mgm1, the earliest sex-specific germline marker in Drosophila, reflects expression of the gene esg in male stem cells

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ABSTRACT The pathway that controls sex in Drosophila has been well characterized. The elements of this genetic hierarchy act cell-autonomously in somatic cells. We have previously shown that the sex of germ cells is determined by a different mechanism and that somatic and autonomously acting elements interact to control the choice between spermatogenesis and oogenesis. A target for both types of signals is the enhancer-trap mgm1, which monitors male-specific gene expression in germ cells. Here we report that mgm1 reflects the expression of escargot (esg), a member of the snail gene family, which are transcription factors with zink finger motifs. Genes of this family partially redundantly control a number of processes involving cell fate choices. The regulation of gene expression in germ cells by sex-specific esg enhancers is already seen in embryos. Therefore, autonomous and non-autonomous sex-specific factors that participate in germline sex determination are already present at this early stage. esg is expressed in the male gonad, both in somatic cells and in germline stem cells. We show that esg expression in the male germline is not required for proper sex determination and spermatogenesis, as functional sperm is differentiated by mutant germ cells in wild type hosts. However, somatic esg expression is required for the maintenance of male germline stem cells.

KEY WORDS: mgm1, escargot, germ cells, sex determination, snail

Introduction

In most sexually reproducing organisms, the mature gametes of the two sexes differ dramatically both in size and shape. Major morphogenesis leading to differentiated eggs and sperm occurs in the adult. Sexual dimorphism, however, can be detected much earlier. In Drosophila melanogaster, the cells of the germline are formed during early blastoderm stage at the posterior pole of the embryo. The large, transparent cells, called pole cells, can easily be distinguished from the smaller somatic cells that cover the rest of the surface of blastoderm embryos. At this time, prospective male germ cells are indistinguishable from prospective female germ cells. During gastrulation, pole cells are integrated into the embryo by being moved into the invagination of the forming gut. Somewhat later, germ cells leave the gut and migrate towards the coalescing gonads (reviewed by Williamson and Lehmann, 1996). When the embryonic gonads are formed and the cells of the germline are embedded in the somatic gonadal tissue, germ cells start to display sexual dimorphism. So far, we can detect a sex-specific difference in number of germ cells and in sex-specific gene expression that is visualized by enhancer-trap markers (Poirie et al., 1995; Staab et al., 1996; Jonsson and Steinmann-Zwicky, unpublished).

At blastoderm, a similar number of germ cells are formed in male and female embryos. This number is not maintained, as some cells are lost during germ cell migration. When germ cells are integrated into the gonads, males possess more germ cells than females, which indicates that the germ cells have responded to some sex-specific signal already at this early stage (Poirie et al., 1995). During the first larval stage, male gonads dramatically increase in size because male germ cells undergo a large number of mitoses. Morphologically, male and female germ cells can easily be distinguished at this stage, as male gonads contain spermatogenic cells that display characteristics of spermatogonia (Kerkis, 1931; Aboïm, 1945). Thus, morphological sexual differentiation starts during the early first larval stage. Female gonads remain small, as female germ cells are essentially inert at this stage. Interestingly, gonads of masculinized XX animals lacking the feminizing transformer (tra) function, are indistinguishable from male gonads in first instar larvae both in size and in appearance of the germ cells (Seidel, 1963; Steinmann-Zwicky, 1994a). It has been shown that the sex of XX germ cells is determined by somatic signals (Steinmann-Zwicky, 1994a).

Abbreviations used in this paper: esg, escargot gene; mgm1, male germline marker 1.

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Zwicky et al., 1989). Since tra function is required to feminize the soma, but not the germline (Marsh and Wieschaus, 1978), the observation that XX germ cells developing in masculinized animals are male reveals that signals originating from somatic cells determine the sex-specific behavior of XX germ cells already in first instar larvae.

Just when the embryonic gonads have been formed, sex-specific enhancer-trap markers reveal differences in gene expression between male and female embryonic germ cells. mgm1 (male germ line marker 1; earlier called A507.2A2; Bellen et al., 1989), one of the enhancer-trap lines isolated in Basel in the laboratory of Walter Gehring during the first large screen reported, displays specific ß-galactosidase expression in male, but not in female germ cells (Staab et al., 1996). This suggests that an enhancer exists close to the mgm1 insertion, that drives the expression of a gene in male embryonic germ cells. In embryos, mgm1 expression is found in all male germ cells. During larval stages, it becomes more and more restricted to stem cells, where the expression is also found in the adult testis. Germ cells of females do not show mgm1 expression at any stage. XX animals, however, that are masculinized by tra mutations express mgm1, showing that the expression of this marker is controlled by somatic sex-determining signals (Staab et al., 1996).

mgm1 is the earliest molecular marker that reveals signs of sexual differentiation. Although female-specific expression of the sex-determining gene Sxl can be monitored in somatic cells before gonads are formed, no other early target gene of the sex-determining cascade has been described. mgm1 has been used to assess the sexual identity of germ cells in several studies (Staab et al., 1996; Staab and Steinmann-Zwicky, 1996; Heller and Steinmann-Zwicky, 1998a; Heller and Steinmann-Zwicky, 1998b; Janzer and Steinmann-Zwicky, 2001). To better understand the early steps of germ line sex determination, we wanted to know the gene whose expression is visualized by mgm1. Here we show that mgm1 is regulated by sex- and germline-specific enhancer elements belonging to the gene escargot (esg). esg is one out of six snail related transcriptional regulators with zinc-finger motifs. These genes have high homology and partly redundant functions. esg is expressed non-sex-specifically in several tissues during development, e.g. in the neuro-ectoderm and in imaginal tissues (Whiteley et al., 1992; Hayashi et al., 1993; Fuse et al., 1994; Fuse et al., 1996; Hayashi, 1996; Tanaka-Matakatsu et al., 1996; Yagi and Hayashi, 1997; Yagi et al., 1998). It is thought to be involved in keeping imaginal tissues diploid while most other embryonic cells undergo endoreplication and form polytene chromosomes (Hayashi et al., 1993; Fuse et al., 1994; Hayashi, 1996), but it also has other functions. Our results show that esg is dispensable in the male germline. This suggests that the gene has no essential function in this tissue, or that its germline function can be provided by another redundant member of the gene family.

Results

In mgm1, a Reporter Construct is Inserted in the Promoter Region of the Gene escargot

Germ cells of animals carrying mgm1, display male-specific lacZ expression, because this reporter gene has come under the control of a male-specific enhancer upon random integration into the Drosophila genome.

Our first goal was to determine which gene this enhancer belongs to. We isolated genomic DNA flanking the P-insertion by the plasmid rescue technique (Wilson et al., 1989, see Materials and Methods) and obtained a clone containing approximately 11kb of genomic DNA (fragment A, Fig. 1). Hybridization to polytene chromosomes showed that the sequence originated from cytological region 35 on the left arm of the second chromosome. This is in agreement with the original mapping by Bellen et al. (1989), who determined that mgm1 (then called A507.2M2) had a reporter construct inserted at position 35DE. Sequence comparison with the by now almost complete D. melanogaster genomic sequence (Adams et al., 2000) revealed that the construct is inserted 1258 bp upstream of the proposed translation initiation codon of the gene escargot (esg) (Whiteley et al., 1992). The lacZ reporter and esg are transcribed in opposite directions (Fig. 1).

To determine whether the region contains other genes beside esg, we screened about two million plaques of a cDNA library derived from testes (kindly provided to us by T. Hazelrigg) with a 18.6 kb fragment that includes esg as a probe (fragment B, Fig. 1). We isolated four clones representing two different esg cDNAs (cDNA_H and cDNA_K) and eight clones representing two different cDNAs (cDNA_1 cDNA_I) deriving from a second gene, originally predicted as DS07851.6 and now called no hitter (nht). This gene is located more than 14 kb upstream of esg (see BDGP genome annotation database GadFly, http://flybase.bio.indiana.edu/annot).) No further cDNAs were detected, which suggests that the region flanking the P insertion contains only two genes. Our findings are in agreement with the predictions presented in GadFly where, other than esg, the only gene predicted within fragment B is nht (DS07851.6).

![Fig. 1. In mgm1, the reporter construct P-1ArB is integrated near the gene escargot. White boxes represent the coding units of esg and DS07851.6 respectively. Arrows designate the direction of transcription. Fragment A is the region isolated in the plasmid rescue experiment. Fragment B is the probe used to isolate the various cDNAs. Construct C2 is described in Yagi and Hayashi, 1997. For details see text.](image-url)
A Reporter Construct Containing esg Promoter Sequences Mimics the Expression Pattern of mgm1

The embryonic expression pattern of esg is dynamic and relatively complex. Most prominent is the expression in the neuroectoderm and in some prospective imaginal tissues (Whiteley et al., 1992; Fuse et al., 1996; Yagi and Hayashi, 1997). The control region driving this expression pattern is known to extend to at least 13 kb upstream of the sequence coding for esg (Yagi and Hayashi, 1997). Thus, virtually the entire sequence between esg and nht contains enhancer elements that direct the expression of esg in a number of tissues. Since on the mgm1 chromosome, the P element is inserted within the esg promoter region, it is likely that mgm1 reflects a specific expression of esg in male germ cells. During a conversation about our preliminary results, C. Schulz and M. Fuller (Stanford University) pointed out to us, that one of the published constructs, in which a sequence from the esg promoter drives the expression of a lacZ reporter, is expressed in germline stem cells. The construct which contains 3.5 kb just upstream of the esg transcription start has been described (construct C2, Fig. 4B in Yagi and Hayashi, 1997), but the expression in the germline was not mentioned.

S. Hayashi kindly sent us transgenic flies containing the construct C2 (strain YT010). We assessed the lacZ expression of both males and females of this line throughout development, to see whether similarities to the expression pattern of line mgm1 could be seen. We found that, in embryos, construct C2 drives lacZ expression in male (Fig. 2A, compare to 2B) but not in female germ cells. During larval stages, the expression of C2 becomes, like that of mgm1, restricted to male germline stem cells. In the adult testis, it is expressed in a pattern indistinguishable from mgm1 (Fig. 2 C,D). Expression in female germ cells was found neither in early stages of development nor in the adult ovary. These results show that 3.5 kb from the esg promoter are sufficient to confer tissue- and sex-specificity to a reporter gene, which now shows the same expression pattern as mgm1. This region thus contains enhancer sequences that direct male-specific gene expression in germ cells.

In Embryos, esg is Expressed in Somatic Gonadal Cells

The gene esg is known to be expressed in germline stem cells of adult males (Kiger et al., 2000). Antibodies directed against the esg protein (kindly provided by S. Hayashi), provided no evidence for esg expression in ovaries of adult females. Thus, esg is regulated by sex-specific control elements. We performed RNA in situ hybridization experiments, using cDNA_K as a probe, to test whether esg RNA can be detected in embryonic germ cells. To our surprise, we found a strong signal in embryonic germ cells in addition to the published embryonic esg expression pattern, which includes the neuro-ectoderm and imaginal tissues (Whiteley et al., 1992; Hayashi et al., 1993; Fuse et al., 1996; Yagi and Hayashi, 1997). The signal, however, was clearly located in the somatic cells of the gonads. It was found in most animals having formed gonads, which suggests that the expression is not restricted to gonads of one sex. Since esg is known to be one of six genes, containing the conserved snail-box (see discussion), we repeated in situ experiments with a probe corresponding to esg codons 131-291, which does not contain the conserved snail-box, to exclude cross hybridization with sequences from the other genes. Furthermore, we sectioned embryos to test, whether the signal is in fact present in somatic cells and whether it can also be detected in germ cells. The esg expression could readily be seen in somatic cells of the gonads, but not in germ cells. However, we would not have detected a low level of esg expression in germ cells, since the level of expression in the different somatic tissues is high, which forced us to stop the detection reaction early.

To test whether nht is expressed in embryonic germ cells, we performed in situ hybridization using cDNA_1 as a probe. No RNA from this gene was detected in embryos, suggesting that nht is required at later stages.

No Evidence for a Role of esg in Controlling Endoreplication in Gonadal Cells

esg prevents polytenization of imaginal somatic tissue in larvae (Hayashi et al., 1993; Fuse et al., 1994). As esg is not expressed in female germ cells, which differentiate polynete nurse cells, but is expressed in male germ cells, which do not undergo endoreplication, we reasoned that, in the germline, esg might be implicated in the control of polytenization. Thus, in the absence of esg, polynete nurse cells might be seen in testes. We analyzed mutant animals that were trans-heterozygous for two hypomorphic alleles of esg (esg05729, esg35Ce-1). These die as pupae that lack tergites and sternites, because abdominal histoblasts become polynete (Hayashi et al., 1993). The animals possessed well-differentiated testes, which contained no cells displaying signs of polytenization. Rather they were filled with sperm. In wildtype, stem cells and spermatogenic cells of various stages are seen at the tip of the testes. These cells were missing in mutants, whose testes only contained sperm bundles and motile sperm (see below).
In gonads, polytenization is not only seen in the female germline. Somatic follicle cells also undergo some rounds of endoreplication (Calvi et al., 1998). To test whether ectopic esg expression prevents endoreplication either in nurse cells or in follicle cells in female gonads, we analyzed females carrying a hs::esg construct (a kind gift from S. Hayashi) that were given a heat shock at various stages of development (see Materials and Methods). The hsp70 promoter is inducible in somatic cells and in germ cells, where it is expressed in response to heat stress at embryonic cycle 13 and 12 respectively, as well as thereafter. In the germline, the promoter becomes repressor to heat induction at about stage 10 of adult oogenesis (Wang and Lindquist, 1998; Zimmermann et al., 1983). At this time, nurse cells are fully polyten. Therefore, decisions that lead to the differentiation of polyten nurse cells are taken at a time when esg can be induced in germ cells. In our experiment, we found no evidence for abnormal development of cells that expressed esg. Oogenesis was normal, nurse cells and follicle cells were differentiated, eggs were laid and yielded progeny. The ovaries did not contain abnormal or degenerating cells, which could point to problems with polytenization in some egg chambers. Since esg expression is not sufficient to disturb polytenization in cells of ovaries, this gene might not control the endoreplication process, or it might not control this process alone.

esg is Not Essential in the Male Germline, but Somatic esg Expression is Required for Maintenance of Male Germ Cells

As mentioned above, testes of animals that were trans-heterozygous for two hypomorphic alleles of esg contained motile sperm, but lacked younger spermatogenic cells. Since a mutation in the gene esg (esg^pho) causes the loss of male germ cells, it had been speculated that esg might be required for maintenance of male germ cells. In fact, males carrying this particular mutation are initially fertile, but they become sterile after a few days (Hime and Fuller, manuscript in preparation; cited in Kiger et al., 2000).

We now wondered whether maintenance of male germ cells requires esg expression in somatic gonadal cells or in germ cells. We transplanted pole cells, which are the prospective germ cells, from donor embryos deriving from heterozygous esg parents into agametic hosts (offspring of osk^307 mutant mothers) which are wild type at the esg locus. To obtain homozygous donor animals lacking the esg function, we selected a molecular null allele, esg^{G668}, for our experiment (Whiteley et al., 1992). The surviving host flies were mated to appropriate partners (for details see legend to Table 1). Half of the surviving host animals that had integrated germ cells were expected to be sterile because functional gametes are only obtained when XY germ cells develop in males or when XX germ cells develop in females (Van Deusen, 1977; Steinmann-Zwicky et al., 1989).

In our experiments, we found two fertile males and four fertile females that had integrated homozygous esg germ cells (Table 1). The genotype of the donor embryos could be unequivocally identified due to the genetic markers seen in the progeny (for details see legend to Table 1). Our results thus show that esg is dispensable for gametogenesis in both the male and the female germline. It is possible, that XY; esg/esg germ cells are not fully functional. In fact, when dissecting the sterile males, we found two cases, with degenerative germ cells different from those seen in XX germ cells developing in males (no nurse cell-like cells were found). Nevertheless, the experiment demonstrates that germ cells lacking esg can be functional.

To test whether homozygous esg germ cells are maintained, we crossed the fertile males several times to new females. They sired a high number of progeny with all females, also with those that were added after several days. Test crosses confirmed that the esg mutation was present in the germline, which obviously do not require esg for maintenance. These results show that somatic and not germ cell expression of esg is required for the maintenance of male germ cells.

Discussion

mgm1 Reflects the Expression of esg in Male Germine Stem Cells

Here we show that the sex-specific germline marker mgm1 reflects a specific expression of esg in stem cells of the male germline. Several lines of evidence allow us to draw this conclusion. 1) In the enhancer-trap line mgm1, the reporter construct is integrated within the esg promoter, 1258 bp upstream of the proposed translation initiation codon. 2) A reporter construct consisting of 3.5 kb of the esg promoter and a lacZ gene gives, in testes of transgenic flies, a pattern of expression that is indistinguishable from that of the enhancer-trap mgm1. In both cases, lacZ expression is seen in germ cells of male, but not female embryos, in a subset of spermatogenic cells of larvae and in germline stem cells of adult males. 3) In adult males, germline stem cells express esg.

In testes of adult males germline stem cells express esg (Kiger et al., 2000). This indicates that tissue- and sex-specific enhancers responsible for this expression pattern of esg do exist. Since, in embryos, the expression of a reporter gene is controlled by these enhancers of esg, all sex- and tissue-specific transcription factors required for the expression of esg in male germline stem cells are present already at this early stage. On the other hand, we could not detect esg transcripts in male embryonic germ cells by in situ hybridization. As both mgm1 and the reporter construct C2, that contains a piece of the esg promoter, are expressed weakly, we expect to find only weak expression of the endogenous esg gene in germ cells at this early stage. Low levels of esg, however, cannot be detected by in situ hybridization, due to strong somatic signals. From these results we conclude that, in embryonic male germ cells, esg is most likely expressed at a low level. Alternatively, mgm1 expression could exist prior to esg expression. In embryonic germ cells of males, esg could be repressed by a factor that cannot act on either of the two different reporter constructs.

We found esg transcripts in the entire somatic part of the embryonic gonad, an expression pattern that had not been de-
scribed before, maybe because earlier studies focused on embryonic stages prior to gonad formation. In adults, esg mRNA is detected by in situ hybridization in the cells of the somatic hub, to which germ cells of males attach (Kiger et al., 2000). Although this expression of esg was only reported recently, several enhancer-trap lines had already given hints that esg might be expressed in somatic hub cells of the testes. At least three lines that are believed to reflect part of the expression pattern of esg are expressed in the somatic hub cells of adults and in some cases already in the embryonic precursors of these cells (line G66, (Whiteley et al., 1992); line 254, (Gonczy et al., 1992); line M5-4, (Gonczy and DiNardo, 1996)). Since we now find that, in embryos, esg is expressed in the entire somatic gonad and not just in a subset of cells that represent precursors of hub cells, it seems that the enhancer-trap lines mentioned above reflect a more restricted expression pattern of esg normally seen later.

esg is Not Required for Sex Determination or Maintenance of Male or Female Germ Cells, but may Act Redundantly with Other Genes

Transplanting pole cells, we could show that esg is not essential in male germ cells and that functional gametes can be produced in the absence of esg. This, however, does not mean that esg does not perform a specific function in male germline stem cells. Rather, it is likely that other genes can substitute for esg, in absence of this gene.

esg belongs to the snail gene family, of which six members are known in Drosophila (reviewed in Hemavathy et al., 2000; Manzanares et al., 2001) The six genes fall into two classes, called snail (sna) and scratch (scrt) that exist in vertebrates and in invertebrates, and that must have separated some 500-1000 Myr years ago. Three of these genes, snail (sna, Grau et al., 1984), esg (Whiteley et al., 1992), and worniu (wor, Ashburner et al., 1999; Ashraf et al., 1999), have arisen by gene duplications from an ancestral sna gene. They are all located very closely, in cytological position 35C-D of the second chromosome. The other three genes, scratch, scratch-like1 and scratch-like2 (Roark et al., 1995; see also Manzanares et al., 2001) have originated by duplication of an ancestral scratch gene. They are located on the third chromosome, although they are less grouped than the genes of the sna class. The genes code for transcription factors with 5 to 6 zinc fingers which act as repressors. They all share a conserved organization, consisting of a very similar C-terminal half (the snail-box), and a divergent N-terminal portion.

The genes of the snail family are involved in very different processes, such as specification of mesoderm, CNS development and wing cell fate determination. For the latter two processes, it could be shown that several genes act redundantly. Whereas deleting region 35C-D with all three genes, esg, worn and sna results in severe CNS defects, transgenic expression of each of the three genes can rescue at least part of this CNS phenotype (Ashraf et al., 1999). The two genes sna and esg redundantly determine the fate of wing cells. In double mutants, the wing marker vestigial is absent, but in single mutants it is present (Fuse et al., 1996). Since genes of the sna family are known to be able to substitute for each other, it is quite possible that an important function of esg required in male germ cells is redundantly provided by at least one other gene of the family.

What is the function of esg in the germline? In imaginal tissue of larvae, esg is required to maintain diploidy of the cells. In absence of esg, abdominal histoblast cells undergo endoreplication and their chromosomes become polytene, as do larval cells. esg may thus control differentiation decisions that preclude endoreplication (Edgar and Orr-Weaver, 2001). Our experiments show that esg does not play a decisive role in the control of endoreplication in germ cells or in somatic cells of ovaries. Since ectopic esg expression did neither disturb the differentiation of nurse cells nor that of follicle cells, esg does not seem to be a crucial switch gene as far as endoreplication in cells of the ovary is concerned. Male cells that were mutant for esg did not become polytene. Rather they differentiated functional sperm. This, however, is expected if a gene of the sna family provides a redundant function. Thus, if esg is involved in controlling endoreplication in gonads, it does not perform this function alone.

A possible function for esg in the male germline is the control of cell adhesion. Tracheal tip cells were shown to require esg to adhere to each other (Tanaka-Matakatsu et al., 1996). esg could thus be required for male germ cells to adhere to the somatic hub cells. Within the somatic hub cells, where esg is also expressed, this gene performs such a function. Males carrying a male-specific mutation in the esg gene loose their germline stem cells and become sterile after an initial phase of fertility (Hime and Fuller 1994 and 1995, unpublished but available from fly base; G.Hime and M. Fuller, manuscript in preparation, cited in Kiger et al., 2000). We now show that this is due to the lack of somatic and not germ cell expression of esg, as our host males with germ cells completely lacking esg maintained male germline stem cells, but whole animals carrying hypomorphic mutations of esg did not.

**TABLE 1**

<table>
<thead>
<tr>
<th>Total no. of surviving host flies</th>
<th>No. of host flies with transplanted germ cells</th>
<th>Sterile, with germ cells</th>
<th>Fertile, germ cells of genotype esgSM5</th>
<th>Fertile, germ cells of genotype esg/esg</th>
</tr>
</thead>
<tbody>
<tr>
<td>males</td>
<td>58</td>
<td>24</td>
<td>11 (12)</td>
<td>11 (8)</td>
</tr>
<tr>
<td>females</td>
<td>74</td>
<td>28</td>
<td>17 (14)</td>
<td>17 (9)</td>
</tr>
</tbody>
</table>

To assess whether germ cells lacking esg are functional, we transplanted germ cells from embryos deriving from a cross that yields homoyzogous esg mutants into agamic hosts. The number of recipient animals having integrated germ cells of various genotypes can be compared with the expected number given in parenthesis (1). Genotype of the donor cross: the allele esg²⁴⁴ was caused by a local transposition of a P-element, which carries a mini white marker gene (Whiteley et al., 1992). This w' allele on the transposon therefore marks the esg mutation. Both parents of the donor cross carry mutant w alleles on their X chromosomes. As far as the second chromosome is concerned, their genotype is esg²⁴⁴, w'/SM5,Cy. We thus expect that 25% of the donor embryos are homoyzogous for esg, 50% heterozygous, with esg and the SM5 balancer, and that 25% are homozygous SM5/SM5. Since pole cells from these latter embryos do not differentiate into functional gametes (Schupbach, 1982), the latter class can be neglected. Therefore, if homoyzogous esg pole cells can differentiate functional gametes, one third of the transplanted animals that are fertile should contain esg germ cells. The transplanted host animals were allowed to grow up and they were mated to partners. Animals, which have incorporated pole cells homoyzogous for esg, can be recognized, when fertile, because their progeny all have red eyes (w') and normal wings (Cy'). Animals having incorporated heterozygous pole cells give rise to 50% offspring with red eyes and normal wings and 50% with white eyes (w) and curled wings (Cy). Sterile animals were dissected after several days and the gonads were visually inspected for the presence of partially differentiated germ cells as described by Steinmann-Zwickly, (1994a) and Steinmann-Zwickly et al., (1989). When germ cells were found, they were deduced to have had the wrong karyotype. For our calculation of the expected frequency, the possibility that some hosts had integrated SM5 homoyzogous germ cells was neglected.
Male sterile alleles of scratch have also been described. Null alleles produce defective spermatids (Roark et al., 1995). This shows that more than one gene of the sna family is involved in male fertility.

**Germline Expression of esg is Controlled Both by Cell-Autonomous and Somatic Sex-Determining Signals**

The male-specific enhancers of esg are regulated both by autonomous and by non-autonomous factors. Somatic regulators exist, as XX germ cells become masculinized and express mgm1 in a male environment (Steinmann-Zwicky et al., 1989; Staab et al., 1996). A difference between XX and XY germ cells, i.e. an autonomous sex-specific regulator also exists, as XY germ cells developing in a female environment become male and express mgm1 (Steinmann-Zwicky et al., 1989; Staab et al., 1996; Janzer and Steinmann-Zwicky, 2001).

Within germ cells, esg could be repressed by signals deriving from a female soma, or it could be activated as a consequence of a male soma. We favor the first hypothesis, because we have isolated mutations that lead to constitutive expression of mgm1 in females, suggesting that repression does not function in these mutants (Jonsson, Cruz and Steinmann-Zwicky, unpublished). In females, a somatic signal could ensure that XX germ cells produce a repressor that negatively controls esg expression. XY animal that are feminized by tra activity (XY; hs::tra pseudofemales) also have this somatic signal, since XX germ cells become oogonic and differentiate into eggs when transplanted in such animals (Steinmann-Zwicky, 1994a). Contrary to XX germ cells, XY germ cells are not responsive to this feminizing somatic signal. XY germ cells display male features, including mgm1 expression not only when developing in XY hs::tra females, that are feminized by tra activity, but even when transplanted into true XX females (Steinmann-Zwicky et al., 1989; Staab et al., 1996; Janzer and Steinmann-Zwicky, 2001; Staab et al., 1996). Therefore, we think that these cells are unable to produce or activate the repressor. They must thus express male-specific genes by default.

**Sex Determination of Germ Cells Begins in Embryos**

A number of genes are known to be required in ovaries of adult females for proper oogenesis. Mutations like ovo, ovarian tumors (otu) or Sex-lethal (Sxl) produce ovaries that are either empty or filled with undifferentiated small cells. In some mutants, cells resembling spermatocytes are observed. Therefore, it was assumed that these genes are implicated in germline sex determination (Oliver et al., 1994; reviewed in Cline and Meyer, 1996). However, none of the described mutations masculinizes germ cells prior to metamorphosis (Staab and Steinmann-Zwicky, 1996; Steinmann-Zwicky, 1994b). Therefore, other sex-determining factors must create a difference between XX and XY germ cells in embryos.

These other factors act on the target gene esg. Recently, we have identified a second target gene. Similarly to esg, gene transcription in germline stem cells of males had been visualized by an enhancer-trap that shows an expression pattern indistinguishable from that of mgm1 (Avakian, Jonsson and Steinmann-Zwicky, unpublished). The early control by sex- and germline-specific enhancers is therefore not only seen for esg. Other genes with similar enhancers do exist. All sex-specific proteins that ensure proper regulation of these enhancers in the germline are present already in embryos. The enhancers of esg, lead to male-specific control of the mgm1 marker both in XY germ cells and in XX germ cells that develop in a male environment. In both cases, there are further signs of masculinization, and male germ cells differentiate cells that can clearly be recognized as being spermatogenic during larval stages. Sex-determining decisions are therefore taken early. Several observations, however, suggest that, in contrast to somatic cells, the commitment to a sexual pathway which germ cells undergo early in embryogenesis is not irreversible (Janzer and Steinmann-Zwicky, 2001; Cruz, Jonsson, Staab and Steinmann-Zwicky, unpublished).

We have learned a few general features about how sex is determined in germ cells. We here report the identification of a target gene, esg, whose expression within germ cells is regulated both by somatic signals and by autonomous factors. This regulation already occurs in the embryo, when first sexual dimorphism is seen. It therefore reflects an early response to primary sex-determining signals. At the moment, most players that participate in the process are still unknown. To identify such genes, to study their effect on germ cells and to analyze hierarchical relationships between different genetic elements, it is extremely useful to know a target gene with sex-specific enhancers. If we learn how these enhancers are regulated, we will have largely progressed in our understanding of how the sex of germ cells is determined.

**Materials and Methods**

**Standard Molecular Techniques**

Standard protocols were followed as described by Sambrook et al., (1989)

**Plasmid Rescue**

A fragment of 11147 base pairs (Fragment A Fig. 1) flanking the insertion site of mgm1 and extending upstream with respect to esg was cloned (clone pMG22) following the principle described by (Wilson et al., 1989). 50 mgm1 flies were homogenized on ice in 400 µl solution A (0.1 M Tris HCl, pH 9.0; 0.1 M EDTA; 1% SDS; 1% DEPC) and then incubated at 70°C for 30 minutes. 56 µl of 8M potassium acetate were added followed by an incubation on ice for 30 minutes. The samples were centrifuged at maximum speed in a micro centrifuge at room temperature for 15'. The supernatant was extracted with phenol:chloroform (1:1) and the DNA precipitated by adding 0.5 volumes of isopropanol. The pellet was washed with 70% ethanol and dissolved in 100 µl water. 5 µg of the resulting DNA were digested with 10 units of HindIII at 37°C for 90 minutes in the presence of 3 µg RNaseA in a total volume of 70 µl. The digest was followed by an extraction with phenol:chloroform (1:1) and precipitation with ethanol.

The entire sample was incubated at room temperature overnight in the presence of 4 units of T4 DNA ligase in a volume of 400 µl followed by an extraction with phenol:chloroform (1:1) and ethanol precipitation. The pellet was dissolved in 10 µl water. 3 µl of this DNA were used for a standard electro-transformation reaction into E. coli CMK (Sambrook et al., 1989).

**Sequencing**

Probes were submitted to Mycrosynth (Basel, Switzerland) or to the departmental sequencing facility for sequencing.

**RNA In Situ Hybridization**

Probe preparation, hybridization and detection were done as described in Sergeev et al., (2001)

**β-Galactosidase Staining**

β-Galactosidase staining was performed as described by Poirie et al., (1995)
Isolation of cDNAs

We received an oligo(dT) and random primed cDNA library in Lambda ZAP II (Stratagene) enriched for testis cDNA from T. Hazeldr (Columbia University, New York). The library was probed using random prime labeled Fragment B (Fig. 1) following standard procedures (Sambrook et al., 1989). Fragment B (an XbaI fragment extending from 16149bp upstream to 2457bp downstream of the esg translation start) was gel purified prior to labeling. The insert with the pBluescript plasmid backbone was isolated from the positives phages by “in vivo excision” using the corresponding kit from Stratagene and following the manufacturer’s instructions. A total of 12 positive clones which represented four different cDNAs were isolated: All positions are given with respect to the translation start point of the esg gene as it was proposed by (Whiteley et al., 1992). For this purpose the position of the AUG was set 20000 arbitrarily. The predicted esg stop codon is at position 21410. The coding unit of the predicted gene DS07851.6 (a TFID like transcription factor) extends as a single exon from position 4752 (start) to 4018 (stop) (information available from flybase, http://flybase.bio.indiana.edu/). cDNA_K: belongs to esg and extends from position 20137 to 21853. It ends in a poly(A) tail. However it must be noted that this poly(A) addition site is not preceded by a standard poly(A) signal and that Whiteley et al., (1992) have isolated a cDNA which extends further (3'). This poly(A) tail may therefore be due to a cryptic polyadenylation site or to a missplicing of the oligo-dT during the reverse transcription reaction. cDNA_H: belongs to esg and extends from position 19975 to 21627. cDNA_1: belongs to DS07851.6 and extends from position 4776 to 3941. cDNA_1: belongs to DS07851.6 and starts at position 4720. The end point was not determined.

Ectopic Expression of esg

Animals carrying a hs:esg construct were kept at 25°C. A heat shock of one hour at 37°C was applied when the animals were 1) 0-48 hours old, 2) after 3 days (2nd larval instar), 3) after 4 days (3rd larval instar), 4) after 5-6 days (3rd larval instar and pupal stage), 5) after 7 days (pupal stage) 6) after 9 days (pupal and adult stage), and 7) after 10 days (in adults). Adult females were dissected in ringer solution earliest 4 days after heat shock, and their ovaries were inspected under a compound Axiophot microscope (Zeiss).

Pole Cell Transplantation

Pole cell transplantation has been described by Van Deusen, (1977) and Steinmann-Zwicky et al., (1989). In our case the host embryos were obtained by crossing two strains heterozygous for osk<sup>(D07)</sup>, which had been maintained separately, with each other (this enhances the viability of osk homozygous animals). The resulting progeny were transferred to 18°C at the third larval instar. When homozygous osk<sup>(D07)</sup> females are raised at this temperature, their offspring are viable but they do not have pole (germ) cells. Homozygous adult females (non virgin) were selected and embryos from these females were collected at blastoderm stage (about 4 h after egglaying at 18°C) and used as recipients. As donor for pole cells we collected offspring of w; esg<sup>(G668)</sup>, w;+SM5.Cy parents at blastoderm stage. esg<sup>(G668)</sup> is a molecular null allele, which was caused by a local transposition of a P-element, which carries a mini white marker gene (Whiteley et al., 1992). This w<sup>+</sup> allele on the transposon therefore marks the esg mutation. The genotype of the collected embryos is either esg<sup>(G668)</sup>, w homozygous (25%), SM5.Cy homozygous (25%) or identical to the parents (50%). At the blastoderm stage neither the genotype nor the sex of the embryos is detectable. Therefore the poles cells of an individual randomly chosen donor were transplanted into the posterior end of a recipient (detailed information about the transplantation procedure and the hard ware used is available upon request). Transplantation and recovery of the transplanted embryos were done at 18°C. After hatching, the transplanted animals were allowed to grow up to 25°C and mated to w partners. Fertile animals, which have incorporated esg homozygous pole cells, can be recognized by their uniform progeny with red eyes (w+) and normal wings (Cy+). Animals which have incorporated w+/SM5 pole cells give rise to 50% offspring with red eyes and normal wings and 50% with white eyes (w) and deformed wings (Cy). Animals with SM5 homozygous pole cells would be expected to produce uniform progeny with white eyes (w) and deformed wings (Cy) however they are sterile because these germ cells cannot differentiate into functional gametes (Schupbach, 1982). Whenever possible, sterile animals were dissected after a few days to assay for the presence of non differentiated germ cells in their gonads.

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References


