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Cell-autonomous and somatic signals control sex-specific gene expression in XY germ cells of *Drosophila*

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Abstract

When XX germ cells develop in a testis they become spermatogenic. Thus, somatic signals determine the sex of genetically female germ cells. In contrast, XY germ cells experimentally transferred to an ovary do not differentiate oogenic cells. Because such cells show some male characteristics when analyzed in adults, it was assumed that XY germ cells autonomously become spermatogenic. Recently, however, evidence showing that a female soma feminizes XY germ cells was reported. The conclusion was drawn that the sex determination of XY germ cells is dictated by the sex of the soma. We monitored the fate of XY germ cells placed in a female environment throughout development. Here we report that such germ cells respond to both cell-autonomous and somatic sex-determining signals, depending on the developmental stage. Analyzing the expression of sex-specific molecular markers, we first detected autonomous male-specific gene expression in XY germ cells embedded in female embryos and larvae. At later stages, however, we found that sex-specific regulation of gene expression within XY germ cells is influenced by somatic gonadal cells. After metamorphosis, XY germ cells developing in a female soma start expressing female-specific and male-specific markers. Transcription of female-specific genes is maintained, while that of male-specific genes is later repressed. We show that in such XY germ cells, the female-specific gene *Sex-lethal* (*Sxl*) is activated. Within the germline, *Sxl* expression is required for the activation of a further female-specific gene and the repression of male-specific genes. We thus report for the first time the existence of downstream targets of the gene *Sxl* in the germline.

Keywords: Sex determination; Germline; Sex-specific gene expression; *Sex-lethal*

1. Introduction

In *Drosophila*, the sex of somatic cells is determined cell-autonomously. A series of well characterized genes acting in a cascade ensure, through a mechanism that largely uses sex-specific splicing, that cells with one X-chromosome (XY or XO) display male, and cells with two X-chromosomes display female features. Three genes that are expressed in a sex-specific manner play a crucial role. *Sex-lethal* (*Sxl*), the key gene for sex determination in the soma, is specifically activated in cells that have a female X:A ratio (reviewed by Cline, 1993). Its product enforces a female-specific splice on the transcript of *transformer* (*tra*), whose product in turn regulates the sex-specific splicing of *double sex* (*dsx*) (Sosnowski et al., 1989; Inoue et al., 1990, 1992).

Most of the genes of this sex-determining cascade are dispensable in germ cells (Marsh and Wieschaus, 1978; Schüpbach, 1982). *Sxl* is necessary for proper development of female germ cells (Schüpbach, 1985; Steinmann-Zwicky, 1988; Steinmann-Zwicky et al., 1989), but this gene, which has several functions in the germline, may not be required until metamorphosis in this tissue (Steinmann-Zwicky, 1994a; Bopp et al., 1999). Since sex-specific differences between male and female germ cells are already found in embryos (Poirié et al., 1995; Staab et al., 1996), other genes must determine the sex of germ cells.

Experiments involving the transplantation of pole cells, the progenitors of the germ cells, indicated that sex determination of germ cells is complex (Steinmann-Zwicky et al., 1989). In these experiments, XX pole cells transplanted into male hosts became spermatogenic when scored in adults, which shows that XX germ cells non-autonomously differentiate according to the sex of the surrounding somatic cells. That somatic cells determine the sex of XX germ cells was confirmed by other results. Masculinized XX animals often possess spermatogenic germ cells that can differentiate non-functional sperm (Nöthiger et al., 1989; Steinmann-Zwicky, 1994b) and that can express a male-specific molecular marker as soon as gonads are formed in the embryo (Staab et al., 1996).

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When XY germ cells were transplanted into XX females, the results were less clear. Scored in adults by morphological criteria, germ cells were found to be either very small and undifferentiated or larger and spermatogenic (Steinmann-Zwicky et al., 1989). Because of these spermatogenic cells, the conclusion was drawn that XY germ cells autonomously choose the male pathway, irrespective of the sex of the surrounding somatic cells. The experiments showed that XY germ cells developing in a female do not differentiate oogenic stages when analyzed by morphological criteria. They did not prove, however, that the analyzed germ cells are male. Small undifferentiated germ cells might be asexual, or they might be undifferentiated female cells, or they might express both male- and female-specific genes. Even cells that appear to be spermatogenic might not truly be male when other criteria are scored.

Recently, it was reported that XY germ cells developing in a feminized XY animal can express female-specific gene products (Waterbury et al., 2000). This led to the conclusion that surrounding somatic cells dictate the sexual identity to XY germ cells. We here show that this is only partially true and that the situation is more complex. In the former studies, germ cells were scored in adults only. We used molecular markers to study the sex-specific gene expression of individual XY germ cells that are placed in a female environment through all stages of development. We found that the pattern of gene expression varies depending on space and time and that it is not possible to draw general conclusions from a quantitative analysis of just one developmental stage.

We find that in a female soma XY germ cells initially autonomously express male-specific genes. Later, somatic signals control sex-specific gene regulation in such germ cells. Our results thus show that there is a cell-autonomous signal that makes XY germ cells different from XX germ cells and that there is a somatic sex-determining signal that acts on XY germ cells.

2. Results

2.1. XY germ cells express a male-specific marker when developing in a truly female environment

We used *mgm1*, the earliest male-specific germline marker described so far, to test for sex-specific gene expression in embryos. *mgm1* is expressed in male embryonic germ cells and in male germline stem cells throughout development. The germline of females does not express the marker, but germ cells of XX animals with a masculinized soma do. This shows that in XX germ cells *mgm1* is controlled by somatic signals (Staab et al., 1996).

Our aim was to test whether genetically male germ cells (XY) that develop in a female environment express *mgm1*. We transplanted pole cells from embryos carrying *mgm1* into agametic host embryos that could be sexed due to a paternally introduced X-linked marker, and analyzed the resulting female XX embryos. The percentage of host females with donor germ cells that all expressed *mgm1* corresponded exactly to the percentage of females expected to have integrated XY germ cells carrying *mgm1* (Table 1, Fig. 1A,B). Therefore, XY germ cells developing in a truly female XX environment autonomously express the male-specific marker *mgm1*.

2.2. Male-specific gene expression is initiated, but not maintained, in germ cells of feminized XY animals

We monitored the sex-specific identity of XY germ cells developing in a female environment at different developmental stages, using pseudofemales of genotype *XY;hs::tra/+*. In these animals, the hsp70 promoter drives the expression of *tra*, which results in feminized XY flies. The promoter, which is expressed in somatic cells, has a constitutive basal activity at 25°C that transforms XY animals into phenotypical females, called pseudofemales (McKeown et al., 1988). In the germline and in particular in primary spermatocytes, the promoter is inefficient even after heat shock treatment (Michaud et al., 1997; Rørth, 1998). The XY germ cells of pseudofemales appear to be spermatogenic as they can differentiate spermatocytes (Steinmann-Zwicky et al., 1989). The somatic cells, however, possess all the qualities required to ensure the oogenic differentiation of germ cells, as XX germ cells that are transplanted into such pseudofemales become oogenic and produce eggs (Steinmann-Zwicky, 1994b).

To assess the sexual identity of the tested XY germ cells at the molecular level, we monitored the expression of three male-specific and one female-specific enhancer-trap lines (this chapter), as well as that of the female-specific gene *Sxl* (see later). Here is a list of the sex-specific enhancer-trap lines used: (1) *mgm1* is expressed in male germline stem cells throughout development (Staab et al., 1996); (2) 606 is expressed in male germline stem cells of third instar larvae and adults (Gönczy et al., 1992); (3) P20 shows expression in male gonial cells in all three larval stages and in adult testes (see Fig. 3G) (Janzer and Steinmann-Zwicky, unpublished data); (4) Q13d is expressed in early stages of adult oogenesis (see Fig. 3H) (Fasano and Kerridge, 1988), but not during spermatogenesis (Gönczy, 1995). In prospective ovaries, we first detect it in second instar larvae.

Germ cells of *XY;hs::tra* pseudofemales revealed that male-specific gene expression is initiated, but not maintained in these cells. The earliest marker, *mgm1*, is expressed in all germ cells of the embryonic gonads. All three male markers, *mgm1* as well as 606 and P20, whose expression is detected somewhat later, were expressed during larval stages. The analysis of animals of different stages of development revealed that the number of gonads with male-specific gene expression dramatically decreased with age, such that none of the markers were expressed anymore when flies hatched (Figs. 1C–G and 2).

In XY pseudofemales, female-specific gene expression

Table 1
XY germ cells expressing *mgm1* in XX embryos

Transplantation series	Genotype of donors (%)	Expected staining in XX host[a]	Observed staining in XX host (%) (*n*)
A	*XX;mgm1/mgm1* (12.5)	−	
	XX;mgm1/Sco (25.0)	−	
	XX;Sco/Sco (12.5)	−	
	***XY;mgm1/mgm1* (12.5)**	**+**	
	***XY;mgm1/Sco* (25.0)**	**+**	**35** (20)
	XY;Sco/Sco (12.5)	−	
B	*XX;mgm1/+* (25.0)	−	
	XX;Sco/+ (25.0)	−	
	***XY;mgm1/+* (25.0)**	**+**	**24.3** (74)
	XY;Sco/+ (25.0)	−	

[a] Expected if XY germ cells autonomously express *mgm1*. If expression is non-autonomous, no staining of XY germ cells is expected in XX hosts.

was found neither in the second nor third larval instar, nor shortly after eclosion (Fig. 2), although in XX animals, Q13d expression is found in all gonads at these stages.

In a later chapter, we will see that XY germ cells of adult pseudofemales start expressing both male and female markers. But before we report these results, we will describe experiments exploring why the expression of male-specific markers is lost.

2.3. Germline stem cells are present in adult XY pseudofemales

In wild-type, the male markers *mgm1* and 606 are expressed specifically in germline stem cells. The lack of stem cell staining in pseudofemales carrying this marker could be due to a progressive loss of stem cells, or to the fact that stem cells lose their stem cell identity, or to the loss

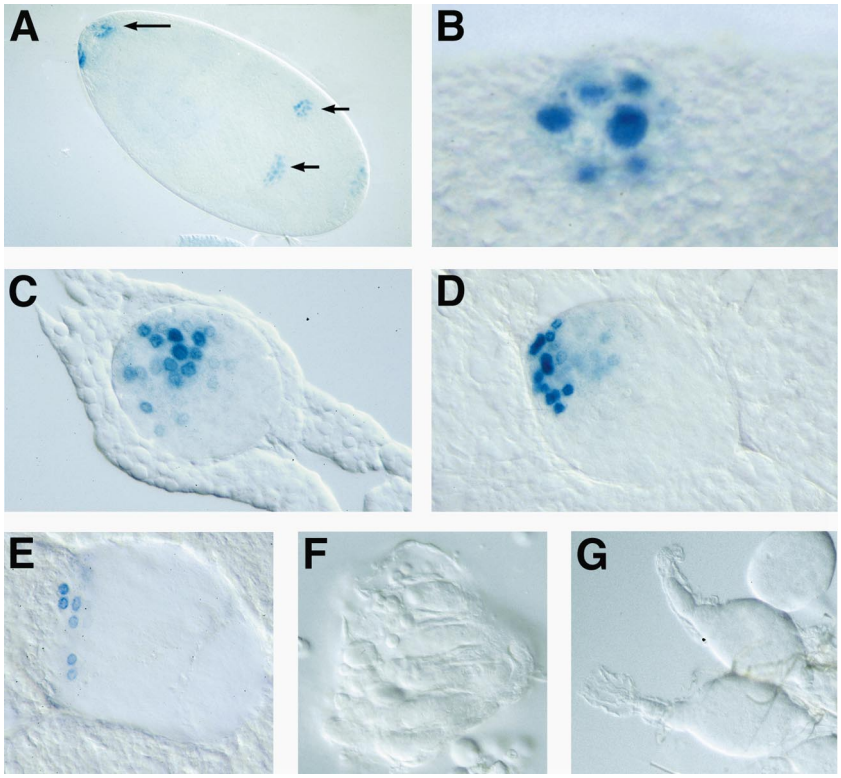

Fig. 1. Initial autonomy of male-specific *mgm1* expression is progressively lost in later stages of development. (A) XY germ cells transplanted into female XX embryos, identified as such by *Dfd::lacZ* expression (long arrow), autonomously express *mgm1* (short arrows); (B) same situation as in (A), but at a higher magnification. (C–G) Progressive loss of autonomous *mgm1* expression in XY germ cells developing in *XY;hs::tra/+* pseudofemales. Gonads of second instar larvae (C), third instar larvae (D), young pupae (E), 2-day-old pupae (F) and freshly eclosed flies (G). Similar results were obtained with the male-specific markers P20 and 606.

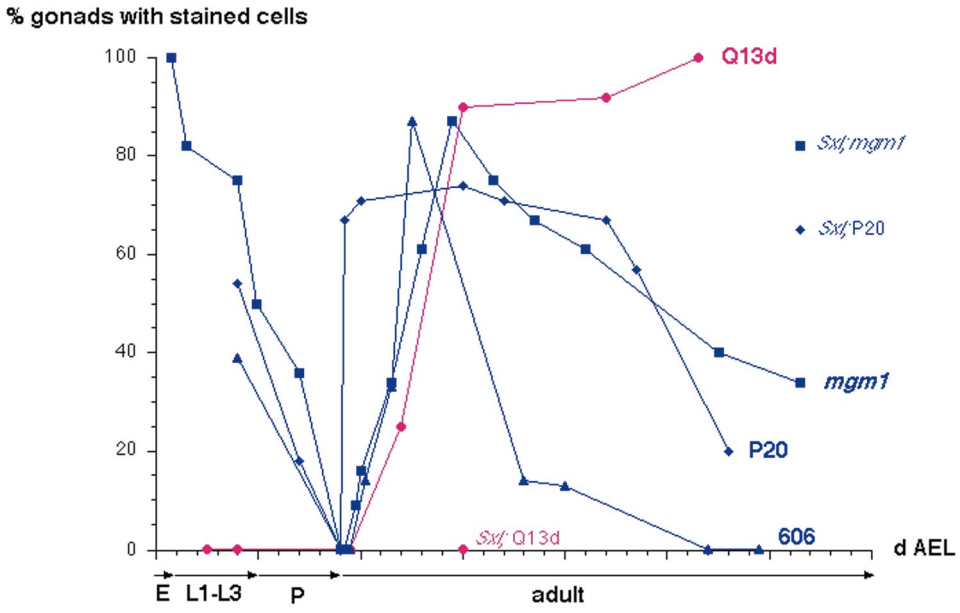

Fig. 2. Percentage of ovaries of XY pseudofemales with germ cells expressing sex-specific markers at various stages of development. Blue, male-specific markers *mgm1*, P20 and 606; pink, female-specific marker Q13d. Initial autonomous male-specific gene expression (*mgm1*, P20 and 606) is progressively lost, and female-specific expression of Q13d is not initiated till metamorphosis. No sex-specific gene expression is found when flies eclose. After about 0.5 days, male- and female-specific genes are activated. Similar expression profiles are found for all tested lines till 6 days after eclosion. Then, a sex-specific difference can be observed: male-specific gene expression is progressively lost, but the number of ovaries expressing Q13d continues to increase. After 17 days, the female-specific marker is expressed in all analyzed gonads. Q13d expression is not initiated in XY ovaries lacking *Sxl* (allele Sxl^{f1}). Furthermore, male-specific gene expression is not lost in adult XY ovaries that do not have a functional *Sxl* allele. Thus, *Sxl* activates female-specific and represses male-specific genes in XY germ cells. Number of gonads tested: embryos, $n = 50$; larvae, $n = 30$; pupae, $n = 10$; adults, $n = 25$. dAEL, days after egg laying.

of male identity. To distinguish between these alternatives, we checked whether germline stem cells are present in the analyzed pseudofemales.

During gametogenesis, germline stem cells normally divide asymmetrically to yield again a stem cell and a daughter cell. This daughter cell will undergo four mitotic divisions, producing a syncytium of 16 cells that are all connected by intercellular bridges called ring canals (reviewed by Fuller, 1993; Spradling, 1993; de Cuevas et al., 1997). The different stages can be distinguished, as all cells of a syncytium are connected by an organelle called fusome whose form and size is stage-specific, and that can be detected by staining with anti-HTS or anti-α-Spectrin antibody (Pesacreta et al., 1989; Lin et al., 1994; Lin and Spradling, 1995). In germline stem cells and in their daughters, these antibodies detect a spherical structure called spectrosome which represents an early stage of fusome development. A typical feature of stem cells is the apical location of the spectrosome which occasionally stretches towards the basal terminal filament cells forming a tail-like structure (Lin and Spradling, 1997). In later stages, the spectrosome elongates and branches to form a fusome connecting all 16 cells of a syncytium. At this stage, fusomes are easily detectable in males, but hardly visible in females. At the tips of germaria of *XY;hs::tra* ovaries stained with anti-HTS antibodies, we found apically located spherical spectrosomes which sometimes exhibit the typical tail-like structure (Fig. 3A). In more posterior regions, cells of ovaries of adult *XY;hs::tra* pseudofemales mostly had branched fusome structures that connected up to 16 cells. In some cases, spectrosomes were also detected. This experiment shows that stem cells are present in ovaries of adult *XY;hs::tra* pseudofemales, but do not express the male stem cell markers *mgm1* and 606.

2.4. No loss of germline-specific gene regulation in ovaries of pseudofemales

Since sex-specific gene expression in germ cells of XY pseudofemales is abolished at metamorphosis, we wondered whether, under our experimental conditions, abnormal development of germ cells leads to a general loss of germline-specific gene expression. To test this, we analyzed ovaries of pseudofemales for the expression of two germline-specific constructs, which are normally expressed in germ cells of both sexes: BC69 in which *lacZ* is driven by the *vasa* promoter (Couderc, J.-L. and Laski, F., pers. commun.; Rodesch et al., 1995; Heller and Steinmann-Zwicky, 1998a) and *ovo::lacZ* (Mèvel-Ninio et al., 1995). Both germline-specific markers are expressed in germ cells of all *XY;hs::tra* animals immediately after eclosion and also later, 2, 4, 9 and 20 days after hatching (Fig. 3B).

In the next chapter, we will see that sex-specific gene expression is reinitiated in aged adults. To test whether multicellular cysts of adult pseudofemales start expressing *lacZ* because of a general and unspecific activation of gene

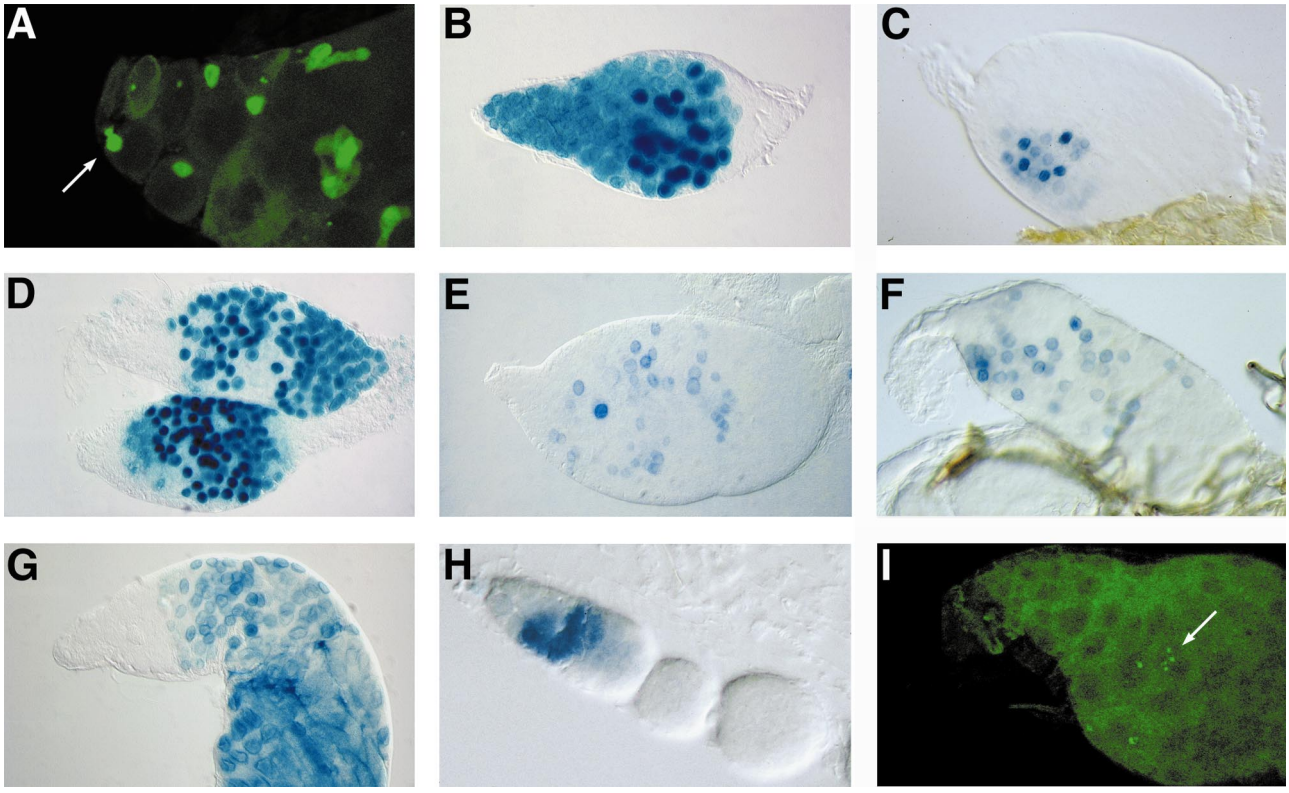

Fig. 3. Monitoring germline-specific gene expression. (A) Anti-HTS labeling (arrow) reveals the presence of germline stem cells in adult *XY;hs::tra/+* ovaries (identified due to the apically located spectrosome with tail-like structures). (B) Germline-specific gene expression as revealed by BC69 (vasa) is maintained in germ cells of *XY;hs::tra/+* pseudofemales at eclosion. (C–F) Multicellular germ cell cysts of XY pseudofemales express *mgm1* (C), P20 (D), Q13d (E) and 606 (F). (G) Expression pattern of P20 in adult wild-type testis. (H) Expression pattern of Q13d in adult wild-type ovary. (I) Anti-SXL antibodies identify SXL protein in cytoplasm and nuclear foci (arrow) of XY germ cells in ovaries of pseudofemales.

expression in our experiments, we further analyzed flies carrying a *lacZ* reporter construct under the control of three different promoters that normally drive the expression of genes exclusively in somatic cells: *hedgehog* (*hh*), *engrailed* (*en*) and *hunchback* (*hb*). In each case, we analyzed pseudofemales that were 2–8 days old. While we found specific staining of somatic cells, as reported by others (Forbes et al., 1996a,b; Zhang and Kalderon, 2000), we never detected stained germ cells in these animals. These results show that germline-specific regulation of genes in XY germ cells developing in a female environment is intact.

2.5. In adult ovaries, XY germ cells express both male- and female-specific markers

Several hours after eclosion, germ cells of *XY;hs::tra* flies begin displaying both male- and female-specific gene expression (Figs. 2 and 3C–F). For the three male-specific markers, similar results were obtained. Blue cells appeared after eclosion; the number peaked around 4–6 days and severely dropped thereafter. The two stem cell markers, *mgm1* and 606, are not expressed within the germarium where stem cells are located, but in the middle of multicellular cysts (Fig. 3C,F). To test whether the cells expressing *mgm1* in 4–8-day-old adult XY pseudofemales have stem cell properties, we co-labeled XY ovaries expressing *mgm1* with anti-β-galactosidase (anti-β-Gal) and anti-HTS antibodies, which reveal the spectrosome structure. None of the cells expressing β-Gal possessed a spectrosome. Thus, cells that do not have general stem cell properties can activate the male marker *mgm1*.

The temporal pattern of expression of the female marker Q13d in adults was different from that of the three male-specific markers. Until about 6 days after eclosion, its expression was similar. Then, the number of ovaries displaying female-specific gene expression continued to increase, such that after 17 days all animals contained cells expressing Q13d, while male-specific expression was nearly gone (Fig. 2).

We tested for the presence of a second female-specific germline marker, the product of the gene *Sxl*. In ovaries of wild-type females, non-differentiated germ cells show a strong cytoplasmic accumulation of SXL protein. As the germ cells differentiate, protein also accumulates in specific nuclear foci (Bopp et al., 1993). To test whether *Sxl* is expressed in germ cells of pseudofemales, we stained XY ovaries with anti-SXL antibody. We found cytoplasmic SXL protein in all XY germ cells (Fig. 3I). Furthermore, we found some cells displaying Sxl accumulation in nuclear foci, a characteristic of more differentiated XX germ cells

(Fig. 3I, arrow). These data show that female somatic cells induce female-specific gene expression in XY germ cells.

2.6. Female-specific gene expression requires Sxl

The gene *Sxl* has many different functions in both somatic cells and germ cells. In the soma, it is the switch gene whose product controls sex determination and dosage compensation to achieve female-specific gene expression (reviewed by Cline, 1993; Cline and Meyer, 1996; Schütt and Nöthiger, 2000). In the germline, *Sxl* is required for cells to become oogenic (Schüpbach, 1985; Steinmann-Zwicky et al., 1989) and later, during meiosis, for recombination (Bopp et al., 1999), which only occurs in females.

Studying the expression of the female-specific marker Q13, we found that this marker is not expressed in Sxl^{f1}/*Y;hs::tra* pseudofemales which lack *Sxl* activity. We therefore conclude that *hs::tra* expression triggers the female-specific expression of Q13d by a mechanism that involves *Sxl*. Besides alleles that totally abolish *Sxl*, mutations of *Sxl* are known that specifically destroy germline functions, while somatic functions are intact. In XX ovaries homozygous for the mutation Sxl^{f4}, SXL protein is found in somatic cells and in the cytoplasm of germ cells. The characteristic foci of more differentiated cells, however, are missing (Bopp et al., 1993). Pseudofemales of genotype Sxl^{f4}/*Y;hs::tra* strongly expressed Q13d in their ovaries, showing that the localization of SXL in specific foci is not required for the expression of Q13d. Double staining XY ovaries carrying a wild-type allele of *Sxl* and Q13d with anti-β-Gal antibodies as well as with anti-SXL antibodies, we found, besides the ubiquitous cytoplasmic SXL, many cells with SXL-positive foci, but none of these expressed Q13d. Other cells were found to express the female-specific enhancer-trap. This confirmed that the presence of localized foci of SXL protein is not a prerequisite for Q13d expression.

To determine whether somatic or cytoplasmic germline SXL protein causes female-specific gene expression within the germ cells, we analyzed pseudofemales with Q13d, but carrying either the mutation *ovarian tumor*^{1} (otu^{1}) or *sans fille*^{1621} (snf^{1621}). In XX germ cells mutant for otu^{1} or snf^{1621}, *Sxl* is spliced in the male-specific mode. Therefore, no SXL protein is made in the germline (Pauli et al., 1993; Bopp et al., 1993). Somatic SXL protein, however, is present and unchanged compared to females carrying a wild-type allele of *otu* or *snf*. In XY animals, somatic SXL protein, which is triggered as a result of a female X:A ratio, is absent. However, there is a remote possibility that *Sxl* is activated in somatic cells of XY pseudofemales that are feminized by *hs::tra*, and that this somatic *Sxl* activity is required for germ cells to express Q13d. If this were the case, pseudofemales that lack *otu* or *snf* functions should express Q13d because they would possess the somatic, and only lack the germline activity of *Sxl*.

In both types of pseudofemales, genotype otu^{1}/*Y;hs::tra* and snf^{1621}/*Y;hs::tra* with Q13d, the female marker was not expressed. This was not due to a general repression of genes, as in flies of the same genotype, the male-marker *mgm1* was expressed as in controls. In rare cases, ovaries contained one to five cells expressing Q13d, which might be expected because both mutations otu^{1} and snf^{1621} are partial loss-of-function alleles. In contrast, all control ovaries contained a large number of cysts with up to 70 cells expressing Q13d. These results show that cytoplasmic SXL is required in germ cells to express the female marker Q13d, and that it is not a somatic *Sxl* function that feminizes the XY germ cells.

2.7. Sxl represses male-specific germline markers

To determine whether the downregulation of male-specific germline markers requires *Sxl*, we analyzed XY pseudofemales carrying *mgm1* or P20, but lacking *Sxl* activity (Sxl^{f1}). We found that the first downregulation which occurs during larval stages is retained, but that the second downregulation that we observe in older adults (Fig. 2) is abolished. After 22 days, 82% of pseudofemales carrying *mgm1* and 65% of pseudofemales carrying P20 possessed staining cells (see Fig. 2). Similar results were obtained with flies carrying otu^{1} or snf^{1621} mutations, which lack germline activity of *Sxl* (data not shown). Thus, in the absence of *Sxl*, the number of ovaries expressing male markers remains constant, while in flies expressing *Sxl* in the germline, the number decreases dramatically. Therefore, *Sxl* represses male-specific gene expression in XY germ cells of adult pseudofemales.

3. Discussion

Evidence published some time ago suggested that XY germ cells autonomously enter the male pathway when placed in a female soma (Steinmann-Zwicky et al., 1989). Recently, however, it was reported that XY germ cells respond to somatic sex-determining signals (Waterbury et al., 2000). The present analysis shows that sex-specific gene expression of XY germ cells placed in a female environment varies according to the developmental stage, and that both cell-autonomous and somatic signals determine the sex of XY germ cells.

3.1. XY germ cells autonomously initiate male-specific gene expression

We used sex-specific molecular markers to assess the sexual identity of XY germ cells that develop in a female environment. Our results show that all XY germ cells transplanted into a true female at blastoderm later express a male-specific marker gene. Furthermore, we show that in feminized larvae, XY germ cells express three male-specific markers, but do not express a female-specific marker. Analyzing feminized XY animals can be tricky. If male-

specific gene expression is found in such animals, it can always be argued that the feminization was not complete (Cline, 1979; Gergen, 1987; Steinmann-Zwicky, 1988, 1994b; Bernstein et al., 1995). XY embryos that are feminized by the mutations Sxl^{M1} or Sxl^{M4} express the male-specific germline marker *mgm1* (Staab et al., 1996). So do germ cells of *XY;hs::tra* pseudofemales (this paper) which display a second spermatogenic feature, as they have a male-specific rate of germ cell divisions during early larval stages (Steinmann-Zwicky, 1994b). To exclude the possibility that XY germ cells become masculinized by male somatic signals before somatic feminization takes place, it was important to identify the sexual identity of XY germ cells in a truly female XX environment. Our results show that such cells are male.

Evidence for autonomous male development of genetically male germ cells had been obtained previously. XY and XO germ cells transplanted into XX females at blastoderm and analyzed in adult ovaries were sometimes scored as spermatogenic by morphological criteria. A prominent male-specific feature was the appearance in XO germ cells of specific crystals that are only found in spermatogenic germ cells lacking a Y chromosome (Steinmann-Zwicky et al., 1989). Using antibodies detecting female- or male-specific components of the fusomes, Hinson et al. (1999) found that in adult pseudofemales a subset of germ cells has initiated spermatogenic development containing male-like polyfusomes. Mostly morphological criteria that are scored in adults only were often thought of as being too imprecise to score the sex of germ cells. However, molecular markers were not available in early studies, and the experimental conditions did not allow for an analysis of the initial sex-specific development of the XY germ cells in female embryos and larvae. We here report that male- but not female-specific genes are expressed in XY germ cells developing in female embryos and larvae. Thus, XY germ cells initially enter the male pathway irrespective of the sex of their environment.

3.2. Somatic tra activity controls the expression of sex-specific genes in the germline

At some point of development, *tra* activity feminizes XY germ cells. In germ cells of adult pseudofemales, we detect Q13d transcription and SXL protein. Since the hsp70 promoter driving the feminizing *tra* gene is ineffective in the germline (Michaud et al., 1997; Rørth, 1998), and since *tra* is dispensable in germ cells (Marsh and Wieschaus, 1978), the *hs::tra* construct must act in the soma rather than in the germline to feminize germ cells of XY flies. There, TRA controls the differentiation of the ovary and the production of feminizing somatic signals.

A much stronger feminization than the one we found was described by Waterbury et al. (2000). Besides the amplification by RT-PCR of female-specific *Sxl*, *orb* and *bruno* transcripts in XY ovaries, the authors also report that differentiated nurse cells, oocytes and mature eggs have been seen in exceptional cases in ovaries of pseudofemales. The karyotype of such cells, however, cannot be analyzed, because the authors are unable to reproduce the observation, as they state themselves in the paper in which they describe their finding. In the course of this study, we have analyzed more than 2000 ovaries of pseudofemales feminized by different *hs::tra* constructs including the ones also used in the other report. Furthermore, we have analyzed numerous cases of XX ovaries containing XY germ cells (Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1993, 1994b). Neither we nor others found any evidence suggesting that XY germ cells could differentiate advanced oogenic stages in ovaries. Therefore, this possibility can be ruled out.

Experiments describing the requirement of germ cells for the gene *otu* already suggested that XY germ cells might respond to somatic feminizing signals. Although only XX germ cells normally require *otu* for survival, it was shown that XY germ cells also require *otu* function when developing in a female environment (Nagoshi et al., 1995). The signals must act at or before pupal stages, as mutant *otu* germ cells die at pupariation (Staab and Steinmann-Zwicky, 1996). The more recent finding, however, that XY germ cells of pseudofemales contain male-like polyfusomes (Hinson et al., 1999) provided no evidence for a feminization of these cells. Since abnormal cells with short fusomes and unusual ring canals were also found, it was thought that a female soma disrupts XY gametogenesis, but that it is not capable of inducing oogenic differentiation.

Now we show that a female soma feminizes XY germ cells during larval and pupal phases. During the feminization process, male-specific genes are repressed and *Sxl* is activated. *Sxl* in turn activates the female marker Q13d. The feminization occurs in spite of the presence of male gene products, such as male fusome structures and male-specific crystals, which are formed during larval stages when XY germ cells autonomously express male-specific genes. Therefore, male and female products are found in such cells. The occurrence of both male and female gene expression was initially not understood. Many scientists have tried to analyze the sex of abnormal germ cells, for example germ cells carrying mutations of *Sxl*, by amplifying sex-specific gene products by RT-PCR. In these experiments, ovaries containing abnormal germ cells are pooled and the presence of transcripts is monitored (Pauli et al., 1993; Bae et al., 1994; Horabin et al., 1995; Waterbury et al., 2000). Our results show that any sex-specific probe is expected to be amplified in such an experiment. This, however, will not reveal the sexual identity of the individual tested cells, and in particular it will not show changes in sex-specific gene expression as flies age. In our study, we tested for sex-specific gene expression in single cells individually and we analyzed animals at different stages of development. Therefore, we could observe that individual cells can express male- or female-specific genes and that the ability

of cells to respond to sex-determining somatic signals depends on the X:A ratio and the developmental stage.

3.3. A specific germline function of Sxl

In undifferentiated germ cells of XX ovaries, SXL protein is present as a ubiquitous cytoplasmic molecule. In later stages, the protein is also found in nuclei localized in specific foci (Bopp et al., 1993). So far, no specific function can be assigned to the cytoplasmic nor to the nuclear SXL protein. Furthermore, no target of *Sxl* was known in the germline. Targets of *Sxl*, such as *tra* or *msl-2*, or *dsx* which is indirectly controlled by *Sxl*, have been identified, but these targets are all controlled by somatic SXL (Boggs et al., 1987; Cline, 1993; Stuckenholz et al., 1999). We here show that Q13d is a target of SXL, and that cytoplasmic but not the localized nuclear SXL protein is required for the activation of Q13d. We predict that the activation by SXL is indirect, because we monitor female-specific transcription. SXL, an RNA-binding protein, does not regulate the transcription of genes. Rather, acting post-transcriptionally in somatic cells, it controls the alternative splicing of the sex-determining gene *tra* (Sosnowski et al., 1989; Inoue et al., 1990) and represses translation of MSL-2 which is required to obtain a male-specific level of expression of X-linked genes (Kelley et al., 1997). Furthermore, SXL has been postulated to interact with specific sites of early expressed X-chromosomal RNAs to achieve an early *msl-2* unrelated form of dosage compensation (Kelley et al., 1995).

The germline function of *Sxl* is required for the activation of a female and the repression of several male markers. Our results show that male gene activity is gradually repressed during larval stages in germ cells of XY pseudofemales. Nevertheless, transient expression of male markers is found in adults. After hatching, germ cells of XY pseudofemales strongly proliferate to yield multicellular cysts. It is likely that sex-determining somatic signals are formed, but that these signals cannot reach all the rapidly proliferating cells immediately. This results in a transient loss of sex-specific gene regulation which is only reestablished later, when signals reach their target genes. We here report a new function for the gene *Sxl*, as we show that within the germline *Sxl* is required to establish a somatically controlled sex-specific gene regulation.

3.4. What do our observations teach us about the wild-type situation?

Our results show that the maintenance of male-specific gene expression in the germline depends on interactions with a male soma. There are two alternative explanations as to why the expression of male-specific germline genes is progressively lost in XY germ cells that develop in ovaries. Somatic testis cells might send signals required for maintenance, or somatic ovarian cells might send signals to repress male-specific gene expression. In male and female embryos that do not have gonads, germ cells can express the male-specific enhancer-trap *mgm1* (Heller and Steinmann-Zwicky, 1998b). This suggests that an ovary-derived repressing signal prevents male-specific gene expression in germ cells of wild-type females. However, not all germ cells express the male marker in XX embryos lacking gonads, and *mgm1* expression is always weaker than in germ cells that are integrated into embryonic testes. Thus, a male somatic signal must enhance male-specific gene expression in germ cells. This shows that interactions between somatic gonadal cells and germ cells are of fundamental importance not only in XX but also in XY animals.

We further learn that *tra*-dependent signals that must originate from somatic cells activate female-specific and repress male-specific germline genes. This does not only happen in XY germ cells developing in an ovary, but also in XX germ cells. In XX females, the signals act earlier than in XY pseudofemales, as in wild-type ovaries we see Q13d expression already in second instar larvae. This is probably because in wild-type females, there are clear, non-conflicting signals, while in our experimental situation autonomous male signals determine the fate of germ cells before female somatic signals are formed. Another alternative is that in somatic ovarian cells of wild-type females, feminizing signals are not only controlled by *tra* but also by *Sxl*. The hypothesis that somatic SXL might be required for feminization of the germline has emerged before. XX germ cells that develop in an XY male will become spermatogenic. XX germ cells that develop in an XX male (e.g. genotype *XX;tra/tra*) become either spermatogenic or oogenic (Nöthiger et al., 1989; Oliver et al., 1993). The difference between the two types of males is that XX males have somatic *Sxl* activity. In fact, XX males lacking the somatic sex-determining function of *Sxl* had purely spermatogenic cells (Nöthiger et al., 1989). These observations led to the view that an early step of germline sex determination which occurs in the embryo might be followed by a second step: "The production of a late feminizing signal could be controlled by the somatic function of *Sxl*, through a pathway that is unrelated to *tra*, *tra-2*, *dsx* and *ix*" (Steinmann-Zwicky, 1992). The idea that the feminizing signal should act late was based on the observation that *XX;tra/tra* gonads are initially male, that they appear to be feminized during larval stages and that nurse cells are differentiated during metamorphosis (Seidel, 1963; Steinmann-Zwicky, 1994a).

Although we here report the late feminization of germ cells, we also show that it is somatic *tra* and not somatic *Sxl* that feminizes the germ cells. Feminizing *tra* function is sufficient to activate *Sxl* in germ cells, but *tra* function is not necessary for this activation, as in *XX;tra/tra* pseudomales, oogenic germ cells and *Sxl* activity can be detected (Nöthiger et al., 1989; Horabin et al., 1995). There must therefore be a certain redundance in the genetic control of feminizing signals, which ensures that the sex of germ cells is properly differentiated.

3.5. Conclusions

XX germ cells were shown to adapt to their environment. In females they express female-specific and in males they express male-specific genes. Now it becomes clear that XY germ cells also respond to somatic signals. Here we show that XX and XY germ cells differ in an initial phase during which XX germ cells adapt to the sex of the surrounding cells and XY germ cells autonomously enter the male pathway. After this initial phase, however, both types of germ cells develop in a non-autonomous way.

In mammals, XX and XY germ cells do not behave differently early in development. Both types of germ cells are masculinized by somatic signals emanating from the male genital ridge from which the testis is differentiated in mice (McLaren and Southee, 1997). In *Drosophila*, sex-determining signals do not only come from one tissue, the testis. Rather it seems that different sex-specific signals act in a complex and partly redundant way to control the expression of sex-specific genes in the germline. To understand how somatic signals derived either from the testis or from the ovary control gene expression within germ cells, it will be interesting to learn the nature of the molecules that form and transmit the sex-determining somatic signals.

It will also be interesting to learn the molecular nature of the autonomous difference between XX and XY germ cells. It is known that the elements counting the X:A ratio of somatic cells do not perform a similar function in germ cells (Steinmann-Zwicky, 1993; Granadino et al., 1993). Other elements must thus form the molecular basis of the germline X:A ratio, the autonomous signal which causes XX and XY germ cells to respond differently to somatic signals.

4. Experimental procedures

4.1. Pole cell transplantation

To transplant pole cells, we largely followed the technique described by Van Deusen (1976), slightly modified by Steinmann-Zwicky et al. (1989). Agametic hosts were produced by crossing homozygous *TMII*^{gs1} females (Erdélyi et al., 1995), which produce progeny lacking germ cells due to a maternal effect, with males carrying the X-linked *Dfd::lacZ*-construct (F1-70.2′; Zeng et al., 1994). The marker allowed us to identify the sex of the host embryos, since all female progeny display *Dfd::lacZ* expression. As donors, we used embryos deriving from our *mgm1* strain (*CyO, mgm1*/*Sco*; series A), or we collected embryos from *w*^{1118} females crossed with *mgm1* males (series B). The marker *mgm1*, which is expressed in male germline stem cells, reveals the earliest sex-specific gene expression in germ cells (Staab et al., 1996).

Host and donor embryos were collected at 18°C 3–4 h after egg laying. Embryos were dechorionated by incubation in 7% Na-hypochlorite for 2 min. Blastoderm-stage embryos were selected, transferred to a coverslip, dried on silicagel for 10 min and covered with Voltalef oil 10S. After pole cell transfer, the oil was removed as completely as possible. The transplanted embryos were raised at 18°C until stage 16–17 and detached from the coverslip by gentle washing with heptane. After X-Gal-staining, the embryos were mounted in Morviol (2.4 g Morviol (Hoechst), 6 g glycerol, 6 ml H_2O, 12 ml 0.2 M Tris (pH 8.5)).

In XY hosts, all germ cells carrying *mgm1* are expected to express this marker. To our surprise, however, we found only very few males among our transplanted hosts. There are two possible explanations for this phenomenon: either our donor cross produces only few males, or we do not select males when picking blastoderm embryos for transplantation. To distinguish between these two alternatives, we performed two control experiments. We counted the sex ratio among progeny from our donor cross that were raised in bottles. No abnormal sex ratio was observed. We also collected embryos from host and donor cross, picked blastoderm stages that are recognized due to a transparent ring that is formed around the embryo and let these develop without further treatment. Fifty percent of the adult donors, but only 10% of the adult hosts were males. We therefore conclude that the male embryos resulting from this particular cross form blastoderm stage are not typical and were therefore not selected. The few XY host embryos that we could analyze in our transplantation experiment (three in series A and seven in series B) confirmed that *mgm1* expression is readily detected in germ cells developing in a prospective testis.

4.2. Fly strains and genetics

Flies were kept on standard food at 22°C, unless stated otherwise. Mutations are described in Lindsley and Zimm (1992) and http://flybase.bio.indiana.edu/ unless listed otherwise.

XY pseudofemales, feminized by a *hs::tra* construct (McKeown et al., 1988) and marked by *y* and *Ki*, were obtained by crossing *y;Df(3L) st*^{j7}*, Ki roe p*^{p} *hs::tra/TM6* females with y^+ males carrying the homozygous *lacZ*-marker of interest: P20, 606, Q13d, BC69, *ovo::lacZ*, *hh::lacZ*, *en::lacZ*, or *hb::lacZ*. XY pseudofemales having inherited the CyO, *mgm1* chromosome were identified due to the CyO mutation.

To positively select for *XY;Ki roe p*^{p} *hs::tra/+;mgm1/+* larvae, we used chromosomes carrying *hs::hid* constructs (Grether et al., 1995; Moore et al., 1998). When induced by heat shock, *hid* triggers apoptosis which makes it possible to eliminate undesired chromosome combinations. *y w/ y;Df(3L) st*^{j7}*, Ki roe p*^{p} *hs::tra/TM3, Sb P[w*$^+$ *hs::hid Sp]* females were mated with *CyO, mgm1/hs::hid Sp* males. Progeny were given a heat shock of 1 h at 37°C 24 h after egg laying. This ensured that all surviving *y* larvae were *y/ Y;mgm1/+;Df(3L) st*^{j7}*, Ki roe p*^{p} *hs::tra/+* pseudofemales.

4.3. Staining procedures and microscopy

X-Gal stainings were performed on embryos, and on larval, pupal and adult gonads fixed for 12 min in 4% formaldehyde/PBS according to standard protocols (staining for 10–12 h at room temperature). For the antibody staining, ovaries were dissected in PBS, fixed in 4% formaldehyde/PBS for 20 min, and washed four times in PBST (0.1% Triton X-100). Permeabilization and blocking was achieved by incubation overnight in 1% BSA/PBST at 4°C. Incubation in primary antibodies diluted in PBST was performed for 3 h at room temperature (ascites fluid of monoclonal anti-HTS (Yue and Spradling, 1992), 1:1000; anti-β-Gal (rabbit; Cappel), 1:1000; hybridoma supernatant of anti-mSXL18, 1:10). After extensive washing steps in PBST, the antigen–antibody complexes were detected using anti-mouse-FITC (donkey, Jackson ImmunoResearch) or anti-rabbit-TRSC (goat, Jackson ImmunoResearch) secondary antibodies, both used with a dilution of 1:200 in PBST. For antibody double stainings, mixtures of antibodies were used. After 1.5 h of incubation at room temperature, the antibodies were removed by washing in PBST and afterwards in PBS. Ovaries were mounted in 80% glycerol/DAPKO (Sigma).

Images of X-Gal stainings were obtained on a ZEISS Axiophot using ZEISS Polaroid DMC. Confocal images of antibody stainings were produced on a Leica SP using the TCSNT program. For final processing of pictures, we used Adobe Photoshop 5.5.

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