected to two heat-shocks of 45 minutes at 37°C, separated by 4 hours, and then left at 25°C for 24 hours before dissection. In about one third of egg chambers between stages 4 and 8, expression of *otu::lacZ* was observed in a few follicle cells, most frequently in the polar cells located at the extremities of egg chambers (Fig. 5K,L). We never observed such expression of *otu::lacZ* in many control experiments. This result show that Stil can induce the transcription of *otu* in tissues other than the germline. However, this ectopic activation is rather inefficient, despite the fact that the amount of Stil induced in follicle cells was similar to the amount present in germ cells (not shown). This suggests that the somatic follicle cells may not contain all the components needed for full activity of Stil.

Expression of Stil is not controlled by the *ovo* or *otu* gene products

We next investigated the expression of Stil in male and female gonads of flies mutant for different alleles of *ovo* and *otu.* To determine the possible effect of Ovo, we used two different genetic backgrounds. The first context was in flies homozygous for *ovo* null alleles, in which very few germ cells are present in adult ovaries. These germ cells did not show a clear staining for the Stil protein (not shown). However, because these cells are very unhealthy, lack of Stil expression could be due to the fact that the cells are dying. Therefore, we turned to ovaries of third instar larvae, in which more and healthier germ cells are present. In these ovaries (Fig. 6A), we found *ovo*$^{-}$ germ cells which express Stil similarly to wild-type germ cells. Males mutant for *ovo* null alleles are fertile and their testes do not present any obvious defects. In these testes (Fig. 6B), Stil protein staining appears to be identical to that seen in wild-type testes. We also looked at Stil expression in ovaries from females heterozygous for the dominant antimorphic allele *ovo*^{D1}. This background was tested because this allele has been shown to have a very strong negative effect on the transcription of *ovo::lacZ* and *otu::lacZ* reporter genes (Oliver et al., 1994; Mével-Ninio et al., 1996; Lü et al., 1998). In *ovo*^{D1} heterozygous females, oogenesis stops at about stage 4, but Stil expression seems normal (Fig. 6C).

We also tested whether Stil expression is controlled by *otu*. Both in ovaries and in testes of flies bearing an *otu* null allele, Stil expression was as in wild-type flies (not shown). We conclude from these experiments that neither the *ovo* nor the *otu* gene products are required for expression of the *stil* gene.

Somatic expression of Stil is lethal

In preparation for the experiment of binding of Stil to polytene chromosomes, we experienced difficulties obtaining transgenic lines bearing a *stil* cDNA under the control of the *hsp70* promoter Indeed, only three lines were recovered. They show a relatively weak viability even without heat-shock and we were not able to maintain them as homozygous stocks. This suggested that even low levels of ubiquitous *stil* expression in the soma is lethal. This hypothesis was supported by heat-induction of the *stil* transgene. At every stage of development, except adulthood, a single pulse of *stil* expression (heat shock: 45 minutes at temperatures between 33 and 36°C) was found to be completely lethal. Death occurred within 24-30 hours. Adults appeared to be more resistant since they could survive for several days after either a longer single pulse or 2 pulses given on 2 consecutive days. It should be stressed that these conditions of induction lead to accumulation of Stil protein in somatic cells at levels similar or lower to the levels normally observed in germ cells. Toxicity due to exaggerated overexpression is therefore not likely.

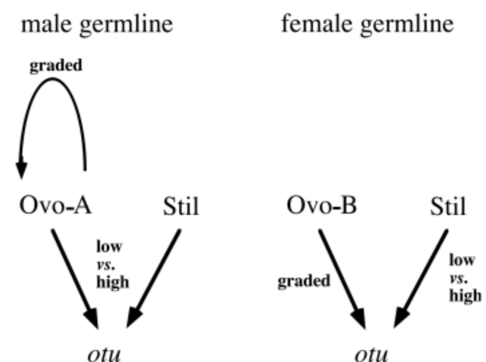

Fig. 7. Model outlining the positive regulatory effects of Ovo and Stil on expression of the *otu* gene in male and female germ cells. Stil has a dramatic dose-independent effect in both sexes. Ovo works differently in the two sexes. In male germ cells, ovo-A has a dose-independent effect on the *otu* promoter and appears to work in a dose-dependent manner on ovo promoters. In female germ cells, Ovo-B has a dose-dependent positive activity on the *otu* promoter, but does not control transcription from *ovo* promoters.

DISCUSSION

Little is known about sex determination in germ cells of *Drosophila*. A few candidate genes, *otu*, *ovo*, *stil*, *Sxl*, *snf* and *fl(2)d*, have been identified on the basis of the phenotypes of mutant alleles and on the altered splicing of *Sxl* pre-mRNAs (see Introduction). In this article, we concentrate on the product of the *stil* gene and on the relationships between *stil*, *ovo* and *otu*.

We show that Stil is expressed in female germ cells throughout development, and transiently in male germ cells, but not in somatic cells. In both sexes, the Stil protein becomes detectable during stage 11 of embryogenesis, shortly after the time at which zygotic transcription starts in germ cells (Van Doren et al., 1998). While expression continues in female germ cells throughout their proliferation and differentiation, male germ cells stop expressing Stil approximately at the time of the last round of pre-meiotic DNA replication (spermatocytes at the beginning of their growth phase). Stil expression in the male germline was not expected since there is no evidence of any requirement for *stil* in the male germline. Interestingly, gratuitous expression has also been found for *otu* and *ovo* (Comer et al., 1992; Oliver et al., 1994; Mével-Ninio et al., 1995), the two other genes whose mutations give phenotypes very similar to those of *stil* alleles.

Ovo and Stil control the transcription of the *otu* gene

The male germline turned out to be a very convenient tissue to test for cross-regulatory interactions between the *otu, ovo* and *stil* genes. Male germ cells have one advantage over female germ cells because null mutations in these three genes have no effect on the male germline (males are fertile), while they kill most if not all female germ cells. Furthermore, surviving female germ cells are not very healthy, which might hinder the analysis of possible alterations of gene expression. Results in male germ cells provide a useful corroboration to the observations made in female germ cells.

We found that the Stil protein is required for high levels of expression of *otu*, but not of *ovo*, in male and female germ cells. Fig. 7 summarizes the data of Oliver et al. (1994), Lü et al. (1998) and results presented in this article. Both Ovo and Stil have positive regulatory effect on *otu* expression in germ cells. However, while Stil has a simple effect (low levels of expression in absence of Stil versus high levels of expression in presence of Stil), Ovo appears to work somewhat differently in the two sexes (Oliver et al., 1994; Lü et al., 1998). The *ovo* gene encodes two types of germline-specific protein isoforms, which all contain a domain of 4 zinc-fingers (Mével-Ninio et al., 1995). The zinc-finger domain binds DNA in vitro in a sequence-specific manner, and several Ovo binding sites have been located in the vicinity of both *ovo* and *otu* promoters, including some sites that overlap with the transcription initiation sites (Lü et al., 1998). The difference between the two types of isoforms results from the use of two different promoters, *ovo*-α (ovo-A isoforms) and *ovo*-β (ovo-B isoforms) (Mével-Ninio et al., 1995). Ovo-B isoforms, which have a shorter N terminus domain, are expressed only in female germ cells and have a dose-dependent positive activity on the *otu* promoter but do not seem to control the transcription of *ovo* promoters (Lü et al., 1998). In male germ cells, Ovo-A isoforms have a dose-independent effect on the *otu* promoter, but appear to work in a dose-dependent manner on their own promoter. Stil does not seem to participate in this autoregulatory effect of Ovo in male germ cells.

We have not been able to show additive or synergistic effect of Ovo and Stil on *otu* expression in double heterozygous mutant females. In other words, the reduction of *otu* expression caused by heterozygosity of *ovo* is not worsened by reducing the $stil^{+}$ gene dosage. This may indicate that in this condition the amount of Stil is not rate-limiting. However, in a situation in which *otu* expression is further reduced (females heterozygous for the dominant antimorphic ovo^{D2} allele), an effect of the amount of Stil was clearly revealed: increased dosage of the $stil^{+}$ gene partially compensated for the negative effect of ovo^{D2}. This observation is fully consistent with the partial phenotypic suppression of the ovo^{D2} mutation by extra doses of $stil^{+}$ previously reported (Pennetta and Pauli, 1997). Genetic interactions had suggested that *ovo*, *otu* and *stil* act in either the same pathway or in parallel pathways (Pauli et al., 1993; Pennetta and Pauli, 1997). Results from Lü et al. (1998) and from this article support the former hypothesis.

Expression of *otu* at the testis apex takes place in mitotically dividing germ cells that contain relatively low levels of the Stil protein. The *otu* gene is not transcribed in the newly formed primary spermatocytes, which express higher levels of Stil. It is possible that Stil has a concentration-dependent effect: low amounts of Stil would activate transcription while higher amounts would turn off the *otu* promoter. Precedent for such concentration-dependent activity has been described for the Krüppel transcription factor (Sauer and Jäckle, 1993).

Chromatin binding proteins, germline development and sex determination

One striking observation described in this article is the widespread association of the Stil protein to chromatin. Analysis of polytene chromosomes, either after ectopic expression in salivary glands or in nurse cells that normally express *stil*, revealed that Stil is preferentially associated with regions of decondensed chromatin (interbands), including puffs. This localization is very similar to that found for RNA polymerase II (Pol II) (Plagens et al., 1976; Krämer et al., 1980), in particular the form of the enzyme containing a hypophosphorylated largest subunit (Weeks et al., 1993). A co-localization of Stil and Pol II on polytene chromosomes is consistent with our observation that Stil controls the transcription of the *otu* gene. The numerous binding sites suggest that Stil probably contributes to the regulation of many genes in the germline.

The importance for germ cell development of proteins that show broad association with chromatin and may exert a general effect on Pol II activity has recently be documented, particularly in *Caenorhabditis elegans*. For example, the product of the *pie* gene contains two putative zinc-finger motifs and is required during early embryogenesis to repress overall Pol II transcription in germline precursor cells and to prevent them from differentiating as somatic cells (Mello et al., 1996; Seydoux et al., 1996). The effect of PIE-1 appears to be mediated by prevention of phosphorylation of the carboxy-terminal repeat domain of the largest Pol II subunit (Seydoux and Dunn, 1997).

Even more striking is the case of the *mes-2*, *mes-3*, *mes-4*

and *mes-6* genes. Maternal-effect mutations in these four genes lead to healthy but sterile offspring characterized by post-embryonic degeneration of the germline. The sex-karyotype of the germ cells is critical for the expressivity of the phenotype since worms bearing 2 X chromosomes are much more sensitive to these mutations than worms bearing a single X chromosome, regardless of the sexual identity of the soma or of the germline (Garvin et al., 1998). This karyotype-dependent requirement is very similar to that found for *ovo* in *Drosophila*, except that *ovo* shows zygotic not maternal requirement (Oliver et al., 1994). A link to gene regulation at the level of chromatin structure became plausible when MES-2 and MES-6 were found to be homologous to two members of the Polycomb group proteins, Enhancer of zeste and Extra sex combs, respectively (Holdeman et al., 1998; Korf et al., 1998). Polycomb group proteins are involved in gene silencing in various situations (reviewed by Pirrotta, 1997; Schumacher and Magnuson, 1997). In *C. elegans*, transgenes arranged in large repetitive arrays are usually active in somatic cells, but silenced in germ cells, a phenomenon that was attributed to some unknown particularity of chromatin organization in the germline (Kelly et al., 1997). This hypothesis received strong support with the recent observation that silenced transgenes can be reactivated by mutations in the four *mes* genes listed above (Kelly and Fire, 1998).

Finally, proteins involved in higher-order chromatin structure are also important in the process of sex determination in mammals: male-to-female sex reversal phenotype has observed in mice mutant for the *M33* gene, an homologue of *Polycomb* (Katoh-Fukui et al., 1998).

The examples reviewed above are meant to stress the likely importance of global gene regulation and higher-order chromatin structure in the development of germ cells as well as in the establishment of particular cell fate such as sexual identity. In the case of *Drosophila*, genetic interactions between some alleles of *ovo* and mutations of either *Polycomb* (Pauli et al., 1995) or *polycomb-like* (B. Oliver and D. Pauli, unpublished data) also point to a role of higher-order chromatin structure in germline sexual identity and differentiation. Given the evidence of a genetic interaction between *ovo* and *stil* (Pennetta and Pauli, 1997), and the extensive chromatin association described in this article, it is plausible that Stil has a rather general effect on transcription. The difference between female and male germ cells would be determined by the interaction with sex-specific partners or by post-translational modifications possibly controlled by the feminizing somatic signals.

Stil is lethal to somatic cells

The *stil* gene is essential in female germ cells for their survival and differentiation. Male germ cells do not require *stil* but tolerate its expression very well. This is in sharp contrast to what we found for somatic cells. Not only do somatic cells not express *stil*, but they are unable to survive with even transient *stil* expression. We suspect that *stil* expression must be under tight silencing in somatic tissues. Further work will be necessary to understand the cause of death of somatic cells expressing *stil*.

We thank L. Glenn for the gift of anti-Otu antibody, B. Oliver for deconvolution of the polytene chromosome immunofluorescences, I. Zhimulev for help with the interpretation of the polytene chromosome labeling and J. Faustino for technical help. We are grateful to J. Andrews, B. Oliver, and U. Schibler for critical reading of the manuscript. This work was supported by a grant of the Swiss National Science Foundation to D. P.

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