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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-

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 germline 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 germline 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 *lac*Z 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).

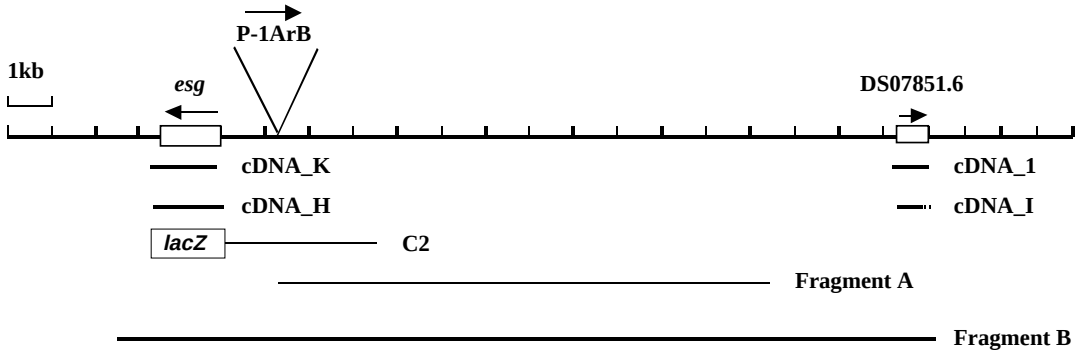

Fig. 1. In *mgm1,* the reporter construct P-1ArB is integrated near the gene *escargot*. *White boxes represent the coding units of* esg *and DS07851.6 respectively. Arrows designate the direction of transcription. Fragment A is the region isolated in the plasmid rescue experiment. Fragment B is the probe used to isolate the various cDNAs. Construct C2 is described in Yagi and Hayashi, 1997. For details see text.*

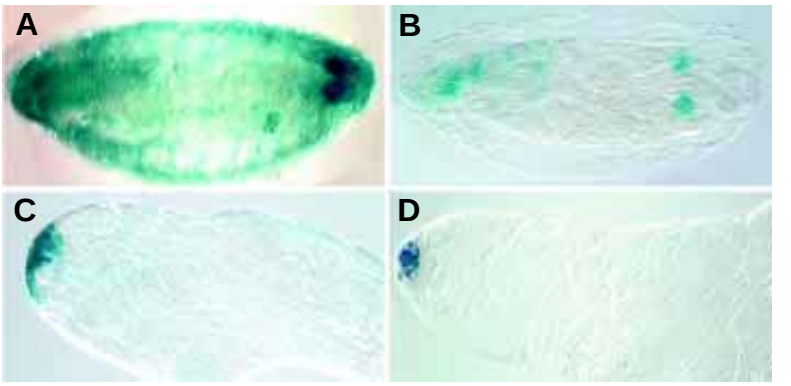

Fig. 2. The *lacZ* expression patterns of *mgm1* and *C2* are highly similar in male embryos and adults. (A) *A male embryo carrying the* C2 *transgene.* lacZ *expression is detected in germ cells and occasionally also in patches of somatic cells, which might be neuro-ectodermal cells of younger embryos. This latter expression is not seen in* mgm1*, which shows that in* C2, lacZ *is not only controlled by the male germline-specific enhancer, but also by other control elements.* **(B)** *A male* mgm1 *embryo showing* lacZ *expression in germ cells.* **(C)** C2 *expression in adult testis.* **(D)** mgm1 *expression in adult testis. In all panels, anterior is to the left.*

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 neuro-ectoderm 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 gonads (Fig. 3) 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 polytene 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*, polytene nurse cells might be seen in testes. We analyzed mutant animals that were trans-heterozygous for two hypomorphic alleles of *esg* ($esg^{05729}/esg^{35Ce\text{-}1}$). These die as pupae that lack tergites and sternites, because abdominal histoblasts became polytene (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).

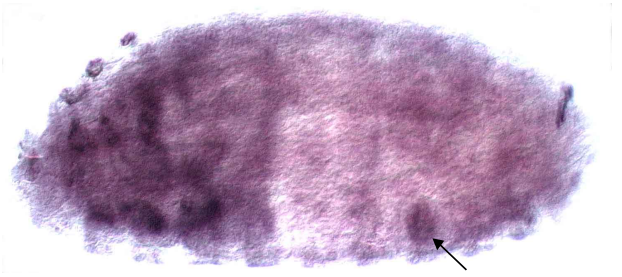

Fig. 3. *esg* is expressed in the somatic gonad of embryos. In situ *hybridization with* esg *cDNA_K as a probe reveals* esg *expression in somatic gonadal cells. Anterior is to the left. The arrow points to the gonad.*

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 recalcitrant 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 polytene. Therefore, decisions that lead to the differentiation of polytene 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*^{shof}) 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*^{301} 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*^{G66B}, 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 germ cells, 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 Germline 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

TABLE 1

***ESG* MUTANT GERM CELLS CAN GIVE RISE TO FUNCTIONAL GAMETES OF EITHER SEX**

	Total no. of surviving host flies	No. of host flies with transplanted germ cells	Sterile, with germ cells	Fertile, germ cells of genotype *esg*/*SM5*	Fertile, germ cells of genotype *esg*/*esg*
males	58	24	11 (12)	11 (8)	2 (4)
females	74	28	17 (14)	7 (9)	4 (5)

To assess whether germ cells lacking *esg* are functional, we transplanted germ cells from embryos deriving from a cross that yields homozygous *esg* mutants into agametic hosts. The number of recipient animals having integrated germ cells of various genotypes can be compared with the expected number given in parenthesis (). Genotype of the donor cross: the allele esg^{G66B} 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 esg^{G66B}, w^+/SM5,*Cy*. We thus expect that 25% of the donor embryos are homozygous 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 homozygous *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 *w* partners. Animals, which have incorporated pole cells homozygous 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-Zwicky, (1994a) and Steinmann-Zwicky *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* homozygous germ cells was neglected.

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, wor* 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.

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 oogenic 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 *Hind*III 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 over night 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 (Balgach, 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. Hazelrigg (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 *Xba*I 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 manufacturers 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 TFIID 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 misspriming 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 where it ends in a poly(A) tail. cDNA_I: 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^{301}, 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^{301} 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 ;* $esg^{[G66B]}$,w^+/*SM5*,*Cy* parents at blastoderm stage. $esg^{[G66B]}$ 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^+ allele on the transposon therefore marks the *esg* mutation. The genotype of the collected embryos is either $esg^{[G66B]}$,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 pole 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 at 25°C and mated to *w* partners. Fertile animals, which have incorporated *esg* homozygous pole cells, can be recognized by their uniformous 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 uniformous 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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