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VIVE LA DIFFÉRENCE: Males vs Females in Flies vs Worms

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ABSTRACT

For 600 million years, the two best-understood metazoan species, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, have developed independent strategies for solving a biological problem faced by essentially all metazoans: how to generate two sexes in the proper proportions. The genetic program for sexual dimorphism has been a major focus of research in these two organisms almost from the moment they were chosen for study, and it may now be the best-understood general aspect of their development. In this review, we compare and contrast the strategies used for sex determination (including dosage compensation) between "the fly" and "the worm" and the way this understanding has come about. Although no overlap has been found among the molecules used by flies and worms to achieve sex determination, striking similarities have been found in the genetic strategies used by these two species to differentiate their sexes.

INTRODUCTION

Beginning in the 1910s, advances in understanding the nature of genes and the way they program development have gone hand in hand with advances in understanding sex determination and the related process of X-chromosome dosage compensation. For example, the discovery that X-chromosome dose determines *Drosophila* sex was coincident with the discovery that genes are on chromosomes (31). Hence, as the twentieth century nears its end and the pace of discovery regarding how genes control development becomes nearly overwhelming, it is not surprising that a significant part of this flood of information

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stems from the study of sexual dimorphism. Some have adopted the view that sex determination is a developmental embellishment peripheral to central problems of embryology and thus can be safely ignored for the time being (17, 136). In fact, confusion over the significance of sex determination can be traced back to the Old Testament, which contains two remarkably different versions of human origins. In one account, the generation of sexual dimorphism is coincident with the creation of humans, while in the other, human sexual dimorphism is an afterthought by the Creator. Admittedly, the program for sexual dimorphism is a genetic overlay on the program for the basic body plan, but this feature gives the study of sexual dimorphism many technical advantages. It is no coincidence that the body of information on sexual dimorphism already covers events in an unbroken logical circle that runs from the earliest regulation of zygotic gene expression soon after fertilization to some of the latest events during gametogenesis in the mature adult. The logical framework that has been developed is unusually robust as well as comprehensive and already intersects studies in many other areas, such as transcriptional control, RNA splicing, translational control, signal transduction, and chromosome structure. Indeed there exists enormous untapped potential for exploiting this understanding to address many general biological and biochemical questions.

Here we compare what is known about the molecular genetic program for sexual dimorphism in the two metazoans most amenable to genetic analysis: the fly *Drosophila melanogaster*, and the worm *Caenorhabditis elegans*. As space permits, we also compare the way in which this information was discovered. Recent discoveries have made it possible to provide a comparative review of fly and worm sex determination that can cover essentially every level of the process and be more than a simple juxtaposition of facts. This information provides a foundation for comparative studies among far more closely related species.

Comparative studies are illuminating in science. The greatest significance is often attached to those situations whose generality is made obvious by a striking conservation of genes and of mechanisms across long periods of evolutionary time. But another rule of science is that much can be learned from a comparison of opposite extremes. From an evolutionary standpoint, sex determination may be such an extreme. Comparison of the elements and mechanisms that direct the sexual dimorphism of various species has revealed a remarkable lack of common genes and mechanisms, despite the fact that metazoans have faced the problem of differentiating their sexes at least as long as they have faced the problem of differentiating top from bottom and front from back (34, 40). Because the rate of evolution of sexual dimorphism is so rapid, changes in sex-determination genes are likely to occur far more rapidly than changes in the biochemical context in which they operate. As a consequence of this simplifying factor,

comparative studies of sex determination seem especially likely to yield unique insights into the process of evolution.

SEXUAL DIMORPHISM IN "THE FLY" AND "THE WORM"

In These Model Organisms, X-Chromosome Dose Determines Sexual Fate, Affecting Nearly Every Feature of the Adult Through a Variety of Developmental Effects on Cell Growth and Differentiation

Both the fly and the worm have two sexes and an X chromosome-counting mechanism that determines sexual fate. The sexes are XY male and XX female for the fly, but XO male and XX hermaphrodite for the worm. The Y chromosome difference between these species is irrelevant to a comparison of their sex-determination mechanisms, since the *Drosophila* Y has no role in this process. The female vs hermaphrodite distinction is also not particularly relevant, since *C. elegans* hermaphrodites are simply self-fertile females whose only male character is the ability to make a limited number of sperm that are used only for internal self-fertilization. *C. elegans* was chosen as a model genetic organism in part because females can, like Mendel's peas, "self" yet can also be outcrossed easily (30). Given the opportunity, the hermaphrodite will mate with a male and use his sperm preferentially. Males are produced either as rare events due to spontaneous nondisjunction of the X chromosome during hermaphrodite reproduction, or as 50% of the outcross progeny from the mating of a hermaphrodite with a male. Hence the population sex ratio for the worm will depend in a complex way on population density, while it will be near 50:50 for the fly.

Sex for both species is determined by the dose of X chromosomes relative to the ploidy, the number of sets of autosomes (32, 142, 160). Thus, regardless of ploidy, flies and worms with an X:A ratio of 1.0 are females and hermaphrodites, respectively, and those with an X:A ratio of 0.5 are males. When X:A = 0.67 (XX AAA), flies are intersexes and worms are males, respectively. Although one commonly refers to an X:A mechanism of sex determination, wild-type animals are diploid and normally only count X chromosomes. Throughout this review, whenever male or female is mentioned without further modifiers, the reference is to chromosomal sex–the X:A ratio–rather than to phenotypic sex.

The adult fly is the reproductive stage (Figure 1*a*) and the stage exhibiting the most striking sexual dimorphism. The species *melanogaster* is named for a male-specific character: the distinctive dark pigmentation of distal regions

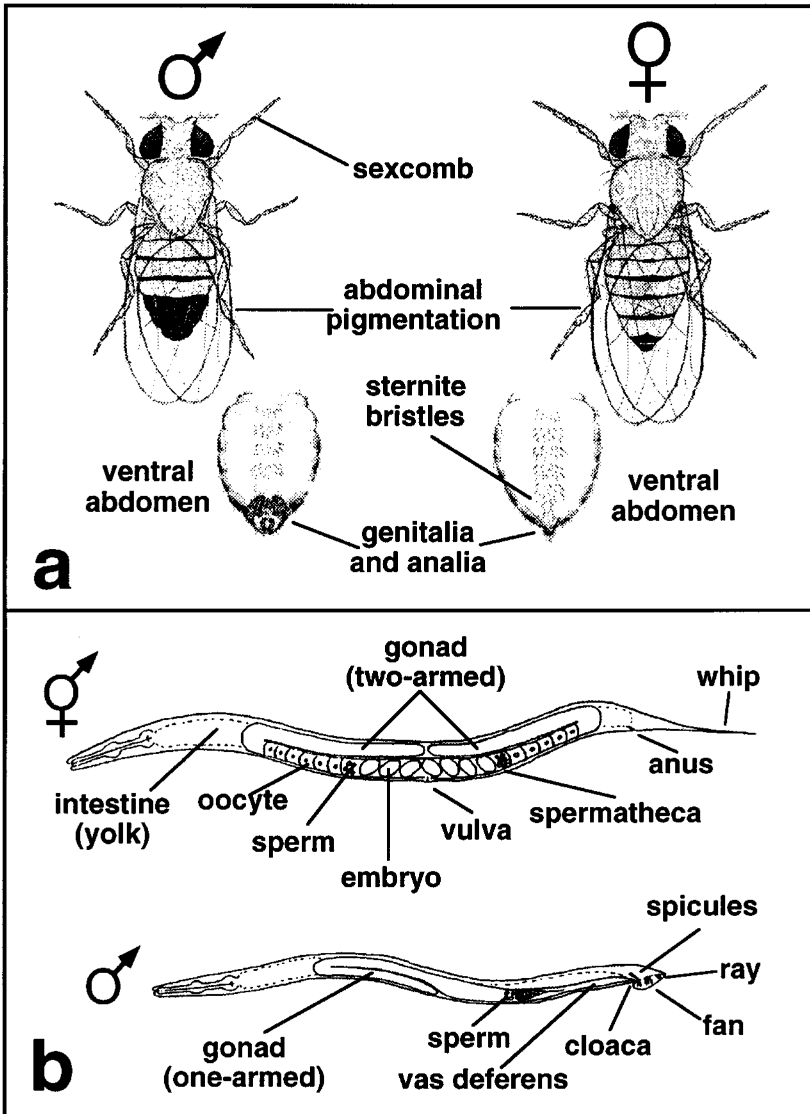


Figure 1 Sexual dimorphism in adult *Drosophila* (*a*) and *C. elegans* (*b*). Only external dimorphisms are illustrated for the fly. Because the worm is transparent, internal dimorphisms are apparent in the intact animal.

of the abdomen. The female is 11% larger than the male in linear dimensions (54). The genitalia are strikingly different. It is this morphological feature of the male that most reliably distinguishes closely related species because it evolves so rapidly (78). External sense organs, called bristles, differ between the sexes. The most distinctive difference involves the giant bristles of the male-specific "sex comb" of the foreleg. Since bristle shafts are generated by the differentiation of single cells, the sex comb region of the foreleg allows unambiguous assessment of sexual phenotype at the level of single cells and hence allows one to distinguish between qualitatively different categories of intersexuality. Not shown in Figure 1*a* are the numerous internal sexual dimorphisms, the most obvious of which involves the gonads and associated reproductive plumbing.

The adult fly is generated by a nearly complete reorganization of the larva during a pupal period lasting four days at 25°C. During this metamorphosis, differentiated larval tissues break down and groups of cells that had been maintained in an embryonic state stop dividing and undergo terminal differentiation. Among these are the imaginal discs and histoblasts that give rise to the exterior adult surface. The larva itself is a feeding and growing stage lasting four days. It forms during an embryonic period lasting a bit less than one day. Generally the egg is fertilized just before being laid, and meiosis and pronuclear fusion occur after the egg is laid.

The first morphological difference to arise between the sexes is a subtle difference in the number of germ cells (178). Equal numbers of germline precursor cells, called pole cells, form in the two sexes at the posterior tip of the young embryo, but by eight hours after fertilization when these migrating cells reach their somatic partners and coalesce into the gonads, males have an average of 16% more germ cells per gonad than females. The gonad continues to be the site of the most obvious sexual dimorphism throughout the larval stages; the male gonad develops far ahead of the female (125). During this same period, the genital imaginal disc becomes distinctive between the sexes as a consequence of the differential proliferation of male and female genital primordia that exist in all embryos (52). In contrast, many other aspects of sexual dimorphism, such as the adult sex comb, arise from the proliferation of a single primordium, the cells of which undergo a sex-specific pattern of terminal differentiation during metamorphosis.

The degree of overt sexual dimorphism in worms is even more extensive than in flies: 30% of the 959 somatic nuclei in the adult hermaphrodite and 40% of the 1031 somatic nuclei in the adult male are sexually specialized. Sexual dimorphism occurs in all tissue types and arises in almost all major branches of the cell lineage (105, 128, 129, 216, 217). The hermaphrodite has a two-armed gonad in which spermatogenesis occurs during the last larval stage, followed by

oogenesis during adulthood (Figure 1*b*). These sperm are stored internally in a specialized compartment, and the oocytes are fertilized as they pass through this compartment into the uterus. Embryos are laid through an opening (vulva) in the ventral hypodermis that also serves as the site of entry for male-produced sperm. In contrast, the male is both shorter (∼ 30%) and thinner than the hermaphrodite and is highly specialized for mating. This specialization is especially evident in the tail, which is equipped with various sensory and copulatory structures that enable him to locate the vulva and inseminate the hermaphrodite (218). The male gonad has only a single arm. Extensive dimorphism also occurs in both the musculature and nervous system. Sex-specific muscles and/or neurons are involved in egg-laying behavior in hermaphrodites as well as mating behavior, copulation, and locomotory behavior in males. The hermaphrodite intestine is functionally specialized for yolk production (129).

Embryonic development is almost identical between the sexes. The first visible sign of sexual dimorphism appears two thirds of the way through embryogenesis, with the programmed cell deaths of two hermaphrodite-specific motor neurons in the male and four male-specific sensory neurons in the hermaphrodite (217). Other sexual dimorphisms arise during the first three larval stages but become most prominent in the last larval stage, L4, and in the adult (216). Diverse strategies are used to generate sexual dimorphism, including sex-specific programmed cell death, generation of sex-specific primordia (blast cells), alternative lineages adopted by a common primordium, and differential gene expression in tissues with identical cell lineages.

For Both Flies and Worms, a Ubiquitous Aspect of Sexual Dimorphism is X-Chromosome Dosage Compensation, Which Is Achieved in Both Organisms by Modulating Transcript Levels of Active X-Linked Genes, Rather Than by Inactivating an X Chromosome

The first aspect of sexual dimorphism to develop for both flies and worms is one shared by all somatic cells: a difference in the average rate of X-linked gene expression, arising as a consequence of dosage compensation (reviewed in 9, 114). This process equalizes the overall amount of X-linked gene products between the sexes in the face of a twofold difference in the dose of genes that comprise one fifth of the fly and the worm genomes. In the 1930s it was inferred from the phenotypic behavior of mutant alleles that both X's of the female fly are active, but X-linked genes are twice as functional per allele in males as in females, regardless of sexual phenotype (155). By the same genetic criteria, it was later shown that this observation is true for the worm as well (57, 147).

For both organisms, subsequent molecular studies revealed that dosage compensation modulates X-linked gene transcript levels to equalize enzyme levels, between the sexes (29, 59, 150). Studies in the fly were facilitated by the giant polytene cells of the larval salivary gland, in which hundreds of copies of each chromosome arm align in register even during interphase (109, 154). The average rate of nascent transcript synthesis per unit DNA was measured by autoradiography in such cells for the approximately 1000 genes of each major chromosome arm. This parameter is elevated for the male X chromosome relative to that for his autosomes and is elevated relative to that for the female X or her autosomes. For the worm, measurements of transcript levels had to be made on a gene-by-gene basis; hence, it cannot be said whether the female or the male rate of X-linked transcript accumulation represents the autosomal average.

The Regulative Character of Fly Development Can Mask Potential Transformations of Adult Sexual Phenotype if They Are Accompanied by Effects on Cell Proliferation; In Contrast, The More Determinative Character of Worm Development Minimizes Such a Complication

Study of sexual dimorphism involves drawing inferences from mutant sexual phenotypes, but such phenotypes can be deceiving when complicated by cell lethality and the premature death of the developing organism. For example, in studies of interactions between a mutation whose effects on sexual fate are masked by its cell and/or organismal lethality and another mutation that can suppress those lethal effects, the second mutation may appear to cause sexual transformations, when in reality it simply allows cells or individuals with potential sexual abnormalities to live. An added complication of cell viability effects for flies stems from this organism's ability to eliminate cells that are growing poorly relative to their neighbors and then compensate for such loss prior to metamorphosis (93, 199, 215). In many mutant situations, a minority of the cells of the developing fly may be disrupted for both sex determination and dosage compensation. Because those abnormal cells can be eliminated and replaced by their unaffected neighbors, not only may escaper adults fail to show evidence of sex-determination upsets, they may not even exhibit evidence of cell death. By contrast, in worms most sexually dimorphic structures arise via specific cell lineages. If specific cells in those lineages die, the structures will generally not be produced. Hence, even though cell death may mask an associated sex transformation, it will at least be obvious that such death has occurred.

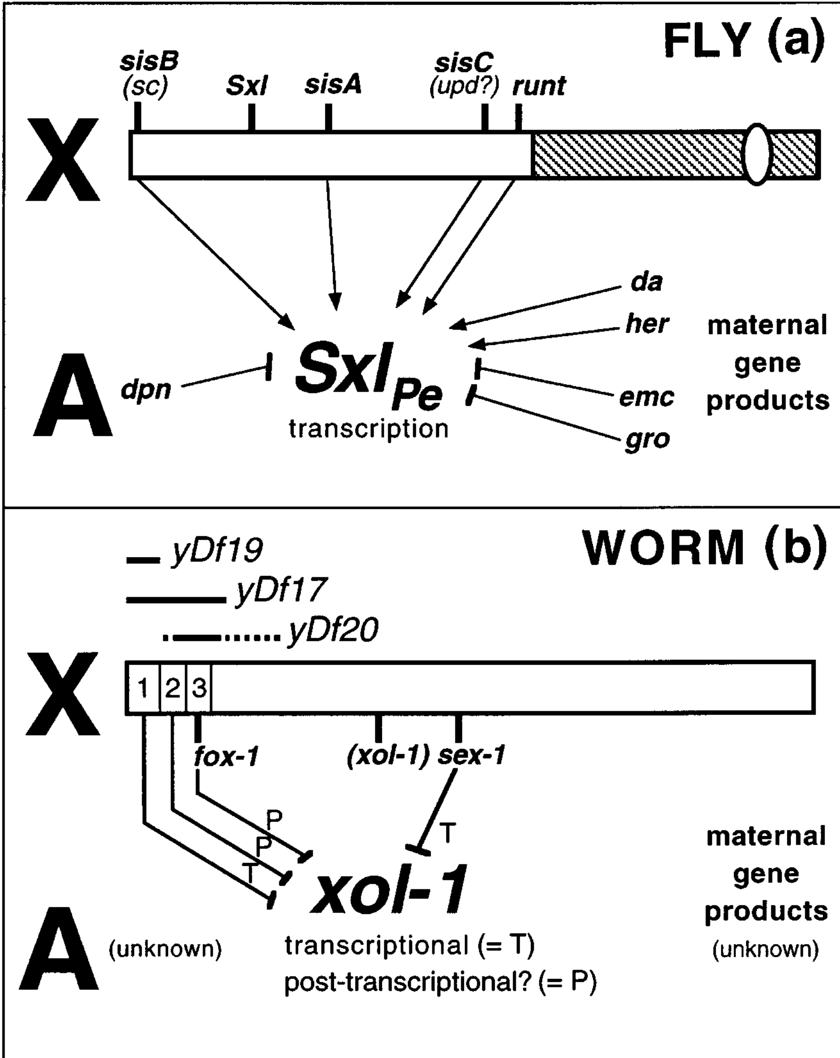
ASSESSMENT OF X-CHROMOSOME DOSE: THE SOMATIC SEX-DETERMINATION SIGNAL AND ITS IMMEDIATE TARGET

For Flies and Worms the Primary Effect of Increased X-Chromosome Dose Is on a Single Gene Target Very Early in Development, Activating It in Flies But Repressing It in Worms

The immediate target of the *Drosophila* sex-determination signal is the X-linked, female-specific switch gene *Sex-lethal* (*Sxl*) (Figure 2*a*) (49). In diploid flies, two X chromosomes activate *Sxl* and thereby initiate the female modes of both sexual differentiation and dosage compensation. The switch-like character of *Sxl* was revealed by the two opposite mutant alleles that established this gene's role in fly development. Sxl^{f1}, a recessive, loss-of-function allele causes female-specific lethality, while Sxl^{M1}, a dominant, gain-of-function allele causes male-specific lethality. Sex-specific lethality of Sxl^{f1} and Sxl^{M1} obscures their effects on sex determination, but those effects can be seen in genetic mosaic animals that survive because only some of their cells are mutant. The first mosaic analysis of *Sxl* showed that Sxl^{M1} feminizes male cells, while Sxl^{f1} masculinizes female cells (45, 46). Moreover, the deleterious effects of these mutations on the growth and differentiation of cells suggested that *Sxl* acts cell autonomously to control dosage compensation as well as sex determination. This hypothesis was confirmed two years later by a study showing the effects of *Sxl* mutations on the morphology and rates of nascent transcript synthesis of polytene chromosomes (140).

The discovery that *Sxl* is a switch gene coordinately controlling sex determination and dosage compensation suggested that it might be the primary target of the X:A signal. This conclusion was confirmed through a series of experiments exploring the genetic and developmental basis for two qualitatively different

Figure 2 The sex-determination signal and its immediate target. For flies (*a*) the target is the X-linked, female-specific switch gene *Sxl* that is activated in response to female dose of X chromosomes. For worms (*b*) the target is the X-linked, male-specific switch gene *xol-1*, that is repressed in response to a female dose of X chromosomes. These targets are controlled by the zygotic dose of the X-linked genes (X:A numerator elements) and autosomal genes (X:A denominator elements) indicated in the figure. Changes in nongenetic parameters such as nuclear volume and total DNA accompany changes in ploidy and may also contribute to the denominator. X:A signal transduction genes such as the maternally acting genes listed establish the thresholds necessary for the X:A elements to exhibit their nonlinear dose effects on their target. They do not exhibit zygotic dose sensitivity in sex determination. Parentheses surrounding the *xol-1* locus indicate that it does not behave as a numerator element, unlike *Sxl*.


FLY (a)
sisB
(sc)
Sxl
sisA
sisC
(upd?)
runt
X
A
dpn
Sxl Pe
transcription
da
her
emc
gro
maternal
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products
WORM (b)
yDf19
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1
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P
P
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xol-1
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kinds of intersexual phenotypes: mosaic and true (50, 143). Mosaic intersex animals are sexually intermediate overall, but individual cells are either normal male or normal female (92). Their sexual ambiguity is in the initial choice of sexual fate by individual cells or in the fidelity with which individual cells maintain that choice. Mutations in regulators of *Sxl* or in *Sxl* itself generate mosaic intersexes if the accompanying lethal dosage-compensation upsets are prevented. Analysis of mosaic intersexes showed that the X:A signal determines the probability that a given cell will activate *Sxl* when cells first form in the fly embryo (49). The same studies revealed a positive feedback loop for *Sxl* that maintains the cells' commitment to the female developmental pathway. The mosaic phenotype of intersexes generated by changes in the X:A signal, or by changes in other specific regulators of *Sxl*, reflects that this feedback ultimately allows for only two stable *Sxl* states: fully on (female) or fully off (male). This feature of *Sxl* regulation is illustrated vividly by the salt-and-pepper SXL antibody staining pattern of XX mutant embryos homozygous for various partial-loss-of-function *Sxl* alleles (22).

In contrast, true intersexes display a sexually intermediate phenotype even at the level of individual cells. Their sexual ambiguity lies in the control of sexual differentiation, not sex determination per se. True intersexes can also be generated by mutations in *Sxl*, but only by mutations that interfere specifically with the control of its downstream gene targets in the sex-determination pathway. True intersexes, but not mosaic intersexes, can also be generated by mutations in those downstream genes (10).

The ability of *Sxl* to respond to the X:A signal can be impaired by mutation without impairing its ability to maintain a female developmental commitment or to direct female differentiation (143, 187). Conversely, the latter two functions can be impaired without loss of the X:A signal response. Molecular characterization of *Sxl* revealed a mechanistic explanation for these observations by demonstrating two different levels of *Sxl* control: activation of *Sxl* by the X:A signal occurs via transcriptional regulation, while positive feedback regulation occurs via alternative pre-mRNA splicing (Figure 3) (18, 126).

These two types of regulation are related to the operation of two separate *Sxl* promoters that function in somatic cells: a so-called establishment promoter, Sxl_{Pe}, which acts very early and transiently, and a maintenance promoter, Sxl_{Pm}, which acts throughout most of development. Transcripts from both promoters encode similar sets of RNA-binding proteins. The two sets have similar if not identical activities and differ only in their N-termini. The role of the X:A signal is to activate Sxl_{Pe} only in females, and thereby to generate a burst of full-length SXL protein only in this sex. Transcription from Sxl_{Pe} occurs only from nuclear cycle 12 to early cycle 14, ending when somatic cells first form in the young embryo (12, 69). As Sxl_{Pe} shuts off, Sxl_{Pm} comes

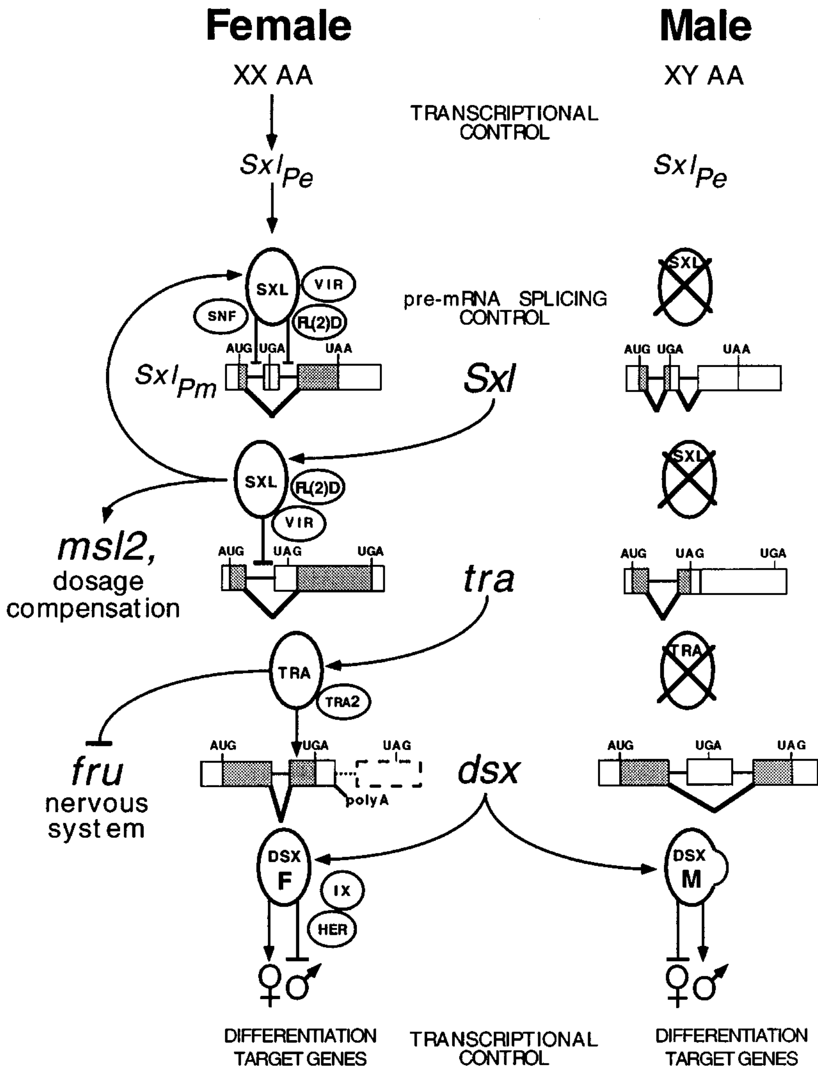


Figure 3 Operation of the coordinate control gene *Sxl* and the regulatory hierarchy that controls *Drosophila* sexual differentiation. This regulatory cascade of switch genes begins and ends with transcriptional control, but control for the intermediate steps is at the level of alternative pre-mRNA splicing. Male RNA splicing occurs by default in the absence of the splicing regulators SXL and TRA. *Sxl* controls both sex determination and dosage compensation, while its targets control only one process or the other. The only *Sxl* target shown on the dosage-compensation branch is *msl2*, although other targets must exist. It is unknown whether *fru* is a direct target for *tra* on the sex-differentiation pathway branch that controls aspects of nervous system sexual dimorphism.

on–in both sexes. However, because Sxl_{Pm}-derived transcripts, unlike those from Sxl_{Pe}, require full-length SXL protein itself to remove a male-specific exon that would otherwise abort translation, only the expression of Sxl_{Pm} in females generates mRNAs that encode full-length SXL protein (27). The rule for Sxl_{Pm} is that if a cell has SXL, Sxl_{Pm} expression will allow it to make more; however, if a cell lacks SXL, it cannot make SXL in response to Sxl_{Pm} expression. The early burst of expression from Sxl_{Pe} in response to the female dose of X chromosomes provides females with the SXL protein needed to initiate this positive feedback loop for Sxl_{Pm} transcript splicing, locking them into the female splicing mode. Because males lack this early burst of SXL and have no other way of removing the translation-terminating exon from Sxl_{Pm}-derived transcripts, they are locked into the male splicing mode.

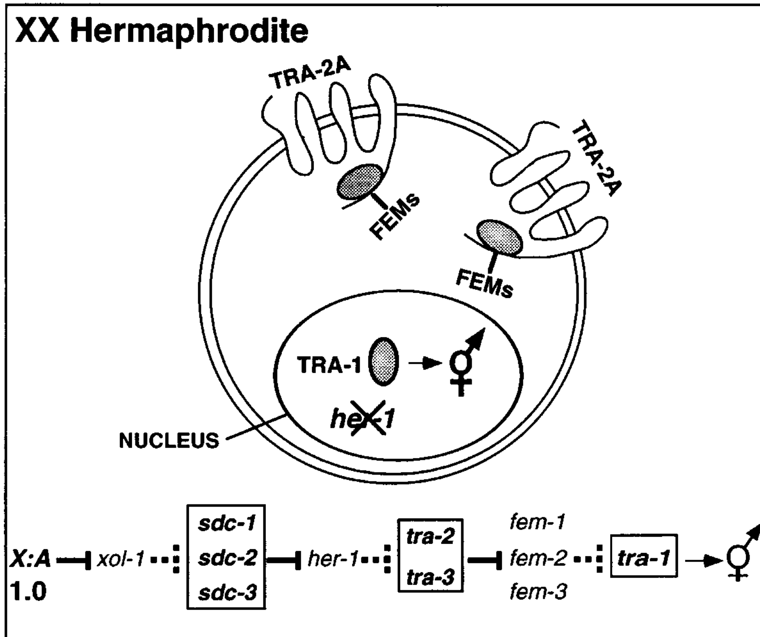
In *C. elegans*, the target of the X:A signal is the male-specific switch gene *XO lethal* (*xol-1*) (Figure 2*b*). *xol-1* is similar to *Sxl* in that it coordinately controls sex determination and dosage compensation and is regulated by the X:A signal at the level of transcription (M Nicholl, C Akerib & B Meyer, unpublished information). Unlike *Sxl*, however, *xol-1* acts in males. Hence X-chromosome dose has the opposite effect on *xol-1* compared to *Sxl*: A male dose of X chromosomes results in a high level of *xol-1* transcripts, and a hermaphrodite dose results in a low level (151, 182).

The gene *xol-1* was discovered after it was already understood that sex determination and dosage compensation are coordinately regulated in worms by a group of hermaphrodite-specific genes, the *sdc* (sex and dosage compensation) genes (58, 163, 234), and that dosage compensation is implemented by *dpy* (dosage compensation dumpy) genes (101, 115, 177), which function in hermaphrodites to down-regulate expression of both X's. Genetic epistasis studies placed *xol-1* upstream of the *sdc* and *dpy* genes, suggesting that *xol-1* is the earliest-acting gene in the hierarchy controlling the male-hermaphrodite decision and therefore may be the direct target of the X:A signal (151) (Figure 4).

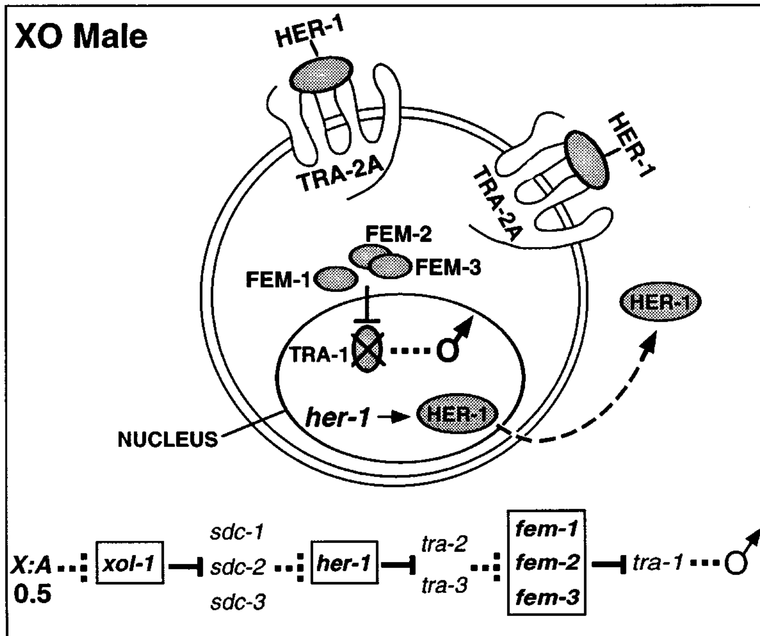
Null alleles of *xol-1* have no effect on otherwise wild-type XX animals but cause the feminization and death of XO animals. This XO-specific lethality results from inappropriately low X-chromosome gene expression. Lethality

→

Figure 4 Operation of the coordinate control gene *xol-1* and the regulatory hierarchy that controls *C. elegans* sexual differentiation. Genes that are functionally active are boxed and bolded. A dark bar indicates a negative regulatory interaction, and a dashed bar indicates the lack of negative regulation because the upstream regulator is inactive. The most likely direct target of *xol-1* is *sdc-2*, which together with *sdc-1* and *sdc-3* controls *her-1* at the level of transcription. The dosage compensation-specific branch of the hierarchy diverges from the sex-determination branch after the *sdc* genes.



XX Hermaphrodite
TRA-2A
TRA-2A
FEMs
FEMs
TRA-1
her-1
NUCLEUS
X:A
1.0
xol-1
sdc-1
sdc-2
sdc-3
her-1
tra-2
tra-3
fem-1
fem-2
fem-3
tra-1



XO Male
HER-1
TRA-2A
HER-1
TRA-2A
FEM-2
FEM-3
FEM-1
HER-1
TRA-1
her-1
HER-1
NUCLEUS
X:A
0.5
xol-1
sdc-1
sdc-2
sdc-3
her-1
tra-2
tra-3
fem-1
fem-2
fem-3
tra-1

but not feminization is suppressed by the dosage-compensation *dpy* mutations, indicating that lethality is caused by inappropriate activation of the XX-specific dosage-compensation machinery in XO mutants. Rescued XO *dpy*; *xol-1* animals are non-Dpy phenotypic hermaphrodites that express nearly wild-type levels of X-linked transcripts. Indeed, the first *xol-1* mutation arose spontaneously in a *dpy* mutant culture as just such an XO animal masquerading as a wild-type hermaphrodite. Mutations in the *sdc* genes fully suppress both the feminization and lethality of *xol-1* mutations, resulting in viable XO males. Together, these genetic interactions demonstrated that *xol-1* promotes male development by ensuring that downstream genes controlling hermaphrodite sexual development and dosage compensation are inactive in XO animals (151). The primary sequence of the 417 amino acid XOL-1 protein provides no clue as to its mechanism of action (182).

The discovery that *xol-1* acts as an early switch in the sex-determination decision indicated that *xol-1* might be a direct target of the X:A signal (182). Ectopic expression of *xol-1* transcripts in XX animals triggers male development and causes death by disrupting dosage compensation. The time in development during which expression of *xol-1* transcripts from a heat shock promoter kills XX animals is the same as that required to rescue *xol-1* null XO mutant animals– from the 28-cell stage through gastrulation, indicting that the effect of *xol-1* on XX animals mimics the action of *xol-1* in XO animals. This timing also agrees with the time of *xol-1* transcription in XO animals as assayed by a *xol-1*::*lacZ* reporter transgene. The very early time of *xol-1* action is additional evidence that it is the direct target of the primary sex-determination signal. The fact that *xol-1* expression becomes dispensable toward the end of gastrulation suggests that an irreversible commitment to a particular sexual fate has occurred by then and that assessment of the X:A ratio is no longer necessary or consequential. Thus, in contrast to *Sxl* in flies, the X:A signal target in worms does not appear to be involved in maintaining the sexual commitment.

Although *xol-1* produces three alternatively spliced transcripts that differ in their 3′ ends, these three *xol-1* transcripts are present in both sexes, and only a single transcript mediates all *xol-1* functions in XO animals (182). Hence alternative splicing does not play an essential role in the sex-specific control of the worm X:A target, in contrast to the situation for *Sxl* in flies.

For Flies and Worms, a Small Set of Specific X-Linked Numerator Genes Communicate X-Chromosome Dose to the Primary Targets Sxl and xol-1, Respectively

Once the gene target of the X:A signal was identified and its roles in sex determination and dosage compensation were appreciated, criteria were established for recognizing the X:A signal genes whose relative dose communicates

X-chromosome number (51). Increases in the zygotic dose of X:A numerator elements should specifically kill males, while decreases should specifically kill females. Changes in the dose of denominator elements should display the opposite sex specificity. These sex-specific lethal dose effects should be suppressed by the appropriate class of mutation in the X:A signal target. For example, increasing the zygotic dose of X:A numerator elements in flies should specifically kill males, and that lethality should be suppressed by a female-lethal mutation in *Sxl*, whereas decreasing the zygotic dose of these same genes should specifically kill females and be suppressed by *Sxl* alleles of the male-lethal class. In worms, increasing the dose of the X:A numerator elements should also specifically kill males but in this case be suppressed by hermaphrodite-lethal transgenes that constitutively transcribe *xol-1*, whereas decreasing the zygotic dose of these same numerator genes should specifically kill hermaphrodites and be suppressed by male-lethal null alleles of *xol-1*.

Sets of X-linked genes identified in flies and worms have satisfied these and other criteria for X:A numerator elements (Figure 2). Moreover, the ability of mutations in the known X:A signal targets to completely suppress the effects of changes in these X:A signal genes indicates that there is only a single signal target in both species. Thus, for both organisms, sex is determined by a polygenic zygotic signal acting on a single switch-gene target. These signal genes interact in a semiadditive fashion: Changes in the dose of any one gene of a class enhance the effects of similar changes in another gene and suppress the effects of the opposite dose change. Synergism is indicated by the fact that simultaneous changes in the dose of two elements have more effect than twice the change in the dose of a single element. This behavior has been exploited in suppression screens for isolating X:A element mutations. Point mutations are available for all fly numerator genes: *sisterlessA* (*sisA*), *sisterlessB* or *scute* (*sisB* or *sc*), *sisterlessC* (*sisC*), *runt*, and *Sxl* (51, 62, 68, 171, 223, 225; P Sziber & TW Cline, unpublished information). Point mutations exist for only two worm numerator genes: *fox-1* (feminizing locus on X) (3, 107; M Nicoll, C Akerib, & B Meyer, unpublished information) and *sex-1* (signal element on X) (I Carmi, J Kopczynski, & B Meyer, unpublished information). Dose effects of chromosome rearrangements have identified two additional regions that harbor numerator elements (3).

For flies, mutations generating even small changes in the X:A signal do not by themselves generally cause unambiguous changes in sexual phenotype. Even though the effect on Sxl_{Pe} of incremental changes in the dose of X:A numerator elements appears to be graded, any such graded effect is translated into an all-or-none effect on *Sxl* by the middle of embryogenesis. Because of the peculiarities of *Sxl* regulation, a small change in the X:A signal has a

large and generally lethal effect on relatively few cells, instead of a small effect on the sexual differentiation of a large number of cells. Larger changes in the X:A signal will simply kill the individual before it reaches the adult stage. At least for small changes in the signal, cell lethality can be suppressed and sex-determination effects revealed by mutations in the downstream genes that implement dosage compensation (49). Alternatively, sex-determination effects of signal changes can be monitored in triploid intersexes (X:A = 0.67) (48). Lethality is less of a factor for them, since the magnitude of X:A signal-induced dosage-compensation upsets is necessarily less than for diploids. Moreover, the ambiguous X:A signal of 0.67 sets their cells on a threshold for stable *Sxl* activation, as evidenced by their mosaic intersex phenotype, so that a small genetic nudge in the signal can have a large effect on the proportion of cells that choose one sexual pathway over the other.

All the fly X:A signal elements act on *Sxl* at the level of transcription. The two strongest elements, *sisA* and *sisB*, affect *Sxl* transcription throughout the embryo. They encode a basic leucine zipper protein and a (bZIP) basic helix-loop-helix (bHLH) protein, respectively (69, 156, 233). Their transcription starts by cycle 8, the earliest yet documented unambiguously for a wild-type fly gene. In contrast, *runt* is weaker, more spatially restricted in its effects, and later expressed. It encodes a protein homologous to the viral transcription factor PEPB2 (121, 122). The gene *sisC* is comparable in strength to runt (223). By itself, loss of *sisC* is like loss of *runt* in affecting *Sxl* transcription only in the middle of the embryo; however, changes in *sisC* dose synergize with mutations in *sisA* and *sisB* to affect *Sxl* expression throughout embryo. The product encoded by *sisC* is not informative (F Béranger, Y Zhang, JR Timmer, & TW Cline, unpublished information).

Dose effects of *Sxl* qualify it as a numerator element. Although the gene functions normally in an autosomal location, X-linkage increases the fidelity of the X:A signal by ensuring that there are more copies of the X:A signal target in the sex that must activate at least one copy in order to develop normally. In contrast, the X-linkage of the worm X:A signal target, *xol-1*, would be expected to operate against its proper functioning.

Are the five numerator elements of Figure 2*a* the entire complement of such genes? If so, duplicating just these five genes should induce a male embryo to produce a female level of SXL protein. Unfortunately, the necessary transgenes do not exist for this test. An alternative approach is to examine X-chromosome deficiencies and duplications for lethal synergistic interactions with known numerator elements to determine if there are interacting regions not already identified as harboring numerator elements. In a screen examining rearrangements covering 79% of X, no new regions of this kind were identified (189).

For worms as for flies, the multigenic nature of the X:A signal delayed initial discovery of mutations in individual elements. Duplications and deletions had been obtained that covered most of the worm X-chromosome, and none caused sex-specific lethality. Although these duplications and deficiencies were found to include some X signal elements, they did not perturb the X:A ratio enough to cause lethality. Moreover, any X-chromosome rearrangement that was sufficiently large to affect the dose of several elements simultaneously and thereby cause sex-specific lethality would not have been recovered, since most of the schemes used to recover these rearrangements demanded that both XO and XX animals be viable.

With more understanding of sex determination, it was possible to devise strategies that would allow the recovery of duplications (3, 106, 107) and deletions (3), regardless of how many numerator elements they might contain. Duplications were recovered in XO animals carrying a mutation in an XX-specific dosage-compensation gene (either an *sdc* or *dpy* gene) that prevented males from activating the XX mode of dosage compensation, while deletions were recovered in XX animals carrying a *xol-1* mutation that prevented hermaphrodites from activating the XO mode of dosage compensation. Subsequently, deletions of signal elements were obtained as suppressors of the male-lethal effect of duplications (3).

In this way, a region was identified near the left end of X that contributes so strongly to the X:A signal that duplications of it cause nearly all males to die from misregulation of *xol-1* (3, 107). The increased dose of three subregions within this duplication is responsible for male lethality (3). Since most or all males survive if only one or two of these regions are duplicated, a screen was undertaken to isolate point mutations in the numerator elements of this region as suppressors of the duplication male lethality. The first loss-of-function mutation in a worm numerator gene was identified in this way, despite the fact that it has no phenotype by itself in XX animals (3) (M Nicoll, C Akerib, B Meyer, unpublished information). An independent molecular approach had already identified a cosmid in the vicinity of this mutation that causes *sdc*-suppressible male lethality when present in multiple copies in transgenic animals, behavior suggesting that the cosmid harbored a numerator element (107). The activity within this cosmid was referred to as *fox-1*, although the gene or genes responsible for the male lethality had not been determined (107). When DNA sequence analysis of the new loss-of-function numerator mutations (3; M Nicholl, C Akerib, B Meyer, unpublished information) revealed that they altered a gene within this cosmid that encoded a putative RNA-binding protein (107), the gene identified by these mutations was given the name *fox-1*. Mutations in a much stronger numerator gene *sex-1* were obtained in a genetic screen

for mutations that activate a *xol-1*::*lacZ* transcriptional fusion reporter gene inappropriately in XX animals (I Carmi, J Kopczynski, B Meyer, unpublished information). Not only does the loss of two copies of *sex-1* cause severe phenotypes in XX animals, the gain of two copies causes extensive male-specific lethality.

In contrast to the situation in flies, simply lowering the dose of numerator elements in XX worms can cause overt changes in sexual phenotype (3). As expected, the mutant phenotypes are suppressed by male-lethal null mutations of *xol-1*. Differences in the extent of masculinization and lethality of changes in different elements indicate that worm numerator elements, like those in flies, are not all equally effective. Synergism among them is shown by the fact that in XX animals the *trans*-heterozygous combination of a *sex-1* mutation and a deficiency (*yDf20*) that eliminates two other elements causes masculinization and lethality, yet neither heterozygous mutation by itself has any obvious effect (I Carmi, J Kopczynski & B Meyer, unpublished information). XX animals heterozygous for a deletion of the three elements included in *yDf17* are masculinized and display a Dpy phenotype indicative of a nonlethal perturbation of dosage compensation (3). The observation of incomplete masculinization and nonlethal dosage-compensation upsets in XX animals with a reduced numerator-element dose suggests that *xol-1*, in contrast to *Sxl*, is capable of stably functioning at intermediate activity states.

The various worm numerator elements vary not just in their strength, but also in the molecular mechanism by which they influence *xol-1*. These differences are revealed by differences in the effects of signal element changes on the expression of an *xol-1*::*lacZ* transcriptional reporter transgene (3). The effects on *xol-1* of *sex-1* or the *yDf19* region are transcriptional, since deletion of these genes dramatically increases expression of this *xol-1* reporter in XX animals, while their duplication in XO animals decreases it (3; M Nicoll, C Akerib, I Carmi, J Kopczynski, & B Meyer, unpublished information). In contrast, *fox-1* and the *yDf20* region have no effect on the reporter in either sex, indicating that these genes do not control the transcription of *xol-1*, despite robust genetic evidence that these elements are *xol-1* regulators (M Nicoll, C Akerib, B Meyer, unpublished information). For example, elevating the expression of *xol-1* by increasing its copy number suppresses male lethality caused by duplications of these elements. Since neither the introns nor the 3′UTR of *xol-1* are present in the transcriptional reporter gene, they are potential targets for this regulation by signal elements. Since *fox-1* encodes a putative RNA-binding protein, a posttranscriptional effect of this gene on *xol-1* functioning would not be unexpected. Thus it appears that *xol-1* is like *Sxl* in being controlled

sex-specifically at two different levels, transcriptional and posttranscriptional, but unlike *Sxl*, both levels of control serve only in the initial assessment of the X:A signal.

To what extent is the functioning of the genes that serve as the X:A signal in these two organisms specific to sex determination? Although neither *fox-1* nor *sex-1* appears to have other functions, three of the five fly numerator genes clearly do. The strong element *sisB* has a key role in neurogenesis as the gene known as *scute*, a role that is evolutionarily conserved. Nevertheless, it is surprisingly easy to destroy most of this gene's sex-determination activity while leaving its proneural function largely intact. The molecular explanation for this fact may reveal how *sc* was recruited as a sex signal. The gene *runt* has important roles in neurogenesis and pattern formation, and *sisC* may be allelic to *unpaired* (*upd*), a complex gene involved in early pattern formation.

What is the "A" part of the X:A signal? A genome-wide screen for fly denominator-element mutations identified only one such negative regulator of *Sxl*, a gene named *deadpan* that had already been recognized as a denominator element serendipitously and shown to encode a negatively acting bHLH transcription factor (12, 23, 238). This gene is pleiotropic, with a non-sex-specific, semivital role in neuronal function. The sex-specific lethality of null *dpn* alleles is much weaker than that of the stronger numerator elements, and changes in *dpn* dose have less effect on Sxl_{Pe}. Although *dpn* is only marginally strong enough to allow efficient study, it may be the only denominator element of such individual significance. Searches for worm denominator elements are in progress.

General models have been proposed for how the extremely nonlinear dose response of Sxl_{Pe} to the nuclear concentration of the transcription factors of the X:A signal element class might arise. Some investigators have found titration models appealing in which competing heterodimerization reactions between negative and positive transcription factors in the nucleoplasm allow positively acting dimers to form only when there is a double dose of numerator genes (172). Another category of model that is likely to be far less sensitive than titration models to random fluctuations in the concentrations of individual factors posits the existence of multiple binding sites for multiple positive and negative transcription factors on the DNA (70). Nonlinearity would result from a combination of causes, including competition among factors for mutually exclusive sites, as well as interactions among proteins at different sites on the DNA itself to affect both their binding affinity and the transcriptional consequences of their binding (95). By this model, interactions among transcription factors off the DNA might also be a significant factor in determining the availability of positive

and negative dimers for binding, but not in the all-or-none sense of a titration model. There are precedents in *Drosophila* for small concentration differences in key transcription factors determining whether a target gene is switched on or off (reviewed in 211). The molecular analysis of these systems can serve as a useful guide for studies of the larger and more complex regulatory region for Sxl_{Pe}. In none of these systems does titration per se appear to play a role.

For Flies and Possibly for Worms, "Signal Transduction" Genes Enable Numerator Genes to Act Appropriately on Their Target

For genes that qualify for the X:A numerator or denominator label, effects on their regulatory target are seen for increases as well as decreases in zygotic gene dose. These key dose-sensitive genes function, however, in a biochemical context defined by other genes that do not display such zygotic dose sensitivity. It is this context that establishes the threshold for activation or repression that is central to the operation of the system. The zygotic dose of such genes may be irrelevant because it is only their maternally generated products that are involved in sex determination, but even for zygotically expressed regulators, the specific zygotic gene dose may not be important for sex determination if the gene products are normally present in excess or if their biochemical activity is limited by the dose of some other locus. Such context genes have been termed X:A signal transduction genes (51). Since a variety of basic cell functions are required for X:A signal assessment, some housekeeping genes may be assigned initially to this class, even though they might have little or no functional specificity for sex determination. The appropriateness of such assignments must be reassessed once the molecular nature of the genes' involvement in sex determination is ascertained.

For flies, four maternal X:A signal transduction genes have been identified and their products have been characterized. The genes *da* and *hermaphrodite* (*her*) are positive regulators of *Sxl*, while *extramachrochaetae* (*emc*) and *groucho* (*gro*) are negative regulators (44, 173, 180, 238). The *da* product is a bHLH transcription factor, while that from *emc* is an HLH protein lacking the basic region generally required for DNA binding (39, 55, 64, 79). Proteins like EMC are thought to exert their negative effects on transcription by sequestering activator bHLH proteins such as DA in heterodimers that cannot bind DNA (232). The gene *gro* encodes a protein that is thought to act as a corepressor by binding to negatively acting bHLH proteins such as DPN. The *her* product is a zinc finger protein (138). A fifth maternal effect gene that probably belongs in this class is *snf* (*splicing necessary-factor*), a gene whose effects have been reported to be on Sxl_{Pm} transcript splicing rather than on Sxl_{Pe} expression (4), although recent discoveries regarding the nature of *snf* mutant alleles (71) have complicated the picture (see next section).

REGULATORY GENES THAT COORDINATELY CONTROL SOMATIC SEX DETERMINATION AND DOSAGE COMPENSATION

The Only Fly Coordinate-Control Switch Gene, Sxl, Maintains its Own Activity State and Those of Its More Specialized Downstream Targets through Effects on RNA Splicing with the Assistance of Non-Switch Regulatory Genes that Have Other Roles in Development

For flies, *Sxl* is the only switch gene downstream of the X:A signal that coordinately controls sex determination and dosage compensation. It does so by regulating pre-mRNA splicing for itself and for at least two functionally more specialized switch genes, *tra* (*transformer*) and *msl2* (*male-specific-lethal-2*) (Figure 3). The gene *tra* heads the branch of the genetic hierarchy that specifically controls somatic sexual differentiation (146, 158), while *msl2* heads a branch of the pathway that controls the dosage compensation of most but not all dosage-compensated genes (see also Figure 5) (16, 124, 241). These two genes rely on *Sxl* to maintain their female-specific splicing mode; for *tra* that mode is functional, while for *msl2* it is nonfunctional.

SXL protein controls *Sxl* and *tra* transcript splicing directly by binding to poly U-rich stretches in pre-mRNA (111, 118, 186, 188, 201, 231). Control of *msl2* is also likely to be direct, since the sex-specifically regulated intron of this gene's primary transcript also has two SXL binding sites (16, 124, 241). For all three genes, the effect of SXL binding is to block a male splice, although the mechanism of that block and its specific molecular consequences differ. For *tra* and *Sxl*, the male splice blocked by SXL generates nonfunctional mRNAs that abort translation, while the male splice for *msl2* removes an intron in the $5'$ untranslated region that appears to interfere with translation if present in females. This *msl2* translational block may be in part a consequence of SXL binding to the same two sites within the female-specific intron that may be involved in the splicing block (16, 124).

SXL does not work alone to control RNA splicing. The gene *snf* (with the caveats mentioned below), *vir* (*virilizer*), and *fl*(2)*d* [*female-lethal*(2)*d*] generate products that facilitate SXL's positive autoregulatory splicing regulation (Figure 3) (71, 87, 96). These genes have other vital functions in development; hence, strong loss-of-function alleles are recessive lethal to both sexes. Nevertheless, female-specific mutant alleles exist for *snf* and *vir* that appear only to interfere with *Sxl* functioning. The gene vir^{2F} is a recessive, female-specific, zygotic lethal that masculinizes escapers. Lethality and masculinization are enhanced by a reduction in Sxl^+ dose and are partially suppressed by Sxl^M alleles.

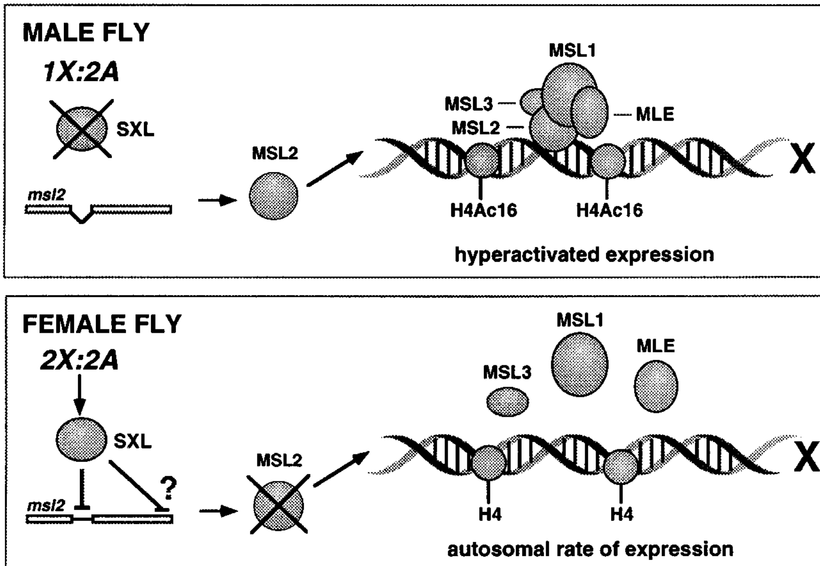


Figure 5 Control of *Drosophila* dosage compensation by the four *msl* (*male-specific lethal*) loci. By default, all four MSL proteins are made in males and assembled on the X chromosome. Hyperactivation of transcription ensues perhaps as a consequence of a histone modification induced by the MSL complex. Female SXL protein prevents the removal of a female-specific intron in the 5′ untranslated region of *msl2* mRNA, thereby reducing synthesis of MSL2 protein. Without MSL2, the other MSL proteins fail to assemble on the X chromosome, and the hyperactivation of a large fraction of dosage-compensated genes is thereby prevented. It is not known how SXL controls the dosage compensation of X-linked genes that are not regulated by the *msl* genes.

In an otherwise wild-type background the female-specific allele snf^{1621} is simply a Sxl^M-suppressible, recessive female-sterile; however, a profound somatic effect of snf^{1621} in females is revealed at low culture temperatures when the zygotic dose of Sxl^+ is reduced to one copy (167, 206). Under these conditions, snf^{1621} has a dominant, female-specific lethal maternal effect, and a dominant masculinizing zygotic effect. This dominant synergism with *Sxl* was first described for deletions of the *snf* region of X (209, but see 51). The subsequent discovery that snf^{1621} exhibited effects of the same magnitude suggested both that *snf* was the dose-sensitive gene responsible for the dominant deletion phenotype and that snf^{1621} might be a null. Surprisingly, when a null *snf* allele was finally isolated, it displayed none of these dominant interactions (71). Thus snf^{1621} must be a gain-of-function mutation, and experiments based on it must be evaluated with that in mind.

Although there is no allele of *fl(2)d* comparable in its sex-specificity to vir^{2F} or snf^{1621}, one can deduce from the behavior of a temperature-sensitive hypomorphic allele that *Sxl* female-specific splicing control is likely to be the aspect of *fl(2)d* function most sensitive to disruption (88). In their interactions with *Sxl*, mutations in *fl(2)d* behave like those in *vir*.

Do snf^{1621}, *vir*, and *fl(2)d* affect only the functional interaction of SXL protein with its own RNA, or are they also involved in its interactions with other target RNAs? This question can be answered by examining the feminizing phenotype of an hsp70::*Sxl* cDNA expression transgene in males that have no endogenous Sxl^+ allele (18). For such males, the transgene phenotype does not depend on *Sxl* autoregulation; hence, any modification of that phenotype will reflect an effect on the functioning of the transgene-encoded, female SXL protein on its downstream targets. The female-specific vir^{2F} allele completely suppresses viability and feminization effects of this transgene in males, thus *vir* is likely to work with *Sxl* on its downstream targets *tra* and *msl2* (96). By contrast, snf^{1621} does not have such interactions (TW Cline, unpublished information); hence, this allele appears to be specific for *Sxl* autoregulation. This test has not yet been reported for *fl(2)d*, but one can infer from published results that it is likely to be like *vir* in working with SXL on other splicing targets.

Is the functional relationship between *Sxl* and *snf*, *vir*, or *fl(2)d* specific, or instead might these three genes simply encode components of the general splicing machinery on which a large number of gene-specific splicing regulators depend for their functioning, with effects on fly sex-determination simply being the most sensitive metric for minor disruptions in general RNA splicing? Since one would expect null mutations in general splicing factors to be cell lethal, the reported survival of clones homozygous for non-sex-specific lethal alleles of *fl(2)d* and *vir* argues against these genes encoding general splicing factors (87, 96), although the alleles examined have not been shown to be truly null. A priori one might not expect to be able to recover mutant alleles with truly sex-specific phenotypes for genes that only encode general splicing factors, yet snf^{1621} is such a functionally female-specific allele of a gene now known to encode a general splicing factor *Drosophila* U1A/U2B″ snRNP protein (71, 179). Recent observations on dose effects of snf^+ transgenes suggest that *snf* is likely to be pleiotropic and to have a function that is indeed specific to *Sxl*. Increases in either the maternal or zygotic dose of snf^+ above the wild-type level dramatically enhance the deleterious male somatic phenotypes of some weak Sxl^M alleles that would not otherwise interfere with male development (TW Cline, unpublished information). Even more significant is that extra copies of snf^+ can trigger female *Sxl* transcript splicing in the male germline (see discussion on the germline, below). These dose effects demonstrate that SNF

can be rate-limiting for SXL splicing even when all housekeeping components of the general RNA splicing machinery are present at wild-type levels, and the effects suggest that a specific interaction takes place between *snf* and SXL at some level that is not limited by other proteins.

The Sxl^M alleles analyzed so far appear to owe their gain-of-function character to lesions in or around the *Sxl* male-specific exon that relax the requirement for SXL protein in the female-specific splicing of Sxl_{Pm}-derived transcripts (11, 22). In this way, these mutations bypass the requirement for the initial burst of SXL protein that only a high value of the X:A signal normally elicits. The primary effect of Sxl^M lesions on transcript splicing can be surprisingly small even for alleles that eventually approach a female level of expression in males. Many such alleles still depend largely on the autoregulatory effect of the wild-type SXL proteins they generate in order to ramp up to female levels of expression. This accounts for the observation that even the snf^{1621} mutation, which is specific for *Sxl*-autoregulation, can suppress some Sxl^M alleles. A factor contributing to the constitutive behavior of some Sxl^M alleles may be increased sensitivity of their mutant transcripts to autoregulation.

The Worm Coordinate-Control Switch Gene sdc-2 *Is the Analog of* Sxl *and Also Works with the Assistance of Non-Switch Regulatory Genes, But Ones That Are Not Pleiotropic*

In contrast to the situation in flies, the target of the worm X:A signal, *xol-1*, coordinately controls sex determination and dosage compensation through its negative regulatory effects on another coordinate-control switch gene (Figure 2). This gene, *sdc-2*, functions exclusively in hermaphrodites and is the gene most analogous to the fly gene *Sxl* (163). It is like *Sxl* in that it requires the participation of at least two other coordinate-control genes, *sdc-1* and *sdc-3*, to activate the hermaphrodite mode of sex determination and dosage compensation, but unlike *vir*, *fl(2)d*, and *snf*, these other *sdc* genes function exclusively in XX-specific aspects of development (58, 234, 235).

The first *sdc-2* mutation was recovered in a screen for X-linked, hermaphrodite-specific lethal mutations. Subsequent alleles were recovered as suppressors of *xol-1* XO-specific lethality. Although null *sdc-2* alleles have no effect on otherwise wild-type XO worms, in XX animals they cause complete reversal of sexual fate similar to mutations in the hermaphrodite sex-determination switch gene *tra-1* (99), and cause XX-specific lethality, similar to mutations in the dosage-compensation *dpy* genes (101, 115, 177). The dosage-compensation defect was demonstrated directly by measurements of X-linked transcript levels.

The effect of *sdc-2* mutations on sex determination and dosage compensation is implemented by independent pathways (163). This point is illustrated by the

fact that masculinization but not lethality is blocked by a mutation in *her-1*, a male-specific switch gene (99) that heads the sex-determination branch of the regulatory hierarchy. The functioning of *sdc-2* as a negative regulator of *her-1* is shown more directly by the fact that in *sdc-2* XX mutants, *her-1* transcripts are present at the level normal for males and not hermaphrodites (226). The gene *sdc-2* could not be positioned in the dosage-compensation hierarchy by such epistasis analysis since mutations in *sdc-2* and the dosage-compensation *dpy* genes have the same effect on dosage compensation. A position for *sdc-2* upstream of the *dpy* genes could be inferred from the fact that *sdc-2* controls both sex determination and dosage compensation, while the *dpy* genes have a direct effect only on dosage compensation. Recent molecular experiments (see below) confirm this placement and demonstrate that SDC-2 is sex-specifically localized to the hermaphrodite X chromosomes (D Lapidus, H Dawes, B Meyer, unpublished information) and activates dosage compensation by localizing the dosage-compensation DPY proteins to the hermaphrodite X chromosomes (P-T Chuang, J Lieb, B Meyer, unpublished information).

Recent experiments also demonstrate that *sdc-2* is a hermaphrodite switch gene (H Dawes, D Lapidus, B Meyer, unpublished information). Antibody staining revealed that SDC-2 is made exclusively in XX animals, confirming that this gene is regulated by the X:A signal. Moreover, ectopic expression of *sdc-2* transcripts in XO animals causes extensive ($\sim$ 90%) XO-specific lethality that is suppressed by XX-specific dosage-compensation mutations. Rescued XO animals develop as hermaphrodites. These results indicate that the death of XO animals induced by ectopic SDC-2 is a consequence of dosage-compensation upsets. As a hermaphrodite-specific switch gene, *sdc-2* is likely to be the target for negative regulation by *xol-1* in males. In support of this hypothesis, an extrachromosomal array carrying numerous copies of a truncated *sdc-2* gene was found to have no adverse effect on males because it made no functional *sdc-2* product, yet it partially suppressed the XX-specific lethality caused by overproduction of *xol-1* transcripts (182). The truncation left intact the 5′ *sdc-2* regulatory region and three fourths of the structural gene; hence, that portion of the *sdc-2* gene may contain the *xol-1* target. SDC-2 protein is huge (350 kDa), more than eight times larger than the largest known product of the analogous fly gene *Sxl* (D Berlin, C Nusbaum, B Meyer, unpublished information). Though *sdc*-2 initiates all aspects of hermaphrodite development, it is unknown whether it is involved in maintaining that developmental mode.

The first *sdc* gene to be discovered was *sdc-1* (234, 235). It revealed that worms were like flies in coordinately controlling sex determination and dosage compensation. It acts at the same place in the hierarchy as *sdc-2*, but it is maternally rescuable and its null phenotype is relatively weak: Not all XX animals are masculinized, and the masculinization itself is incomplete. Moreover, null

sdc-1 alleles cause no significant XX-specific lethality, despite causing overexpression of X-linked genes. Nevertheless, there is synergism between alleles of *sdc-1* and *sdc-2* that demonstrates the importance of their joint participation in development. The combination of a weak *sdc-2* allele that causes little or no lethality by itself, and a null *sdc-1* allele that is also nonlethal, results in complete XX-specific lethality. Temperature-shift experiments demonstrated that *sdc-1* is required in the first half of embryogenesis for proper sex determination and for establishing the XX mode of dosage compensation. Consistent with a role for *sdc-1* as a negative regulator of *her-1* transcription, the gene encodes a 139-kDa protein that contains seven zinc finger motifs of the TFIIIA variety and may therefore be a DNA-binding protein (161).

Another partner for *sdc-2* is *sdc-3*. The intimate relationship between these two genes is illustrated dramatically by the fact that the localization of SDC-2 protein to the hermaphrodite X chromosomes requires *sdc-3* activity (H Dawes, D Lapidus, B Meyer, unpublished information); conversely, localization of SDC-3 to X requires *sdc-2* (T Davis, B Meyer, unpublished information). The stability and/or synthesis of SDC-3 is reduced by mutations in *sdc-2*.

Analysis of *sdc-3* was complicated by its unusual genetic properties, which nevertheless ultimately shed considerable light on its function (58). This gene differs from the other coordinate-control genes in that its sex-determination and dosage-compensation activities are separately mutable, indicating that they function independently. Three different classes of mutant *sdc-3* alleles were identified genetically. One class masculinizes XX animals by elevating *her-1* transcript levels but has no effect on dosage compensation. A second class disrupts dosage compensation and causes more than 95% XX-specific lethality but has little or no effect on sex determination. These two classes of mutations complement each other as if they represented two separate genes. However, a third class comprised of true null alleles fails to complement alleles in either of the first two classes, indicating that all three classes are defective in the same gene. Ironically, the null phenotype itself is misleading, since it does not reflect the gene's involvement in sex determination: Escapers are not masculinized. Extensive genetic and molecular analysis revealed that the dosage-compensation defect of *sdc-3* null alleles suppresses their own sex-determination defect as a consequence of a feedback between sex determination and dosage compensation that is discussed in the next section.

Molecular analysis of *sdc-3* (131) confirmed the genetic conclusions and revealed that the sex-determination mutations cluster to a region of the 250-kDa SDC-3 protein that has limited homology to the ATP-binding domain of myosin, while dosage-compensation mutations eliminate a pair of TFIIIA zinc finger motifs at the carboxy terminus. Null mutations all abort translation of the *sdc*-3 protein prior to its sex-determination and dosage-compensation domains.

The zinc finger motifs are essential for the localization of SDC-3 to X for dosage compensation (T Davis, B Meyer, unpublished information). The mechanism by which SDC-3 represses *her-1* in sex determination is unknown.

REGULATORY GENES THAT SPECIFICALLY DIRECT SOMATIC DOSAGE COMPENSATION

For Both Flies and Worms, A Set of Sex-Specific Vital Genes Controls Dosage Compensation But Has No Direct Effect on Sex Determination; However, the Fly Set Elevates Male X-Chromosome Expression While the Worm Set Reduces Hermaphrodite X-Chromosome Expression

Genes whose mutant alleles cause lethal phenotypes that depend on X-chromosome dose have been the key to understanding not only sex determination, but also dosage compensation in both flies and worms. Characterization of these female-specific and male-specific lethals has shown that the regulation of dosage compensation is a sex-specific process for both organisms, contrary to earlier models in flies (reviewed in 139) that had no place for null mutations with these phenotypes. Sex-specific lethals have moved analysis of dosage compensation out of the realm of a twofold quantitative difference in gene expression–the end-point of the process–and into the more experimentally robust realm of on/off qualitative differences in gene functioning. Moreover, sex-specific lethals have been exploited to develop experimental designs that overcome the pitfalls of some classical dosage-compensation studies, as well as more recent studies modeled after them (9; see debate in 24, 123). These strategies use a disruption in the normal dosage-compensation mechanism to make cells healthier rather than sicker, and/or they manipulate the experimental situations so that only a minority of cells are abnormal, thereby minimizing upsets in cell and organism physiology (84).

The relevance of sex-specific lethals to dosage compensation was suggested in connection with the discovery of the female-specific lethal effect of *da* that led to an understanding of *Sxl* (43). Four years later, the results of the first systematic screen for autosomal mutations that differentially affect male and female viability were reported (20). The recessive *msl* (*male-specific lethal*) genes identified are required for hyperactivation of the male X chromosome (19). Soon thereafter similar experiments established that *Sxl* represses this X-chromosome hyperactivation in females (140). The discovery of an additional *msl* (229) brought the total to four: *msl1, msl2, msl3,* and *mle*. A screen for X-linked, male-specific lethals recently identified a fifth *msl* gene that is much like the others (97).

Genetic and developmental characterization of the four *msl* genes revealed no interactions among them and suggested that these genes were likely to participate in the same cell-autonomous developmental process. Mosaic analysis showed that the *msl* genes were unlikely to be involved in sex determination and on this basis could be placed downstream of *Sxl* in a regulatory branch separate from *tra* (Figure 3). In view of this placement, it was unexpected that the *msl* lethal periods were much later than that for Sxl^- females, since aneuploid studies had shown that flies tolerate an excess of gene products (the predicted effect on females of loss of Sxl^+) better than a deficit (the predicted effect of *msl* mutations on males). This disparity presaged the fact that the *msl* genes carry out only a subset of the dosage-compensation functions of *Sxl*.

The correlation in flies between sex-specific lethality and upsets in dosage compensation was influential in the interpretation of similar phenotypes in worms several years later. In the first report concluding that worms might dosage compensate (101), *dpy-26* mutations were shown to cause a maternal-effect, XX-specific lethal phenotype. The gene was named for the fact that rare XX escapers have a distinctive dumpy phenotype (short and fat). In contrast, *dpy-21* mutations were found to cause a recessive, XX-specific Dpy phenotype without lethality. The *dpy-21* mutants are killed, however, by one extra X, whereas it takes two extra X chromosomes to kill an otherwise wild-type diploid worm. The XX-specific phenotypes of *dpy-21* and *dpy-26* mutants are similar to the Dpy and lethal phenotypes of diploid animals with 3X and 4X chromosomes, animals that might be expected to have an excess of X-linked products. Therefore, the idea was proposed that these mutant phenotypes result from elevated X-chromosome expression and that worms must undergo dosage compensation. Three additional maternal-effect XX-specific lethals (115, 177) were subsequently identified in screens for sex-specific lethal mutations and for suppressors of *xol-1* XO-specific lethality: *dpy-27*, *dpy-28*, and *dpy-30*. Mutations in *dpy-27* and *dpy-28* cause a degree of lethality similar ($\sim$95%) to that of *dpy-26* mutations, but *dpy-30* mutations cause complete lethality.

The phenomenon of dosage compensation in worms, as well as the involvement of these *dpy* genes in that process, was shown using a phenotypic assay similar to the one that first demonstrated dosage compensation in flies (57, 147). By this assay, XX and XO animals exhibit equal expression of X-linked genes; and mutations in *dpy-26*, *dpy-27*, and *dpy-28* elevate this expression in XX but not XO animals. The *dpy-21* mutations also elevate X expression in XX animals, but they have a minor effect on X expression in XO animals as well. At the same time, dosage compensation and its perturbation by mutations in *dpy-21*, *dp-27*, and *dpy-28* were demonstrated at the molecular level through quantification of X-linked transcript levels (150). XX and XO animals have similar levels of X-linked transcripts, and mutations in the *dpy* genes disrupt

dosage compensation, causing elevated X-chromosome transcript levels in XX animals. Similar genetic and molecular assays subsequently demonstrated that mutations in *dpy-30* specifically elevate X-chromosome expression in XX animals (115). Together these experiments indicate that the *dpy* genes equalize worm X-chromosome gene expression between the sexes, most likely by reducing the levels of transcripts produced by both hermaphrodite X chromosomes.

The lack of interactions between mutant alleles of *dpy-26*, *dpy-27*, and *dpy-28* suggest that these genes act together to control X-chromosome expression. However, *dpy-21* again behaves differently: Its mutant alleles alleviate some of the XX-specific lethality caused by the other *dpy* mutations, suggesting that the role of *dpy-21* in dosage compensation is different from that of the other genes.

In summary, although flies and worms both rely on a set of sex-specific vital genes to specifically achieve dosage compensation, the two sets are engaged in the opposite sex and have the opposite effect on X-linked gene expression. The fly genes are male specific and increase gene expression, whereas the worm genes are hermaphrodite specific and decrease gene expression.

Another difference between the *Drosophila msl* genes and the *C. elegans dpy* genes is in their degree of pleiotropy. Thus far, *msl* functioning appears to be limited to dosage compensation, but some *dpy* genes are clearly involved in other aspects of development. Mutations in *dpy-26* and *dpy-28* increase meiotic nondisjunction in both sexes, suggesting a relationship between two very different aspects of chromosome dynamics (101, 177). Mutations in *dpy-30* cause a variety of deleterious effects on the morphology and behavior of even XO animals (115). Perhaps the XX-specific lethal effect of *dpy-30* mutations is so unusually strong because it is a combined result of a standard XX-specific dosage-compensation upset and a non-sex-specific disruption of development that can be tolerated by individuals not also suffering a dosage-compensation upset.

For the fly, there is no evidence for interactions between the dosage-compensation and sex-determination regulatory pathways, but in the worm an inappropriate shift of dosage compensation toward the male mode can cause a shift of sexual differentiation toward the hermaphrodite mode. This feedback of dosage compensation onto sex determination appears to arise as an indirect consequence of perturbations in X-chromosome expression, rather than as a direct action of *dpy* gene products on genes of the sex-determination hierarchy. Perhaps it serves a homeostatic function in the wild-type worm to compensate for chance errors in X:A signal assessment, errors that in the fly are corrected instead by cell death. This feedback is illustrated vividly by *sdc-3* mutations (58). XX worms homozygous for a sex-transforming allele of *sdc-3* develop as fertile males, yet if they are also mutant in any of the dosage-compensation

dpy genes or in the dosage-compensation domain of *sdc-3* itself, they develop as fertile hermaphrodites. Since the level of *her-1* transcripts is appropriate for the phenotypic sex of the animal in all cases, suppression of *sdc-3* masculinization must occur via the sex-determination pathway, either by effects on *her-1* itself or on some gene upstream of it. Other examples of interactions between dosage compensation and sex determination include the fact that mutations in any of the dosage-compensation *dpy* genes suppress the masculinization of XX animals caused by *her-1* gain-of-function mutations (177, 227). Moreover, mutations in *dpy-21*, *dpy-27*, or *dpy-28* cause 2X:3A animals to develop as fertile hermaphrodites instead of as males (104, 177).

In Both Flies and Worms, Proteins Made by Many of the Dosage Compensation Genes Bind to the X Chromosome in a Sex-Specific Fashion to Modulate Gene Expression

All four fly *msl* genes are transcribed in both sexes, but except for *mle*, high levels of their protein products are found only in males (16, 83, 84, 124, 132, 169, 170, 241). In males, these gene products colocalize to hundreds of specific sites along the X chromosome, consistent with their having an active and direct role in hyperactivating X-linked gene transcription (Figure 5). There are some autosomal binding sites as well: 30-40 for MLE, and 10–20 for MSL1 and MSL2. Little or no chromosome binding is evident in females, except for the binding of MLE to some autosomal sites. Loss-of-function mutations in any one *msl* gene reduce or eliminate X-chromosome binding of the other MSL proteins and also lower the level of these proteins, though not necessarily to the level found in females. Loss of *msl1* or *msl2* function has a significantly greater effect on the others than does loss of *mle* or *msl3* function, consistent with the fact that the developmental upsets caused by mutations in *msl1* or *msl2* are more severe than those caused by *mle* or *msl3* mutations. These studies suggest that the four MSL proteins bind as a complex, and indeed MSL1 and MSL2 co-immunoprecipitate.

MSL1 and MSL3 do not resemble any protein whose function is known. In contrast, MLE is closely related to a mammalian RNA helicase. MSL2 has a variety of protein sequence motifs; notable among them is a RING finger putative DNA-binding domain. This motif has been found in a functionally diverse family of proteins whose members mediate transcription, DNA repair, and recombination.

The molecular mechanism of dosage compensation in flies involves an isoform of histone H4 mono-acetylated at lysine 16. Mutation of lysine 16 in yeast histone H4 alters chromatin structure at the silent mating-type loci and leads to derepression of transcription (119). This fly histone isoform is found preferentially on the male X chromosome in a pattern that generally coincides

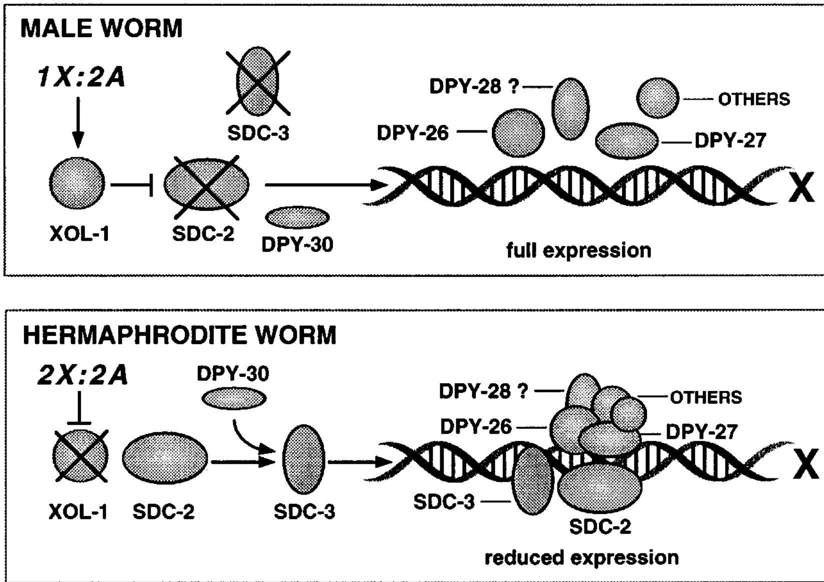


Figure 6 Control of *C. elegans* dosage compensation by the *sdc* and dosage-compensation *dpy* genes. In males, *xol-1* inactivates *sdc-2*, thereby preventing a dosage-compensation complex from assembling on X. In hermaphrodites, *sdc-2* is active and together with *dpy-30* activates *sdc-3*. Both the SDC-2 and SDC-3 proteins localize to X and assemble a DPY protein complex that reduces expression of both hermaphrodite X chromosomes.

with that for the binding of the MSL proteins (228). It is not found on X if any *msl* gene is nonfunctional, and it is found, along with all four MSL proteins, on the X chromosomes of female cells that have failed to maintain *Sxl* in an active state (26).

Like the fly, the worm achieves dosage compensation by localizing dosage-compensation gene products to the X chromosome in only one sex, but it is the opposite sex from flies (Figure 6). Molecular analysis of *DPY*-27 first provided this insight (42). DPY-27 is localized to both hermaphrodite X chromosomes throughout most of development. Although it is also produced and localized to the nucleus in males, it does not bind to the male X chromosome. Thus, as in flies, only the sex that actively implements dosage compensation has its X chromosomes decorated with dosage-compensation proteins.

The localization of DPY-27 to X at the 30-cell stage reflects the onset of dosage compensation in worms. There is no need for dosage compensation to begin earlier, since the onset of embryonic transcription is between the 8- and 16-cell stage (63). Fly dosage-compensation also begins early, as soon as

somatic cells form in the young embryo, at or just after the point when zygotic transcription first reaches high levels (80).

DPY-26 behaves like DPY-27: It is produced in both sexes but localizes to X only in XX animals around the 30-cell stage (J Lieb, E Capowski, P Meneely, B Meyer, unpublished information). The DPY-26 antibody staining pattern is more complex than the DPY-27 pattern, however, reflecting the role of DPY-26 in other processes. Consistent with the involvement of *dpy-26* in meiosis, DPY-26 protein associates with all chromosomes in meiotic germ cells of both sexes. In contrast, DPY-27 is absent from the germline. In young embryos that have not yet begun dosage compensation, DPY-26 is diffusely distributed throughout interphase nuclei, but unlike DPY-27 it associates with all condensed mitotic chromosomes. A few cell divisions later it becomes specifically localized to X for the remainder of (somatic) development.

In worms, as in flies, dosage-compensation proteins assemble on X as a complex (P-T Chuang, J Lieb, B Meyer, unpublished information). DPY-27 antibodies immunoprecipitate DPY-26 from wild-type extracts but not from *dpy-26*, *dpy-27*, or *dpy-28* mutant extracts. Conversely, DPY-26 antibodies immunoprecipitate DPY-27 from wild-type but not from *dpy-27* mutant nuclear extracts. This complex contains at least two other proteins. Based on their size they cannot be the products of previously cloned dosage-compensation genes, but by the following rationale one may be DPY-28: Both DPY-27 and DPY-26 proteins are undetectable in DPY-28 mutants, even though the *dpy-27* and *dpy-26* transcript levels are nearly wild type. This indicates that *dpy-28* affects either the production or stability of DPY-26 and DPY-27. If DPY-27 and DPY-26 function in a complex with DPY-28, both might become unstable in the absence of their DPY-28 partner. There is precedence for such destabilization both in the case of DPY-27 in a *dpy-26* mutant background, and between MSL1 and MSL2 in *Drosophila* (124).

Although *dpy-27* mutations disrupt the stability of DPY-26 and its localization to X, they do not interfere with the role of DPY-26 in meiosis or its association with mitotic chromosomes. In contrast, *dpy-28* mutations interfere with the role of DPY-26 in these three processes (J Lieb, E Capowski, P Meneely, B Meyer, unpublished information). The participation of DPY-26 in meiosis, dosage compensation, and perhaps mitosis may therefore be a consequence of its association with different protein partners in each of these different roles.

An important clue as to the mechanism of dosage compensation in worms came from the amino acid sequence of DPY-27. DPY-27 is a member of the SMC (structural maintenance of chromosomes) family of proteins known to be involved in several aspects of chromosome dynamics (42). Yeast SMC proteins are essential for mitotic chromosome condensation and segregation (185, 212,

213). In frogs, they have been shown to participate in the induction and maintenance of chromosome condensation (98). The similarity of *dpy-27* to the SMC proteins, together with its X localization, suggests that DPY-27 may reduce X-chromosome transcript levels by inducing partial X-chromosome condensation in interphase nuclei. Perhaps worms have adopted an evolutionarily conserved mechanism of chromosome condensation used previously in mitosis to achieve dosage compensation. Although it is too early to speculate, such a mechanism would appear to be different from the mechanism likely to be used by flies, which seems to involve histones more directly.

In Flies, the Coordinate-Control Gene Sxl Regulates Dosage Compensation at Least in Part by Controlling the RNA Splicing of msl2, Whereas in Worms, the Coordinate-Control Genes Regulate Dosage Compensation by Recruiting the DPY Proteins to the X Chromosome

Molecular characterization of *msl2*, the last fly *msl* gene to be cloned, gave the first indication of how *msl* functioning might be controlled by *Sxl* (16, 124, 241). As mentioned above, an intron with two SXL-binding sites was found in the 5′ untranslated region of this gene that is spliced out only in the absence of SXL. One group also reported that *msl2* mRNA is much less abundant in females (241). Several possibilities have been raised for how retention of this intron in noncoding RNA might interfere with translation and, perhaps secondarily, mRNA stability. A second level of control by SXL has been proposed based on the fact that there are four additional optimal SXL-binding sites in the 3′ untranslated region (UTR) of *msl2* mRNA (16, 124). SXL binding to this 3′ UTR might contribute to *msl2* negative regulation. Such an abundance of potential SXL-binding sites in *msl2* is unlikely to be fortuitous. From a total of 1324 *Drosophila* 3′ UTR sequences examined, only 21 were found to contain even 3 such sites, of which 20 belong to X-linked genes (124). Among them is *runt*, a gene whose early dosage compensation is *msl* independent and might therefore be mediated directly by SXL (80). The one autosomal sequence in this group belongs to *msl1*! These findings suggest that SXL might participate directly in dosage compensation by reducing the expression of these 20 X-linked genes in females.

An ectopic expression construct established that *msl2* is a switch gene (124). This construct expresses the *msl2* open reading frame via a heterologous promoter to generate a transcript lacking the potential 5′ and 3′ sites for negative regulation by SXL. The transgene can serve as the sole source of *msl2* function in males, and it escapes negative regulation by SXL, so that substantial amounts of MSL2 protein are generated in females. MSL2 protein induces assembly

of the other three MSL proteins on the female X chromosomes and imparts to those chromosomes the diffuse appearance characteristic of the hyperactivated male X. However, female lethality caused by ectopic MSL2 is incomplete, suggesting that some other dosage-compensation component is limiting. This component is likely to be MSL1, since deleting one copy of *msl1*$^{+}$, a change that has no adverse effect on males, suppresses the MSL2 transgene phenotype.

An understanding of how the worm coordinate-control genes regulate dosage compensation came from analyzing the effects of *sdc* and *xol* mutations on DPY-26 and DPY-27 X-chromosome localization. As predicted, both DPY-26 and DPY-27 decorate the single X of mutant *xol-1* males, consistent with the role of this male-specific switch gene in blocking the hermaphrodite program of dosage compensation. Predictions for the effects of the *sdc* and *dpy* genes were less clear, since it had not been possible previously to order these genes with respect to their functions in dosage compensation. Mutations in *sdc-2*, *sdc-3*, and *dpy-30* cause XX animals to behave like XO males in that they produce DPY-26 and DPY-27 proteins but fail to localize them to X (P-T Chuang, J Lieb, B Meyer, unpublished information). Because the SDC-2 and SDC-3 proteins are themselves localized to X, these two coordinate control genes most likely regulate dosage-compensation gene function by recruiting the DPY proteins to the X chromosome (T Davis, D Lapidus, H Dawes, B Meyer, unpublished information). Consistent with this view, ectopic expression of *sdc-2* in XO animals is sufficient to assemble the dosage-compensation complex on the single male X chromosome (H Dawes, T Davis, B Meyer, unpublished information). In contrast, DPY-30 plays an indirect role in X localization. The novel, 123-amino-acid DPY-30 protein is diffusely distributed throughout the nuclei of both sexes, and its staining pattern is not affected by any dosage-compensation mutations (113). Hence DPY-30 is not part of the dosage-compensation complex. DPY-30 influences dosage compensation through its effects on *sdc-3*, since SDC-3 protein is not made in the absence of DPY-30. Thus, unlike the situation in flies, some dosage-compensation genes in worms act as regulators of others and do not participate in the dosage-compensation process through an association with the X chromosome.

In contrast to mutations in *sdc-2*, *sdc-3*, and *dpy-30*, mutations in *sdc-1* and *dpy-21* have no effect on the DPY-26 and DPY-27 staining pattern (P-T Chuang, J Lieb, B Meyer, unpublished information). These two genes must affect some aspect of the dosage-compensation process other than localizing or stabilizing DPY-26 and DPY-27.

Figure 6 presents a model for the sex-specific assembly of the dosage-compensation complex in worms, based on all information currently available. In hermaphrodites, the XX-specific *sdc-2* switch gene is active and produces

SDC-2 protein in the presence of the maternally supplied SDC-3 and DPY-30 proteins. Around the 30-cell stage of embryogenesis, SDC-2, in collaboration with SDC-3, localizes to the X chromosomes and recruits a protein complex consisting of at least DPY-26, DPY-27, and possibly DPY-28. These dosage-compensation proteins then alter the structure of interphase X chromosomes, thereby causing a reduction in X-linked transcript levels. In males, the XO-specific *xol-1* switch gene is active and represses the *sdc-2* gene, thus preventing the recruitment of a dosage-compensation protein complex to X.

For Flies, the msl Genes Participate in Only Some Aspects of Dosage Compensation Directed by Sxl, Whereas for Worms, the dpy Genes Participate in All Aspects of Dosage Compensation Directed by sdc-2

If the only role of *Sxl* in dosage compensation were to repress the *Drosophila msl* genes and thereby prevent hyperactivation of X-linked genes, and if *Sxl* had no essential female-specific function other than dosage compensation, mutations eliminating *msl* function should suppress the female-lethal effect of mutations that abolish *Sxl* activity. Rescued females should develop as males, since they would still lack the Sxl^+ activity needed to regulate *tra*. In the early 1980s it was shown that *msl* mutations do not rescue Sxl^- females (200, 230). One of two simple explanations could account for this lack of suppression: Either the *msl* genes do not control as many aspects of dosage compensation as *Sxl*, or *Sxl* controls some unknown, embryo-vital, cell-autonomous function that depends strictly on the X:A balance yet is unrelated to dosage compensation. Some researchers felt that Occam's razor favored the first alternative (48), while others held the opposite view (8). First, nothing in the isolation or characterization of the *msl* genes had demanded that they control the compensation of all dosage-compensated genes, or control these genes at all stages of development. Second, the screens in which *msl* genes were recovered were biased against pleiotropic genes and hence could easily have missed key regulators of the process. Third, based on expectations from the study of aneuploids, the relatively late lethal periods of MSL mutations did not mesh with such a comprehensive role in dosage compensation. Last, even with all the information available today, it is still difficult to imagine what sex-specific vital function unrelated to dosage compensation *Sxl* could control.

Any serious question that the role of the *msl* genes in dosage compensation was more limited than initially believed seemed to be eliminated with the report that *Sxl* and *da* control the dosage compensation of a segmentation gene named

runt, while the *msl* genes do not. The validity of these *runt* studies has been questioned recently (9) based on the ironic discovery that *runt* itself is part of the X:A signal that ultimately leads to *runt* dosage-compensation (62). However, the concerns raised had already been discussed and dismissed by others using the *runt* compensation assay in a situation that would have been far more likely to be complicated by such a consideration (21). In any event, the complications that one might reasonably anticipate would conspire against, not in favor of, the clear-cut results obtained. A more limited role for the *msl* genes has become more attractive in light of proposals of how SXL might directly control the dosage compensation of X-linked genes like *runt* (124).

Although mutations in the *msl* genes do not rescue Sxl^- females, they can rescue Sxl^- female cells in situations where females would survive even without such rescue, and a mosaic intersex phenotype results (49). Masculinizing interactions had been observed between *Sxl* and *msl* mutations early on (8, 200, 230) , but understanding why one would expect them to arise from cell rescue rather than from direct effects of *msl* mutations on sexual differentiation required understanding important features of *Sxl* regulation. When *Sxl* regulation is impaired only moderately, flies can develop as epigenetic mosaics of cells that express *Sxl* and cells that do not, but the cells that do not are lost, in some cases with no obvious effects on the adult. Apparently the limited relief from dosage-compensation upsets that the *msl* mutations provide to cells lacking SXL prevents at least some of these cells from dying, but is not sufficient to restore them, much less the whole animal, to full health. Some very special alleles of *Sxl* can be suppressed spectacularly by *msl* mutations, with the XX survivors developing as males (49).

In worms, all evidence indicates that the dosage-compensation *dpy* genes and the *sdc* genes work together in all aspects of dosage compensation, and that males simply inactivate that process rather than activate a male-specific expression system. First, all dosage-compensation genes with XX-specific lethal phenotypes are involved in the sex-specific assembly of the same large dosage-compensation complex on the hermaphrodite X chromosomes. Second, a male will die if it inappropriately assembles that dosage-compensation complex on its X. Such inappropriate assembly will occur if a male lacks *xol-1* or if he inappropriately expresses *sdc-2*, but in either case the lethality can be suppressed by loss-of-function alleles of any *sdc* or *dpy* gene, indicating that this set of genes regulates and implements a single dosage-compensation process. Such suppression could not occur if any significant part of the male lethalty were the result of failure to activate a male-specific X-chromosome expression system rather than the failure to inactivate the XX-specific dosage-compensation system.

REGULATORY GENES THAT SPECIFICALLY DIRECT SOMATIC SEXUAL DIFFERENTIATION

For Flies, a Cascade of Regulated RNA Splicing Determines the Expression State of the Bifunctional Switch Gene dsx Whose Sex-Specific Products Drive Males and Females Away from a Common Intersexual State of Differentiation

The switch gene *tra* (*transformer*), the direct regulatory target of *Sxl*, was discovered in the 1940s. Its relevance to fly sex-determination was obvious, since null *tra* alleles have no effect on males but transform females into sterile pseudomales that are identical to males in every respect except for their nonfunctional gonads and female size (214). The switch character of *tra* was established, and its placement in the sex-determination hierarchy confirmed, through analysis of a *tra* cDNA expression construct that circumvented the SXL splicing control (146). To carry out its feminizing role, *tra* requires a partner, *tra2* (*transformer2*), whose null phenotype in XX animals appears identical to that for null *tra* alleles (74). Unlike *tra*, *tra2* is constitutive in the soma (145). Together, these two genes control the sex-specific alternative RNA splicing for *dsx* (*doublesex*), an unusual bifunctional switch gene (Figure 3) (35, 146, 158). However, not all sex differences that arise from the sex-specific functioning of *tra* and *tra2* are abolished by loss of *dsx* function; hence, *dsx* cannot be the sole target of *tra* and *tra2* (219, 221). The residual differences involve aspects of behavior and the male-specific "muscle of Lawrence." A likely target for *tra* on this minor branch of the sex-determination pathway is *fru* (*fruitless*), since *fru* mutations affect sexual behavior and the male muscle (76, 77).

Elegant genetic analysis showed that *dsx* is downstream of *tra* and *tra2* and that it functions actively in alternative modes in the two sexes to elicit either male or female differentiation (10). The *dsx* bifunctionality is evident from its null phenotype: a somatic sexual intermediate of the true intersex variety for both sexes. Its bifunctional switch-gene character was revealed by mutant alleles that differentially produce male-specific (DSX^M) or female-specific (DSX^F) products (157). Studies using female-specific, dominant masculinizing dsx^M alleles, which generate only DSX^M product regardless of the X:A signal, showed that the presence of DSX^M and DSX^F in the same cell produces a phenotype similar to that of the null allele. Therefore, the sex-specific *dsx* products are mutually antagonistic.

The 427-residue DSX^F protein is made in females as a consequence of TRA and TRA2 assembling at a *dsx* repeat element (*dsxRE*) in the *dsx* pre-mRNA to promote the use of a noncanonical female-specific 3′ splice site and hence

the production of mRNA-encoding DSX^{F} (94, 112, 141, 157, 184). In the absence of either TRA or TRA2, an alternative conventional splice site is used by default, and the resulting mRNA encodes the 549-residue DSX^{M} protein. TRA2 protein binds directly to *dsxRE* RNA, apparently recruiting a complex that includes TRA and general splicing factors of the SR family (222). Like SR family members, TRA and TRA2 proteins contain a domain rich in arginine and serine (5, 25, 82). TRA2 also contains a conserved RRM domain characteristic of a large family of RNA-binding proteins that includes SXL. The two DSX proteins differ only in their C-termini (35). A novel zinc-finger-related DNA-binding motif resides in their common region (67).

DSX proteins work both by repressing gene expression that is only appropriate for differentiation of the opposite sex and by inducing gene expression appropriate to the same sex (53, 120, 220). This behavior is illustrated by the fact that both DSX^{M} and DSX^{F} bind to the same four sites within a 127-bp FBE (fat-body enhancer) element of the (female-specific) yolk genes, yet DSX^{M} binding represses transcription, whereas DSX^{F} binding enhances it (53). Hence these gene products and their targets display the kind of functional flexibility that is compatible with the extremely rapid evolution of sexual morphology.

Yolk-gene regulation represents one of the two basic modes by which *dsx* controls sex differentiation: (*a*) continuous regulation of a target to maintain sexual phenotype or (*b*) regulation only during a relatively brief critical period in development, after which sexual phenotype is irreversible (reviewed in 36). The difference may simply reflect whether the targets are regulatory genes that generate sex-specific structural proteins (e.g. yolk) in a non-sex-specific tissue (e.g. the fat body) or instead genes that specify the differentiation of a sex-specific cell or tissue type (e.g. the male accessory gland).

As with the other switch genes in the fly sex-determination hierarchy, *dsx* is assisted by partners not themselves sex-specifically regulated: *ix* (*intersex*) and *her* (*hermaphrodite*) (10, 38, 180, 181). Neither *ix* nor *her* affects the sex-specific splicing of *dsx* RNA, but both are required for the activity of DSX^{F}. Loss-of-function mutations in either gene cause females–and only females–to differentiate as true intersexes. Ironically, *her* participates in transcriptional events that both begin and end the sex-determination pathway illustrated in Figure 3. The gene *her* has non-sex-specific vital functions, but *ix* may be sex specific.

It is unclear what is responsible for size dimorphism in flies. *Sxl* may play a part, but alternative explanations for the smaller size of XX animals masculinized by leaky Sxl^{f} mutations have not been ruled out, and no Sxl^{M} X(Y) escapers larger than normal males have been found.

For Worms, a Complex Gene Cascade Determines the Functional State of tra-1, a Switch Gene that Imposes the Hermaphrodite-Specific Pattern of Differentiation

All aspects of sexual differentiation in worms are controlled by a complex regulatory cascade that includes several switch genes (Figure 4). This cascade is substantially more complex than the fly cascade and uses more diverse forms of gene regulation, but not the alternative pre-mRNA processing central in flies. The fly and worm cascades are nonetheless similar in that their terminal switch genes encode transcription factors, although the worm gene (*tra-1*) is sex specific in its action rather than bifunctional, at least in the soma. A factor adding complexity to the worm cascade may be the involvement of its genes in germline as well as somatic sex-determination plus the complication that hermaphrodites are programmed to make both sperm and oocytes.

The first three sex-determination genes identified in the worm resembled fly genes in that null mutations transform XX animals into males (108). The most severe null phenotype of these transformer genes (*tra*) is that of *tra-1*: XX *tra-1* worms are transformed into fertile phenotypic males. Four additional genes with no functional counterparts in the fly were discovered subsequently: *her-1* (hermaphroditization) (99) and three *fem* (feminization) genes (61, 102, 127). All four are required for male development, and the *fem* genes are also required for spermatogenesis in hermaphrodites. Null *her-1* XO worms are fertile hermaphrodites, while null *fem-1, fem-2* and *fem-3* XX and XO animals are fertile, spermless females.

The genes *her-1*, *tra-1*, and *tra-2* all function as switch genes, with constitutive activity of their products being sufficient to set sexual fate regardless of the X:A signal. For all three genes dominant alleles exist that have phenotypic effects opposite to those of null alleles. Dominant *tra-1* mutations transform XO animals into either hermaphrodites or spermless females (99, 103). Dominant *tra-2*(eg) mutations transform XO animals into hermaphrodites (106). Dominant *her-1* mutations partially masculinize XX animals (227).

A negative regulatory cascade was deduced from analysis of the epistatic interactions among these dominant and recessive mutations (99, 102). The gene *tra-1* was identified as the terminal regulator because its activity is sufficient to trigger hermaphrodite development, while loss of its activity specifies male development, regardless of the activities of other genes in the pathway. Genetic analysis further indicated that *tra-1* activity is set in the following way: In XO animals, *her-1*, the first gene in the pathway, negatively regulates *tra-2*, thereby activating the *fem* genes. The *fem* genes inactivate *tra-1* to elicit male development. The gene *her-1* must remain active throughout male development to prevent yolk production (192). In XX animals, *her-1* is repressed

and *tra-2* negatively regulates the *fem* genes, thereby activating *tra-1* to elicit hermaphrodite development. The proposed role of *tra*-3 in the pathway is to potentiate *tra-2* activity.

Molecular analysis confirmed this view of the regulatory cascade and revealed the great diversity of molecular mechanisms that operate in it. As in flies, transcriptional control is central for the first and last steps of the pathway, but cell-cell signaling and signal transduction are central for the middle steps to coordinate the sexual fates of the sexually dimorphic cells. The gene *her-1* is regulated at the level of transcription by the *sdc* genes, which function as *her-1* repressors in XX animals (58, 226). This negative regulation can be overcome by mutations in any of the *sdc* genes or by the dominant mutations in the *her-1* promoter region. The functional *tra-1* gene product is a protein with five tandem zinc fingers resembling those in the *Drosophila* CI^D protein and in two human oncogenes associated with glioblastoma tumors, *GLI* and *GLI3* (239). TRA-1 binds DNA and most likely acts as a transcription factor to control downstream sex-differentiation genes (240). No direct TRA-1 targets have been found; however, in response to *tra-1* action, genes required for male-specific development such as those involved in forming the complex tail structures or in mating behavior (66) must be repressed, and genes required for hermaphrodite-specific development such as those essential for vulval development (90) and yolk production must be activated. Dominant *tra-1* mutations render the gene insensitive to negative regulation and cluster to a small region at the N-terminus of the protein, suggesting that the sex-specific regulation of *tra-1* is achieved posttranslationally via inhibitory protein-protein interactions, presumably with one or more of the FEM proteins (56).

The gene *her-1* participates in the signal transduction step (117, 175). It encodes a small novel protein with a predicted signal sequence. Ectopic *her-1* expression directed by a promoter specific to the myosin body wall muscles masculinizes the soma and germline of XX animals, suggesting that HER-1 protein functions as a secreted signaling molecule. The masculinization requires the putative signal sequence. Additional evidence for a signal transduction cascade is the non-cell-autonomous behavior of *her-1* (117; see below).

The likely receptor of the putative HER-1 ligand is the TRA-2A protein, which is present in both sexes. It has nine potential membrane-spanning domains and a putative signal sequence (135, 164). Overexpression of TRA-2A from a transgene driven by a heat-shock promoter transforms XO animals into fertile hermaphrodites, indicating that TRA-2A is normally inactivated in males and that this negative regulation can be overridden by overexpression (134). If HER-1 ligand inactivates the putative TRA-2A receptor protein by binding to its extracellular domain, one might expect to find *tra-2* mutations that interfere with the HER-1 binding but not with the TRA-2A feminizing activity. Such dominant

tra-2 mutations have been identified, and they have the properties expected for constitutively active TRA-2A. The mutations alter a predicted extracellular domain of TRA-2A and transform XO animals into hermaphrodites (106, 133).

It has been proposed that TRA-2A inactivates one or all of the intracellular FEM proteins in XX animals through a physical association with its carboxy-terminal cytoplasmic domain, thereby preventing TRA-1 inactivation (135). By this model, HER-1 binding to TRA-2A releases the FEM proteins from TRA-2A, allowing them to inactivate TRA-1. Support of this proposal is of two types: First, expression of the TRA-2A C-terminal cytoplasmic domain from a heat-shock promoter feminizes phenotypic males, implying that extra copies of this domain titrate a male-specific protein (134). Second, the novel protein FEM-3 (2) physically associates with the TRA-2A C-terminal cytoplasmic domain, as assayed by the yeast two-hybrid system and by co-immunoprecipitation of the two proteins synthesized in vitro (A Mehra, P Kuwabara, L Heck, A Spence, personal communication). It is unknown whether this physical association is sufficient to inactivate FEM-3.

How the FEM proteins inactivate TRA-1 has not been established, although all three FEM proteins are clearly required. For example, do the FEM proteins interact to form a regulatory complex, do they act sequentially to produce one inhibitory activity, or do they function independently? FEM-1 contains six copies of an ankyrin motif, which in other proteins mediates specific protein-protein interaction (202). FEM-2 interacts directly with FEM-3, as demonstrated by the yeast two-hybrid system and by co-immunoprecipitation (41). Finally, FEM-2 is related to Type 2C protein serine/threonine phosphatases, and FEM-2 possesses protein phosphatase activity in vitro (41, 176). Mutational analysis showed that this activity is necessary for FEM-2 to promote male development in vivo (41). Thus protein phosphorylation appears to be involved in the signal transduction steps that coordinate worm sexual fate.

Although *tra-3* potentiates *tra-2*, it does not play a direct role in the signal transduction aspect of TRA-2A's function. TRA-3 is a member of the calpain regulatory protease family (13), and recent experiments indicate that it may affect TRA-2A by inactivating a translational repressor of *tra-2* (B Goodwin, J Kimble, personal communication).

Current molecular models make the regulatory complexities of worm sex-determination seem less bewildering. Nonetheless, several minor branches and feedback mechanisms exist throughout the pathway that can have profound effects on sexual phenotype despite having no molecular explanation. One such subtlety is revealed by the fact that a null mutation in the terminal hermaphrodite switch gene *tra-1* has a greater masculinizing effect on XX animals than a null mutation in the upstream hermaphrodite switch gene, *tra-2*: Null *tra-1* XX animals are fertile males, whereas null *tra-2* XX animals are

nonmating, incomplete males (99). Thus control of *tra-1* by the X:A signal cannot all be exerted through *tra-2*. There must be an additional feminizing activity in hermaphrodites that contributes to *tra-1* activation. Ironically this minor feminizing activity in XX animals is mediated by *xol-1*, the coordinate-control switch gene that sets the male state in XO animals (151). The wild-type appearance of XX *xol-1* mutant animals gives no hint of such a role, but it is revealed in XX animals that have been incompletely masculinized by mutations in other genes such as *tra-2*. The *tra-2*; *xol-1* XX double mutants are fertile males. Similarly, *xol-1* mutations enhance partially masculinizing mutant alleles of *sdc-1*, *sdc-3*, and *tra-3*. This masculinizing effect of *xol-1* mutations is independent of the masculinizing switch gene *her-1*, which lies between *xol-1* and *tra-1* in the hierarchy. Thus this feminizing effect of wild-type *xol-1* must occur via a parallel pathway that intersects the main pathway between *tra-2* and *tra-1*. The feminizing activity of *xol-1*(+) in XX animals is separately mutable from the masculinizing activity in XO animals, and the two activities function at different times in development (182). Hence these two *xol-1* functions act independently and are likely to be mechanistically distinct.

The Fly Somatic Gene Cascade Generally Operates in a Cell-Autonomous Fashion, While the Worm Cascade is Non-Cell-Autonomous

Ever since *Drosophila* gynandromorphs were discovered in the early part of this century, it has been clear that assessment of the X:A ratio and sexual differentiation are cell-autonomous processes, at least for cells of the imaginal discs and histoblasts (153). Gynandromorphs are genetic mosaics that arise as a consequence of X-chromosome loss only during the earliest nuclear divisions in diplo-X embryos, generating animals that are a mixture of XX and XO cells. Because these embryos develop into sexually mosaic adults in which the sexual phenotype of individual cuticular cells invariably reflects their chromosomal sex (224), it came as no surprise that all the somatic switch genes from *Sxl* to *dsx* are strictly cell autonomous in the cuticle (10, 45, 46, 190, 237). Isolated examples of partial nonautonomy have been documented for noncuticular somatic tissues, but they appear to represent peculiarities of sexual differentiation per se, rather than nonautonomy in the operation of the gene hierarchy leading to *dsx*. For example, if female gonadal mesoderm comes in contact with male genital disc cells, the XX cells can be induced to produce male pigment (75). Moreover, male nerve cells can induce female muscle precursors to form the male-specific muscle of Lawrence (137).

What has been learned about X:A assessment does, however, raise the possibility that the perfect correspondence between sexual genotype and phenotype in the adult cuticle could be an exaggeration of the true degree of cell

autonomy of the sex-determination process, since cells that improperly assess their X-chromosome dose can be expected to die prior to the adult stage from dosage-compensation upsets. As long as the fraction of cells involved is relatively low, the animal can eliminate any trace of the problem. Since the border between cells that do or do not express *Sxl* has been found to be quite sharp even in blastoderm-stage gynandromorphs (27), the amount of X:A misassessment is likely to be small; nevertheless, a more rigorous analysis of such mosaics is needed to establish the true degree of cell autonomy in fly X:A signal assessment.

Worms are at the opposite end of the spectrum with respect to the involvement of cell-cell signaling in somatic sex-determination. Analysis of both *sdc-1* genetic mosaics (235) and triploid intersexes (191) showed that worm cells do not to choose their sexual fates independently. Since *tra-1* behaves in a strictly autonomous manner, only genes upstream of it can be responsible for this nonautonomy (116). The gene *her-1* is one of the culprits. Its nonautonomous behavior in mosaic studies is striking (117). Not only can *her-1*(−) cells express a wild-type fate if surrounded by *her-1*(+) cells, the converse is also true: *her-1*(−) cells can force their *her-1*(+) neighbors to express a mutant fate! Not all *her-1*(−) cells are equivalent in their effects on *her-1*(+) cells. Generally it is *her-1*(−) cells derived from the posterior blastomere (P_1) of the two-celled embryo that influence *her-1*(+) cells derived from the anterior blastomere (AB). The converse is rarely if ever true. However *her-1*(−) cells can affect *her-1*(+) cells even within the AB lineage. These results indicate that *her-1* activity in sexually dimorphic cells is neither necessary nor sufficient for their male development. The cell-cell communication indicated by these results can be accounted for by the model in Figure 4 if one assumes that the difference in potency of *her-1*(−) cells to affect *her-1*(+) cells reflects differences among cell types in the concentration of TRA-2A, the putative HER-1 receptor.

It has been suggested that the nonautonomy in nematode sex-determination serves as an error-correction mechanism for an organism that does not have the luxury of a regulatory strategy of development (117). However, it is not obvious how practical such a mechanism would be if mistakes made by a minority of specific cells (derived from P_1) prevent the majority of cells from making the correct choice. Moreover, if the original mistake were caused by misreading the X:A signal, a mechanism for correcting errors in sexual fate would be useful only if it were accompanied by an equivalent mechanism for correcting dosage-compensation upsets. In this connection, it is unknown whether the regulation of dosage compensation, or indeed the assessment of the X:A signal, is cell autonomous.

CONTROL OF GERMLINE SEXUAL IDENTITY

For Flies and Worms, Sexual Differentiation is Determined in the Germline Differently from the Soma, and the Differences are Greater for Flies Than Worms

The way in which the sex of fly germ cells is determined differs from the way sex is determined in the soma. For the worm there are more similarities, but even for them not all genes involved in germline sex-determination participate in the somatic process, and even genes involved in both processes may have different specific roles and regulation in the two cell types. Worms have the added complication that male gametes are made in both sexes. It has been more difficult to discover how germ-cell sex is determined for the fly than for the worm in part because genetic manipulations that interfere with *Drosophila* germline sex-determination cause germ cells to abort growth and/or differentiation, making assessment of their sexual phenotype problematic. Worm germline mutant sex phenotypes are more straightforward, perhaps because gametogenesis in the worm does not require as extensive a collaboration between germline and somatic cells.

The fact that fly gonadal sexual phenotype is determined by the genotype of the gonad itself was established by the observation in 1957 that immature gonads transplanted to larvae of the opposite sex differentiate according to their own chromosomal sex, not that of the host (75). Years later, a sophisticated extension of gynandromorph analysis was used to explore the role played by cells within the gonad in determining their sex (196). This study established that when the somatic cells of the gonad are female, an X:A ratio-sensing system operates cell-autonomously in the germ cells in a fashion that closely resembles its operation in the soma. X-chromosome loss early in the development of XXX AAA embryos generated adults with mosaic gonads in which all somatic cells were XXX and hence female, but all germ cells were XX AAA and thus had a potentially ambiguous sex-determination signal. These XX AAA germ cells exhibited two distinct modes of differentiation even within the same ovary. Some formed normal-appearing ovarian cysts that developed into eggs, while others formed tumorous cysts in which cells proliferated abnormally and ultimately degenerated. The same tumorous cyst phenotype was displayed by diploid Sxl^- XX germ cells transplanted into Sxl^+ female hosts, suggesting that the tumorous cyst phenotype of germ cells with an ambiguous sex-determination signal might arise whenever such cells fail to activate Sxl^+ and therefore attempt to embark on a male developmental pathway while surrounded by female somatic cells. In contrast, XX AAA germ cells that stably activate Sxl^+ follow a normal female pathway of differentiation. By this model,

triploid intersex germ cells in these bimodal gonads would exhibit essentially the same *Sxl*-based mosaic intersexuality observed for triploid intersex cells of the cuticle and shown to result from a reduced but nonzero probability of cells' engaging the *Sxl* autoregulatory feedback loop. This hypothesis was verified by the demonstration that the semiconstitutive allele Sxl^{M1} suppresses tumorous cyst development in triploid intersexes, causing all germ cells to chose the female pathway and differentiate eggs (162).

Diploid germ-cell transplants, however, showed that not all the differences between male and female diploid germ cells can be the result of effects on *Sxl*, since XX germ cells fail to survive in wild-type male hosts regardless of their *Sxl* genotype (196). Moreover, transplanted X(±Y) germ cells grow well when associated with a male gonadal soma but cannot be recovered in wild-type female hosts regardless of their *Sxl* genotype.

The *tra* gene, the somatic sex-determination target for *Sxl*, was shown not to be a *Sxl* target in the germline by the fact that tra^{-} XX germ cells make functional eggs when transplanted to a tra^{+} XX somatic environment (144). In the same way, germline expression of *dsx*, *tra2*, and *ix* was shown not to be required for oogenesis (195). A major difference between somatic and germline targets of *Sxl* is also indicated by the behavior of a fully viable but female-sterile class of mutant *Sxl* allele whose members all have missense changes in a region of SXL common to all isoforms (28; RA Lersch, TW Cline, unpublished information). These alleles appear wild-type with respect to *Sxl* somatic functions, but when homozygous they produce a tumorous cyst phenotype in the ovary like that of Sxl^{-} germline mosaics (187).

Although regulation of *Sxl* in the germline by the X:A signal is cell autonomous if the surrounding soma is female, surprisingly it is not cell autonomous if that soma is male. The literature on *tra* and *Sxl* contained hints of such nonautonomy (49, 144), but the point was established only by an examination of aberrant gonadal phenotypes generated by transplantation of germ-cell precursors between donor and host embryos of opposite sexes (210). Such experiments had been uninformative earlier because sexually mismatched donor germ cells were eliminated in competition with the hosts' own germ cells. This complication was overcome through the use of hosts that had no germ cells of their own.

In this less competitive situation, male germ cells survived well in a somatically female gonad and displayed the male-like morphology expected if germ-cell sexual identity were determined only by the germ cells' own X:A ratio, although they never progressed beyond the spermatocyte stage. Sometimes they developed into germline tumors similar to those observed previously for XX AA, Sxl^{-} germ cells. The very male-like appearance of Sxl^{-} XX germline tumor cells was noted and used to support the argument that a tumorous

cyst phenotype can be indicative of a male germ-cell fate in a female somatic environment.

In the reciprocal transplantation experiment, however, it was found that the gonadal soma can influence the functional state of *Sxl* in XX germ cells. Female germ cells in a male soma failed to follow their own X:A signal but instead embarked on an abortive, male-like developmental program, and their viability was much reduced. Both masculinization and reduced viability could be traced to a failure of these XX germ cells to activate *Sxl*, since both effects were suppressed by Sxl^{M1}. Although these experiments confirmed a major role for *Sxl* in the development of XX germ cells and showed that activation of *Sxl* required somatic input as well as the germ cells' own X:A signal, this study provided no evidence for a switch-gene role for *Sxl*. An earlier report (47) was confirmed that Sxl^{M1} does not impose the female pathway choice on XY germ cells, regardless of their somatic environment. However, since Sxl^{M1} was already known to be incompletely penetrant in the soma, no inferences could be drawn from this negative result.

A curious aspect of these transplantation studies seems to have escaped notice: XX cells require female Sxl^+ function for normal viability in a male somatic environment, since Sxl^{M1} allows them to grow much better than Sxl^+ or Sxl^- cells; nevertheless, XX cells do not require female Sxl^+ function for normal viability in a female (XX) somatic environment. Hence there must be a proliferative factor required by XX germ cells that can be provided either from the XX soma or from female expression of *Sxl* in the germ cells.

The feminizing effect of the XX gonadal soma on XX germ cells requires operation of the same sex-determination gene hierarchy in the gonadal mesoderm as is required in nongonadal cells to determine somatic sex. Loss-of-function mutations in somatic *Sxl*, *tra*, *tra2*, or *dsx* have morphologically masculinizing effects on XX germ cells that resemble the effects of a male soma, although the penetrance of these effects is generally not as high (33, 49, 162, 197). As expected, Sxl^{M1} blocked this masculinization in all cases examined (162). This is a second way in which *Sxl* is "upstream of itself" in a regulatory sense.

The relationship between somatic and germline sex is more complex than first believed. Both a male-specific and a female-specific input are generated from the bifunctional gene, *dsx*, downstream of *tra* (208). Moreover, additional evidence has accumulated for redundancy in somatic signaling from genes downstream of *Sxl*, and not all of this signaling appears to be channeled via *tra* through *dsx* (110, 165, 208).

The ultimate molecular effect of somatic and germline X:A signals on *Sxl* in germ cells appears to be the same as in the soma: removal of the male-specific exon from *Sxl* pre-mRNA (28, 165). Moreover, since ectopic expression of female-specific SXL protein in the male germline can induce removal of the

male exon and engage the splicing feedback loop, just as it does in the soma, autoregulation clearly operates also in the germline yet may not be a primary control point for the somatic and germline signals (91; JH Hager, TW Cline, unpublished information). This male germline autoregulation is blocked by snf^{1621}, just as snf^{1621} blocks female germline autoregulation.

Some genes that participate zygotically in *Sxl* germline autoregulation seem not to do so in the soma. For example, zygotic mutations in *otu* (*ovarian tumor*) or in *ovo* can block *Sxl*-induced autoregulation in male germ cells, yet these mutations do not affect *Sxl* somatic functioning (91; JH Hager, TW Cline, unpublished information). Both of these genetically complex, female-sterile loci play a role in conferring female germline sexual identity at least in part through their effects on *Sxl* functioning (7, 28, 165, 166, 174). Mutations in either gene can generate the characteristic ovarian tumor phenotype and cause *Sxl* germline transcripts to be spliced in the male mode. Moreover, Sxl^M alleles partially suppress the female-sterile phenotype of some *ovo* and *otu* alleles. Analysis of the functional relationships among these genes is complicated by the fact that both *ovo* and *otu* clearly have other female-specific germline functions besides the regulation of *Sxl*. For example, although Sxl^- germ cells proliferate normally, null alleles of *ovo* and *otu* can block proliferation of XX germ cells (130, 168, 183, 203). The gene *ovo* encodes a zinc finger protein (148, 149), while the *otu* protein products show no informative homologies (204, 205).

It is unknown how the female-specific splicing mode of *Sxl* is initiated in the germline. Neither the *sis* genes nor their target, Sxl_{Pe}, appear to be involved (89, 126, 207). The existence of a *Sxl* promoter specifically functioning in the germline was inferred from complementation between female-sterile *Sxl* alleles and female-lethal intragenic *Sxl* deficiencies (187). The subsequent discovery that the female-sterile *Sxl* alleles complemented are not themselves defective in the initiation or maintenance of the female germline *Sxl* RNA splicing mode has weakened the genetic argument that such a germline promoter is likely to be involved in initiating or maintaining female splicing (28).

What might the genes of the germline X:A signal be? Although *snf* has been excluded as a significant somatic X:A numerator element, it may serve such a role in the germline. Simultaneous duplication of Sxl^+ and snf^+ can initiate female *Sxl* splicing in the male germline, and increasing zygotic snf^+ dose shifts XX AAA germ cells in a female somatic environment toward the female pathway (91; JH Hager, TW Cline, unpublished information). This activation must be a gene dose effect in the germline itself, since it does not require *tra* or *tra2*. There is a paradox, however. In the heterosexual germ-cell transplantation experiments described above, female development of XX germ cells was observed only when those cells were Sxl^M; hence, simultaneous duplication of all X-linked genes only in the male germline cannot induce

female germline expression of *Sxl*. Perhaps this activation of *Sxl* by the smaller duplications is a vestige of an ancestral X-chromosome counting system based only on RNA splicing that was later fine-tuned by the addition of other levels of control including negative X-linked elements.

Spermatogenesis appears to proceed normally regardless of whether female-specific *Sxl* germline splicing in males is induced by a *Sxl* cDNA transgene or by snf^{+} duplications, and even though at least the two major SXL isoforms are generated, either one of which is sufficient to feminize somatic cells. In the rare instances in which development is abnormal, no female character to the abortive germ cells is apparent. Although the lack of germline feminization by SXL may result from the absence of some minor SXL isoform, it seems more likely that *Sxl* simply does not function as a switch gene in diploid germ cells. This explanation would account as well for the lack of feminizing effects of even strong Sxl^{M} alleles in the male germline (207).

Expectations that *Sxl* would be a unifying factor in understanding fly germline sex-determination the way it was for the soma have faded somewhat as evidence has accumulated that *Sxl* may not be a switch gene in diploid germ cells and may not even be required in the germline for all aspects of female-specific germline gene expression. Morphological evidence that Sxl^{-} XX germ cells adopt a male fate was confirmed initially by data on molecular markers of sexual differentiation (236), but two recent reports employing another molecular marker of differentiation, *orb* (*oo18 RNA-binding*), raised the possibility that these masculinized cells may be intersexual (7, 110). Unfortunately, the data published so far are not sufficient to establish whether XX germ cells that have never expressed *Sxl* in its female mode can nevertheless express *orb* in its female expression state.

As yet no "master sex-determining gene" has been found for the germline. Perhaps as some have suggested, fly germ-cell sex-determination is more analogous to events in terminal sexual differentiation that occur downstream of *tra* and *dsx* in somatic cells (110). In this case, germline genes that respond directly to primary sex signals might not be sex-specific regulatory loci, but rather genes involved in various specific aspects of gamete differentiation. On the other hand, the fact that an X:A signal clearly operates in the germ cells themselves to control *Sxl* and other genes argues that some kind of sex-specific regulatory hierarchy analogous to that in the soma may operate in germ cells. Perhaps the regulation of fly germ-cell sex is a hybrid situation with characteristics of both sex determination and sex differentiation.

No consistent model for germline sex-determination accommodates all the numerous and sometimes conflicting and/or incomplete observations reported. For that reason, the scheme presented in Figure 7 to summarize the current state of understanding of germline sex-determination does not include the kind

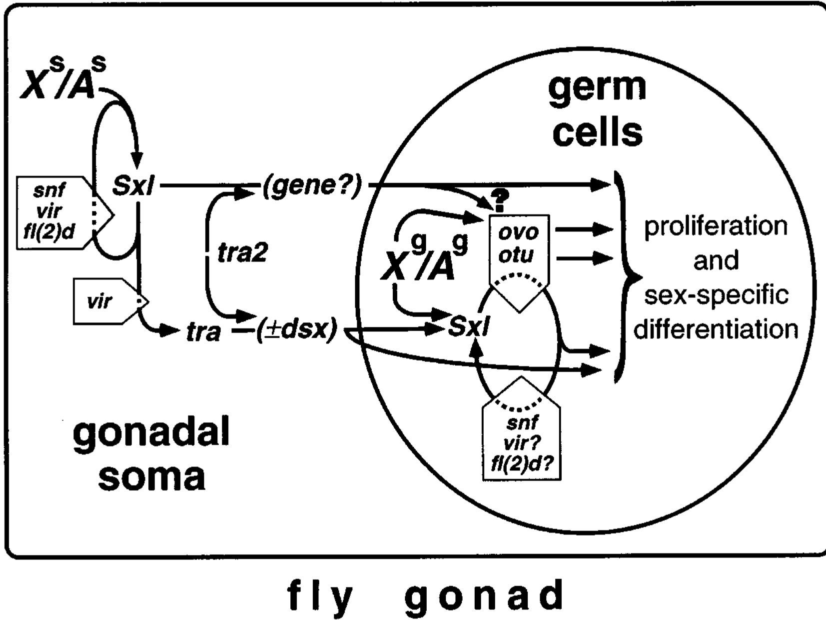


Figure 7 Genes and signals involved in the control of germ-cell sexual identity in *Drosophila*. The sexual identity of germ cells is determined by both the X:A ratio of the germ cells themselves (g), and the X:A ratio of the surrounding soma (s). The genes that comprise these two X:A ratios are different. The somatic X:A signal is communicated to germ cells at least in part through the operation of known switch genes in the somatic sex-determination hierarchy. Among these somatic switch genes, only the expression of *Sxl* is required in the female germline itself. In the germline, *Sxl* is not the only target of somatic and germline sex signals, nor is it a switch gene. *Sxl* operates via a positive RNA splicing feedback loop with the participation of some of the same accessory gene products in the germline as in the soma. Two genes, *ovo* and *otu*, are important for the growth and differentiation of female but not male germ cells and, like *Sxl*, appear to be targets of both somatic and germline sex-determination signals. They also facilitate *Sxl* germline autoregulation. *Sxl* germline targets have yet to be identified.

of male-female comparison presented in other figures. Instead the figure is designed to emphasize general points that have been made about germline sex-determination and to identify some of the genetic players involved.

Worm germline sex-determination has similarities with worm somatic sex-determination, but the two processes still differ in at least four fundamental ways: (*a*) The *her*, *tra* and *fem* genes that are essential for somatic sex determination are also important for germline sex determination; however, *tra-2* and *fem-3* exhibit germline-specific regulation that differs in mechanism from their soma-specific regulation (1, 60, 81, 94). (*b*) Germline sex determination requires genes not needed for somatic sex-determination, the *fog* genes

(feminization of the germline) for spermatogenesis (14, 65) and the *mog* genes (masculinization of the germline) for oogenesis (85, 86). (*c*) In the soma, *tra-1* is a terminal switch gene whose product imposes the hermaphrodite pathway of differentiation, while in the germline *tra-1* does not behave as a switch gene, but participates in both female and male aspects of germline development (103, 193). (*d*) The *fem* genes act at the terminal position in the gene hierarchy (along with *fog-1, fog-3*, and *tra-1*), while in the soma they occupy a position farther upstream (15, 65, 102). The gene *fem-3* acts as a germline switch gene: Active *fem-3* triggers spermatogenesis, and inactive *fem-3* triggers oogenesis (15).

The role of interactions between the soma and germline in setting germline fate is less well understood in the worm than in the fly. In worms, the only such interaction established so far is one that is essential for the transient production of sperm in hermaphrodites (J McCarter, R Francis, T Schedl, personal communication). Laser ablation of somatic gonad lineages feminizes the hermaphrodite germline by preventing sperm production. This result indicates that somatic cells of the hermaphrodite are essential for the production of sperm and may therefore transmit a negative signal to the germline to inhibit oogenesis. This phenomenon can be reproduced by gain-of-function mutations in the *shv-1* gene. By altering somatic gonad lineages, these mutations block spermatogenesis in the hermaphrodite.

Sex determination in the germline of the XO male is regulated somewhat differently from that in the soma, although many of the same genes participate in both processes (Figure 8*a*). One difference is in the role of *tra-1*. Recall that in the soma, *tra-1* is an XX-specific terminal switch gene that promotes hermaphrodite development. Male development ensues in XO animals because *tra-1* is turned off by the *fem* genes, and that is the only known role of the *fem* genes in the male soma. In the germline, however, *tra-1* functions in males to promote abundant spermatogenesis and block oogenesis; in its absence, small amounts of both sperm and oocytes are produced (103, 193). Moreover, rather than simply acting to turn off *tra-1*, the *fem* genes actively participate with *tra-1* in promoting male development. In doing so, the *fem* genes occupy a terminal position in the regulatory hierarchy. In the absence of FEM proteins, males produce only oocytes and do so even in the absence of *tra-1* (61, 102). Hence the block to oogenesis in wild-type males requires both the *fem* genes and *tra-1*. Recall that in the soma, feminization by the loss of the *fem* genes is blocked by loss of *tra-1*, one piece of evidence placing *tra-1* downstream of the *fem* genes in that tissue type. Two additional germline-specific genes, *fog -1* and *fog-3*, are needed in the male germline as terminal regulators to specify the male fate (14, 65). Mutations in *fog-1* and *fog-3* eliminate spermatogenesis in both XX and XO animals and act in both sexes at the same position in the genetic hierarchy as the *fem* genes.

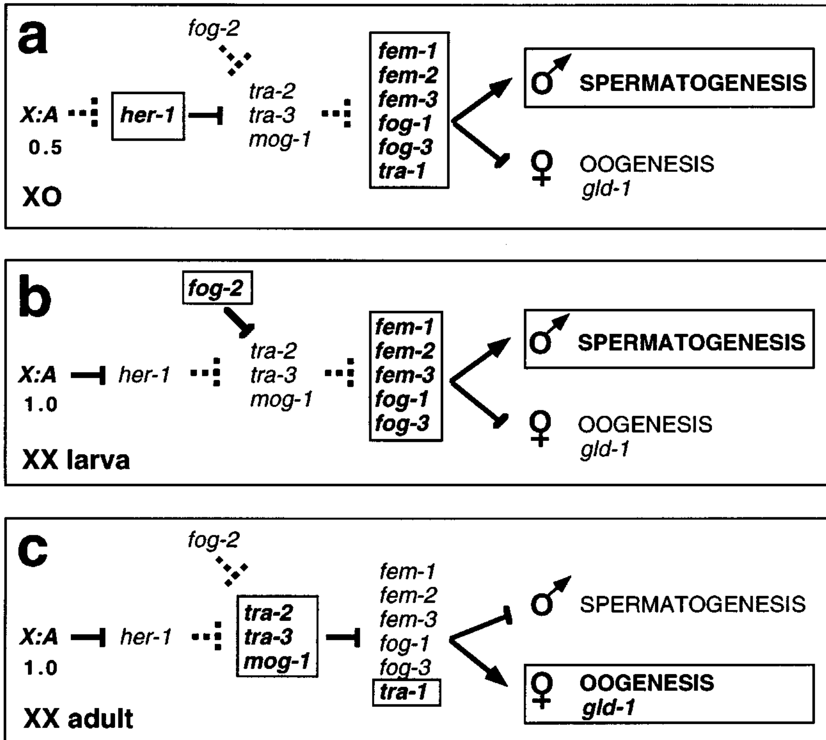


Figure 8 Control of germ-cell sexual identity in *C. elegans*. The shift in gametogenesis in the hermaphrodite from the initial male mode during the L4 larval stage to the subsequent female mode in the adult is mediated by gene-activity changes shown in the two panels for XX animals. Genes that are functionally active are boxed and bolded. A dark bar indicates a negative regulatory interaction, and a dashed bar indicates the lack of negative regulation because the upstream regulator is inactive.

While *tra-1* functions in the male germline, it also functions in the hermaphrodite germline in the opposite capacity: to block spermatogenesis and promote abundant oogenesis, a role more in keeping with its somatic functions (103, 193). Hence, with regard to its role in the germline, it is more appropriate to think of *tra-1* as playing an active but different role in XX and XO animals, rather than being functionally on in XX and off in XO animals, as it is in the soma. The following model for *tra-1* germline functioning is consistent with all the known genetic and molecular data for this gene, but it departs from the conventional presentation of linear pathways and unifunctional elements. The basic idea is that in the germline, *tra-1* has opposite effects on germ cells that depend

on its interactions with the *fem* genes. The gene *tra-1* promotes abundant spermatogenesis and blocks oogenesis when it interacts with (or is modified by) one or more of the *fem* genes, but in the absence of these interactions it promotes abundant oogenesis and blocks spermatogenesis. The *tra-1* gain-of-function (gf) mutants rarely make sperm regardless of the activity state of the *fem* genes. This result can be rationalized by the view that their TRA-1 protein is refractory to interactions with the *fem* genes and hence constitutively functions in its female mode, only promoting oogenesis. A parallel situation may exist for *fem-3*(gf). This allele can fully masculinize the germlines of either XX or XO animals independent of *tra-1*. In the course of eliminating the normal controls on this gene, this gain-of-function mutation appears to eliminate the need for *tra-1* to participate in the masculinizing activities of this gene.

Sex determination in the hermaphrodite germline requires two steps of regulation to achieve the wild-type pattern of spermatogenesis in L4 larvae followed by oogenesis in adults. First, the X:A signal has to be circumvented to initiate spermatogenesis in XX animals. This step requires the down-regulation of *tra-2* (60, 81, 194) and the participation of *fog-2* (194) (Figure 8*b*). Second, male development must be turned off and female development turned on to switch from spermatogenesis to oogenesis in adulthood. This step requires down-regulation of *fem-3* (15) and participation of the *mog* genes (85, 86) (Figure 8*c*).

The idea that down-regulation of *tra-2* is necessary to initiate male germline development came from the discovery of two classes of *tra-2* gain-of-function (gf) mutations, which reduce or eliminate sperm production in XX but not XO animals (60, 194). These mutations are unlike the *tra-2*(eg) dominant mutations described above in that they do not transform XO animals into hermaphrodites and they do not interfere with the negative regulation of *tra-2* by *her-1*. One class of mutations alters a 28-nucleotide direct tandem repeat located in the *tra-2* 3′ UTR (81). The strongest *tra-2*(gf) alleles delete both of the repeats, and the weaker alleles affect a single repeat. The involvement of these direct repeats in the translation of the *tra-2* mRNA was demonstrated by chimeric reporter constructs in which the *tra-2* 3′ UTR was shown to repress β-galactosidase translation in the germline. If the 3′ UTR lacked the repeats, repression did not occur. In addition, the role of the direct repeats in translation was also suggested by the finding that *tra-2*(gf) mRNAs are associated with larger polysomes than wild-type *tra-2* mRNAs. An activity has been reported that specifically binds to the direct repeats and may be the translational repressor (81).

The degree to which translational repression plays a role in *tra-2* control in vivo is unclear, since a different class of *tra-2*(gf) mutations also affects *tra-2* activity by another means. This second class of mutations, *tra-2*(mx), causes dominant feminization of the germline and partial masculinization of the soma in XX animals (60, 194). The alleles are missense mutations in a 22-amino-

acid stretch of the predicted cytoplasmic domain (P Kuwabara, P Okkema, J Kimble, personal communication). A proposed role for this region is to bind a germline negative regulator of *tra-2* and thereby promote sperm development by preventing FEM-3 from binding and being inactivated. A candidate for this *tra-2* negative regulator is the *fog-2* gene (194). The *fog-2* mutations block spermatogenesis in XX but not XO animals and cause XX animals to be females. Although genetic interactions between *fog-2* and the other sex-determination genes are consistent with it being a *tra-2* negative regulator, the available data do not distinguish whether the site of regulation is within *tra-2* or one of the fem genes. Thus *tra-2* appears to be negatively regulated in four separate ways: by HER-1 binding to the excellular domain in male somatic and germline sex-determination, and by three apparently *her-1* independent mechanisms that function in hermaphrodite germline sex-determination. These latter three mechanisms include regulation via a hypothetical negative regulator produced by the somatic gonad, binding of a negative regulator to the region of TRA-2 defined by the *tra-2*(mx) mutations, and translational repression via the 3′ UTR. The relative contributions of these different forms of regulation have not been determined.

Once the *tra-2* activity is repressed, the *fem* genes together with *tra-1*, *fog-1*, and *fog-3* can carry out spermatogenesis. Another gene, *gld-1* (defective in germline development) (72, 73), plays a nonessential role in spermatogenesis in hermaphrodites and acts downstream of *tra-2* and *tra-3* (see below).

Although the control of spermatogenesis in hermaphrodites requires additional levels of regulation that are not necessary in males, the process of spermatogenesis is similar between the sexes, and the spermatozoa made from males closely resemble those from hermaphrodites. Despite the similarities in male- and hermaphrodite-derived sperm, there is a difference in the mechanism of spermatid activation between the sexes. This difference was discovered through the identification of mutations in three genes, *spe-8*, *spe-12*, and *spe-27* (spermatogenesis abnormal), which specifically affect the sperm made by hermaphrodites but not males (152, 198). Mutant hermaphrodites are self-sterile because their spermatids are not activated; however, mutant males are fertile. Their spermatids become activated as usual in both wild-type and *spe* mutant hermaphrodites. Moreover, male seminal fluid made from wild-type or *spe* mutant males can activate the *spe* mutant spermatids made by hermaphrodites. Thus the *spe* mutants reveal two separate pathways of spermatid activation: a hermaphrodite-specific pathway that is blocked by *spe* mutations and a male-specific pathway that is unaffected by *spe* mutations.

Spermatogenesis ceases in the young adult hermaphrodite, and the germ cells undergo oogenesis instead. The switch from spermatogenesis to oogenesis in hermaphrodites requires the negative regulation of the *fem* gene products.

The importance of down-regulating *fem-3* to effect the switch to oogenesis was revealed by *fem -3* gain-of-function mutations that completely masculinize the germline (Mog phenotype) (15). All of these gf mutations affect a small region of the *fem-3* 3′ UTR (1). Disruption of this region appears to disrupt translational control of *fem -3*. The *fem-3*(gf) mRNAs possess longer poly(A) tails than the wild-type mRNAs. More important, overexpression of *fem-3* 3′ UTR in XX transgenic animals appears to titrate a *trans*-acting negative regulator and masculinize the germline. Overexpression of the *fem-3*(gf) 3′ UTR has no effect on the germline. The strongest candidates for the *trans*-acting negative regulators of the *fem-3* 3′ UTR are the six *mog* genes, which are necessary for oogenesis (85, 86). The *mog-1* through *mog-6* loss-of-function mutants fail to switch from spermatogenesis to oogenesis, like the *fem-3*(gf) mutants. Any of these mutants produce more sperm than is normally present in hermaphrodites, indicating that germ cells that would become oocytes in wild-type animals are transformed into sperm. By epistasis tests, the *mog* genes act at the same step in the pathway as *tra-2*. All six *mog* genes also act maternally and affect embryogenesis as well as the sperm-oocyte switch, suggesting that these genes regulate many mRNAs.

A *tra*-2 gene product may also assist the sperm-oocyte switch by sequestering a negative regulatory factor that binds to the region defined by the *tra-2*(mx) mutations. The *tra-2* locus expresses a germline-specific mRNA that potentially encodes the carboxy-terminal cytoplasmic domain of TRA-2A, the likely site of negative regulation involved in repressing *tra-2* in L4 larvae (P Kuwabara, personal communication). No analogous genes to *fog*-1 or *fog*-3 have been found for oocyte development; however, the *gld-1* gene is also involved in the sperm-oocyte switch as well as the specification of oocyte fate and/or oocyte differentiation (72, 73). These conclusions were drawn from two of the numerous classes of *gld-1* mutations [*gld-1*(*mog*) and *gld-1*(tum)]. The *gld -1*(mog) mutations cause continued spermatogenesis and prevent oogenesis. The *gld-1*(tum) mutations are null mutations that cause germ cells to fail to undergo oogenesis and instead form a germline tumor of proliferating cells. These germ cells progress through the early stages of meiotic prophase but then exit meiosis and return to the mitotic cycle. This phenotype occurs only when the germline is set to the female mode (*fem*/*fog* genes inactive in either XX or XO animals), suggesting that *gld-1* directs oogenesis by specifying the oocyte fate or by acting at an early step in oocyte development.

For Neither Organism Is It Yet Known Whether Germ Cells are Dosage Compensated

Since cell death is a hallmark of dosage-compensation upsets in the soma, at least for the fly, one might expect mutations in genes involved in germline

dosage-compensation to cause germ-cell death that depends on the dose of X-chromosomes. By this criterion, *Sxl*, *msl1*, and *msl2* would not be involved in this process, since germline stem cells lacking these functions grow normally (6, 196). In contrast, mutations in *mle* may block spermatogenesis (6), but since there has been no analysis of the phenotypic nature of this apparent block, it is too early to conclude that it has a bearing on germline dosage-compensation. Since mutations in *ovo* and *otu* have XX-specific effects on germline stem-cell proliferation, they would seem to be the prime candidates for regulators of germline dosage-compensation. Moreover, loss of *mle* function has been reported to partially suppress this germ-cell lethality (165). However, even *otu*$^{-}$ XY germ cells die if the somatic environment is phenotypically female (159). This observation is difficult to reconcile with a dosage-compensation role for *otu*, but if there are sex-specific controls on germ-cell proliferation that are unrelated to dosage compensation, they might account for the lethal effects of *ovo* as well.

Mutations in worm dosage-compensation genes can affect the fertility of XO animals in non-wild-type situations, suggesting effects on germline growth. For example, *her-1* and *tra-2*(eg) XO hermaphrodites make many more progeny if they are also mutant for a *dpy-26* mutation (101, 133). However, it is unknown whether it is *dpy* gene expression in the germ cells themselves that is relevant, or even whether elevated X-chromosome expression is involved. The fact that *dpy-27* has a similar effect on *her-1* XO hermaphrodites indicates that this effect is not related to germline dosage-compensation, since DPY-27 appears to be absent from proliferating germ cells and therefore is unlikely to participate in germline dosage-compensation. If the germline is dosage compensated, the genes *mes-2, mes-3, mes-4*, and *mes-6* (*maternal-effect sterile*) (37) are potential candidates for this role, since mutations in them cause germ-cell-specific death that is greater for XX than XO cells (S Strome, personal communication).

The Relationship Between Germline and Somatic Sex-Determination Appears to Limit Genetics and Evolution Far More for the Fly Than the Worm

Worms forced by mutation to follow a pathway of sexual differentiation that is inappropriate to their X:A ratio nevertheless are often able to produce functional gametes, while similarly sex-transformed flies are invariably sterile. As a consequence, newly arising fly sex-transforming mutations cannot be recovered from the individuals in which they appear, and the use of suppressor genetics in the study of fly sexual dimorphism is far more limited than in the worm. For example, mutations in worm dosage-compensation genes can be readily selected as suppressors of the male-specific lethality of *xol-1*, since the rescued XO worms differentiate as self-fertile hermaphrodites (151). The

analogous situation in flies is the rescue of the female-specific lethality of *Sxl* mutations by mutations in downstream dosage-compensation genes, but such rescued females are generally sterile pseudomales and hence a genetic dead end.

Because sex-transformed worms are fertile, it is possible to change the sex-determination system of this organism with remarkable genetic ease (100, 151). For example, with a *xol-1* mutation and two functionally opposite mutations in a single worm gene, *tra-2*, *C. elegans* can be transformed from the normal XX//XO, hermaphrodite/male strain to a ZZ//ZW, male/female strain, which is remarkably healthy and fertile. It is hard to imagine that any comparably simple combination of mutations is likely to be found for the fly that would change its sex-determination mechanism in such a fundamental way. It may be that the fruit fly has evolved itself into a corner with respect to its sex-determination mechanism.

SEX IN THE NEW MILLENNIUM

As the next century progresses, biochemical mechanisms underlying the general molecular themes revealed by the sex-determination studies described here will be explored in detail, with increasing impact on a wide range of general research areas. The potential for uncovering new regulatory mechanisms of general significance seems particularly high in studies of how chromosome-wide gene regulation is achieved by the MSL and DPY dosage-compensation complexes. Analysis of the genetic basis for sexual behavior has already revealed unexpected aspects of the fly sex-gene hierarchy and promises to be an exciting area of future sex-determination research in both the fly and the worm. More effort will be forthcoming to understand how sexual morphology is specifically sculpted during terminal differentiation, a difficult question because the gene targets involved, most of which are not yet known, necessarily function at interfaces between diverse regulatory cascades whose individual inputs may be quantitative rather than qualitative. Understanding how sexual identity is communicated to the germ cells could be challenging for some of the same reasons if, as may be true for the fly, the sex of the cell is never truly determined as it is for a somatic cell. Some aspects of germ-cell sexual identity might be extensions of the sexual differentiation of the surrounding soma. It is hoped that future studies will determine how ploidy affects the sex-determination process–the nature of the "A" of the X:A signal.

Other issues to be addressed include how developmental programs resist environmental and genetic perturbation so effectively. The reproducibility of development over a wide range of growth conditions and in the face of outcrossing and inbreeding shows that flies and worms have effective mechanisms for

buffering their developmental programs against short-term changes. Mutations in individual genes that participate in this buffering may not cause obvious phenotypes in the lab. It is easy to lose sight of this fact when so many successful studies are based on a forward genetic approach that relies on dramatic mutant phenotypes generated by single-gene knockouts.

Study of how the fly and worm gene hierarchies described here have changed among more closely related species will provide insight into the molecular mechanics of evolution and address a number of questions. How are elements of the polygenic sex-determination signals recruited and how rapidly are they decommissioned? Can one infer the circumstances that might favor an evolutionary strategy of pleiotropy over one of gene duplication and functional specialization? How does the dosage-compensation mechanism cope with rapid changes in sex chromosomes? How quickly do changes occur in the relationship between germline and somatic cell sex-determination? For both worms and flies, what is learned on the evolutionary front will surely facilitate progress on developmental problems, and vice versa. Clearly the flood of information being generated from studies of fly and worm sex-determination is not likely to abate any time soon.

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