Exploring the Envelope: Systematic Alteration in the Sex-Determination System of the Nematode *Caenorhabditis elegans*

Jonathan Hodgkin

Genetics Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

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ABSTRACT

The natural sexes of the nematode *Caenorhabditis elegans* are the self-fertilizing hermaphrodite (*XX*) and the male (*XO*). The underlying genetic pathway controlling sexual phenotype has been extensively investigated. Mutations in key regulatory genes have been used to create a series of stable populations in which sex is determined not by X chromosome dosage, but in a variety of other ways, many of which mimic the diverse sex-determination systems found in different animal species. Most of these artificial strains have male and female sexes. Each of seven autosomal genes can be made to adopt a role as the primary determinant of sex, and each of the five autosomes can carry the primary determinant, thereby becoming a sex chromosome. Strains with sex determination by fragment chromosomes, episomes, compound chromosomes, or environmental factors have also been constructed. The creation of these strains demonstrates the ease with which one sex-determination system can be transformed into another.

THE diversity of sex-determination systems in the animal kingdom is a remarkable feature of the natural world. Here it is demonstrated that much of this diversity can be artificially created within a single species, by utilizing control gene mutations and chromosomal rearrangements to modify the sex-determination system of *Caenorhabditis elegans*.

Sexuality is almost universal in the animal kingdom, despite the existence of rare exceptions such as the bdelloid rotifers (Welch and Meselson 2000). Many different sex-determination mechanisms occur, and the particular nature of the sex-determination system can have important evolutionary consequences. One striking example is provided by the order Hymenoptera, containing over half a million species of bees, wasps, and ants, all of which exhibit a common pattern of sex determination by ploidy with haploid males and diploid females (Beukeboom 1995). Social behavior has arisen on multiple independent occasions within the Hymenoptera, almost certainly because the sexual system leads to greater genetic similarity between related individuals, and therefore facilitates evolution of the kinds of altruistic behavior that are essential for sociality. Another, more specific, example is found in the case of brood-parasitizing cuckoos. Within a single cuckoo species, multiple races can exist, each of which is specialized for parasitizing a different host and produces eggs that specifically mimic the eggs of that host. The specialization probably depends on female birds being heterogametic (*ZW*), allowing the female-specific W chromosome to evolve to carry race-specific traits (Gibbs *et al.* 2000).

Chromosome and gene evolution can also be strongly affected by the nature of sex-determination systems. There is abundant evidence for the repeated evolution of sex chromosomes and sex chromosome heteromorphy, followed by phenomena such as dosage compensation and imprinting. The majority of genes on sex chromosomes are usually not involved in controlling sex *per se*, but they nevertheless experience its consequences.

The diversity of animal sex-determination systems is conspicuous (for general reviews, see Bell 1982; Bull 1983). Many groups have the familiar *XX* female, *XY* male system encountered in most mammals, but others, such as birds and butterflies, have heterogametic *ZW* females and homogametic *ZZ* males. Chromosomal sex determination can also be more complex, with multiple X and Y chromosomes, or maternal, as in the carrion fly *Chrysomya rufifacies*. Sex can also be determined by environment rather than by genome, as in the many groups exhibiting temperature-dependent sex determination or sex determination in response to nutritional conditions, crowding, and other social factors. The existence of all these different systems raises many questions about what selective forces may have caused their evolution and how one system can evolve into another.

These problems have been extensively studied at a theoretical level with some success, but it seems likely that adequate understanding will depend also on knowledge of the basic molecular machinery involved in sex determination. Here, too, a surprising diversity is encountered. The molecular genetics of sex has been studied most extensively in three types of animals: mammals, the fruitfly *Drosophila melanogaster*, and the nematode

Address for correspondence: Genetics Unit, Department of Biochemistry, University of Oxford, South Parks Rd., Oxford OX1 3QU, UK. E-mail: jah@bioch.ox.ac.uk

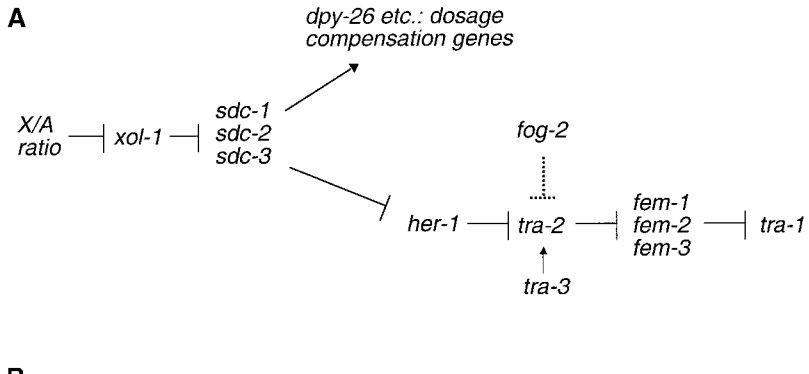

FIGURE 1.—Interactions between sex-determination genes in *C. elegans*. (A) A simplified diagram of the standard model for the sex-determination pathway in *C. elegans*. Genes discussed in this article are illustrated. Pointed arrows indicate positive regulation; blunt-end arrows indicate negative regulation. The dotted blunt-end arrow refers to the germline-specific action of *fog-2*. Genes with minor or uncertain roles such as *egl-41* (DONIACH 1986) and *laf-1* (GOODWIN *et al.* 1997) have been omitted, along with minor interactions and feedback effects. Germline-specific genes and pathways that are not relevant to this article have also been omitted. (B) Proposed activity states in the soma for the major genes (or their gene products) in the two sexes. For further explanation, see text.

C. elegans (for recent comparative review, see ZARKOWER 2001). In each of these systems, many of the key genes involved have been defined, and detailed models have been constructed, most completely in the case of the two model invertebrates. However, the three systems seem to have almost nothing in common in terms of molecular factors. One candidate conserved factor has been identified, but both of its targets, its mode of regulation, and its relative importance appear to vary considerably between different phyla (RAYMOND *et al.* 1998). Overall, the molecular mechanisms are strikingly multifarious.

The diversity of molecules and control networks involved in sex determination suggests that the developmental regulation of sex may undergo especially rapid evolutionary change. Conspicuously high levels of divergence have indeed been observed in comparisons of sex-determining genes in related species of flies, mammals, and nematodes (O'NEILL and BELOTE 1992; WHITFIELD *et al.* 1993; DE BONO and HODGKIN 1996; KUWABARA 1996b).

The sex-determination system of *C. elegans*, and extensive genetic work on its basis, allow construction of strains exhibiting alternative sex-determination systems, thereby demonstrating the ease with which one system may be able to evolve into another. Earlier work has already illustrated this principle (HODGKIN 1983a; SIGURDSON *et al.* 1986; MILLER *et al.* 1988), and this article provides a more extensive gallery of artificially created stable systems. Many of these strains reproduce the properties of sex-determination systems in entirely different organisms, thereby showing that one system can be transformed into another with relative ease. The construction and successful propagation of these strains also provide stringent tests for the correctness of the basic genetic model proposed for *C. elegans*, in terms of regulatory interactions.

The cascade of major regulatory interactions involved in sex determination in *C. elegans* is illustrated in simplified form in Figure 1. Multiple mutations have been created in all of the genes concerned in this pathway, often with diverse properties in different alleles. Relevant mutations and phenotypes are summarized in Table 1. More extensive description and discussion can be found in CLINE and MEYER (1996), KUWABARA (1999), ZARKOWER (2000), and GOODWIN and ELLIS (2002).

The main properties of the regulatory cascade are believed to be as follows. Primary sex determination is a function of the X chromosome dosage, such that *XX* animals develop into self-fertile hermaphrodites, and *XO* animals develop into males. More precisely, the critical variable is the ratio of X chromosomes to sets of autosomes, known as the X:A ratio (MADL and HERMAN 1979). Sexual dimorphism is extensive and has been described in great detail, thanks to exhaustive anatomical and developmental descriptions (for a summary, see HODGKIN 1988). The hermaphrodite sex should be regarded as a modified female sex, which has the ability to differentiate both sperm and oocytes from the same primordial germ cells. Most nematode species have the usual male and female sexes, so it is presumed that the self-fertile hermaphrodite sex has evolved relatively recently, probably as an adaptation allowing rapid population growth and efficient colonization of transient habitats.

The primary determinants of sex on the X chromosome are a small number of genes, most notably *sex-1* and *fox-1*, which act as counting elements to distinguish high from low X dose (AKERIB and MEYER 1994; HODGKIN *et al.* 1994; CARMI and MEYER 1999). In *XX* individuals, the higher dose of these genes and their products results in downregulation of *xol-1*. Low activity of *xol-1* permits high activity of *sdc-2*, which appears to be the most important of the three *sdc* genes; these genes coordinately regulate both X chromosome dosage compensation and sex determination (DAWES *et al.* 1999). The *sdc* gene products have two functions. First, they activate recruitment of dosage compensation proteins to the X chromosome, thereby leading to a general depression of gene activity on both X chromosomes in the *XX* sex.

TABLE 1

Phenotypes of relevant mutations affecting sex determination

Gene	Mutation	*XX* phenotype	*XO* phenotype
Wild type	—	Viable hermaphrodite	Viable mating male
tra-1 III	*e1099*	Poor mating male	Poor mating male
	e1834	Poor mating male	Poor mating male
	e1575	Female	Female
tra-2 II	*e1095*	Nonmating male	Mating male
	ar221	Nonmating male(25°) or hermaphrodite (15°)	Mating male
	q276	Mating male	Mating male
	e2020	Female	Mating male
	e2046e2531	Female	Female
tra-3 IV	*e1767*	Nonmating male (m−z−) or hermaphrodite (m+z−)	Mating male Mating male
her-1 V	*e1518*	Hermaphrodite	Hermaphrodite
	e1561	Hermaphrodite	Hermaphrodite (25°) or male (15°)
fem-1 IV	*e1927*	Female	Female (m−z−)
fem-2 III	*e2105*	Female (m−z−)	Female (m−z−, 25°)
fem-3 IV	*e1996*	Female	Female (m−z−)
xol-1 X	*y9*	Viable hermaphrodite (Tra enhanced)	Dead, feminized
sdc-2 X	*y15*	Dead, masculinized	Viable mating male
sdc-3 V	*y52*	Viable, masculinized	Viable mating male
dpy-26 IV	*n199*	Dead, hermaphrodite	Viable mating male
dpy-27 III	*rh18*	Dead, hermaphrodite	Viable mating male
fog-2 V	*q71*	Female	Mating male

Relevant phenotypes are more fully described at appropriate points in the text. The abbreviation (m+z−) refers to animals homozygous for a given mutation but derived from a heterozygous mother carrying a wild-type allele for the relevant gene; (m−z−) refers to homozygous mutants derived from homozygous mutant mothers.

In the absence of dosage compensation, excess X chromosome gene expression has lethal effects on *XX* animals. Dosage compensation proteins are encoded by a set of genes specific to this process, such as *dpy-26* and *dpy-27* (Hodgkin 1983b, 1987b; Plenefisch *et al.* 1989). These genes do not affect sexual phenotype, only the relative viability of *XX* and *XO* individuals.

Second, *sdc-2* gene activity leads to transcriptional repression of the sex-determination gene *her-1*. This gene is able to encode a diffusible protein, believed to act as a repressive ligand for a membrane receptor encoded by *tra-2* (Kuwabara *et al.* 1992; Perry *et al.* 1993). In the absence of *her-1* activity, however, the TRA-2A receptor is active and able to inhibit one or more of the three FEM proteins encoded by *fem-1*, *fem-2*, and *fem-3* (Spence *et al.* 1990; Ahringer *et al.* 1992; Pilgrim *et al.* 1995). Activity of TRA-2A, and therefore inhibition of FEM proteins, is largely dependent on TRA-3, a regulatory protease encoded by *tra-3* (Barnes and Hodgkin 1996). At least part of the FEM inhibition involves direct protein-protein interaction between TRA-2A and FEM-3, perhaps resulting in sequestration of a FEM protein complex at the cell membrane (Mehra *et al.* 1999). The inhibition or sequestration of FEM proteins in turn prevents them from inhibiting the activity of the transcription factor TRA-1A, encoded by *tra-1* (Zarkower and Hodgkin 1992). TRA-1A is therefore fully active in the soma of *XX* animals, resulting in female somatic development.

Regulation in the germline of *XX* animals is modified to permit both spermatogenesis (early) and oogenesis (late). This modification involves multiple factors and translational regulation, acting at the level of the TRA-2 and FEM proteins (for a more complete review, see Kuwabara and Perry 2001). One important and specific gene here is *fog-2*, which is necessary for the modulation of TRA-2 activity that permits spermatogenesis in *XX* hermaphrodites (Schedl and Kimble 1988).

In *XO* animals, a reciprocal set of regulatory events

occurs. The lower dose of counting elements on the X chromosome results in higher activity of *xol-1*, which in turn represses *sdc-2*. In the absence of SDC-2, *her-1* is transcriptionally active, resulting in synthesis of the secreted protein HER-1A. This probably acts as a direct inhibitory ligand for the TRA-2A transmembrane receptor, which is therefore downregulated in activity. Consequently, the FEM proteins are no longer inhibited and can act to reduce TRA-1A activity. This lower activity results in male development of both soma and germline.

This outline of major regulatory events omits a number of significant additional interactions and effects, some of which need to be considered in the context of this article. First, regulation of *her-1* is not the only event transducing X chromosome dosage to the downstream sex-determination genes, although it is the most important factor. There is good genetic evidence for an additional bypass effect that affects activity of the *fem* genes and/or *tra-1* (Hodgkin 1980). This bypass involves *xol-1* activity, but in this context *xol-1* has a paradoxical feminizing function, in contrast to its masculinizing function at the initial steps of X chromosome dosage assessment (Miller *et al.* 1988). Second, there is evidence for a number of feedback effects within the pathway, which probably reinforce adoption of either the female or the male state (Okkema and Kimble 1991; Delong *et al.* 1993). Third, feed-forward effects, such as a direct interaction between a C-terminal portion of TRA-2 and TRA-1A, apparently bypass the FEM proteins (Lum *et al.* 2000). Fourth, the action of TRA-1A is more complex than the simple binary ON/OFF choice that describes regulation for many of the other genes in the pathway. Completely normal male development and full male fertility require low activity of *tra-1*, as opposed to complete inactivity (Hodgkin 1987a). Consequently, *tra-1(null) XX* animals are fully male in almost all features, but are variable in a few somatic features and have poor male germline development.

These effects and others (Goodwin and Ellis 2002) can legitimately be regarded as minor modulations or reinforcements of the major interactions that direct sexual development along either the male or the female path. Their existence is characteristic of the kind of fine tuning that becomes apparent when any piece of biological regulation is examined in detail. However, their significance can obviously change over evolutionary time. On the one hand, some minor interactions may be relics of regulatory events that were much more important in the past. On the other hand, some may acquire a major role in the future. Recent discoveries in the evolution of development suggest that developmental regulation is in a state of constant flux. This may be especially true of sex determination, as indicated by the rapid evolution of sex-determination genes. The manipulations described here demonstrate that the pathway in *C. elegans* is malleable to a remarkable degree.

MATERIALS AND METHODS

Nematode handling: Standard methods for culture of *C. elegans* were used (Brenner 1974). Strain construction and maintenance were carried out at 20° unless otherwise noted. Irrelevant markers were often used in the construction of these strains, but were removed by segregation from the final stable strains. Genotypes were verified by appropriate testcrosses.

Nomenclature: The nomenclature used is as in Horvitz *et al.* (1979). Allele abbreviations are mostly used in the text, for simplicity: *gf* (gain of function), *lf* (loss of function), *eg* (enhanced gain of function), *am* (amber nonsense), *ts* (temperature sensitive).

Strains and alleles used: The names and full genotypes of strains described are as follows:

Strain 1 (CB3470): females, *tra-1(e1575)/tra-1 (e1575e1816e1828)*
males, *tra-1(e1575e1816e1828)*

Strain 2 (BS508): females, *tra-2(q276); tra-1(oz6)/tra-1(+)*
males, *tra-2(q276)*

Strain 3 (CB5190): females, *tra-2(e2020); tra-1(e1099)/ tra-1(+); xol-1(y9)*
males, *tra-2(e2020); tra-1(e1099); xol-1(y9)*

Strain 4 (CB5081): females, *tra-2(e2046e2531); tra-1(e1099)/ tra-1(+)*
males, *tra-2(e2046e2531); tra-1(e1099)*

Strain 5: see Miller *et al.* (1988)

Strain 6 (CB4734): females, *tra-2(e1095)/tra-2(+); fog-2(q71); xol-1(y9)*
males, *tra-2(e1095); fog-2(q71); xol-1(y9)*

Strain 7 (CB5023): females, *tra-2(e2531 e2046)/+; dpy-26(n199)*
males, *dpy-26(n199)*

Strain 8 (CB4733): T females, *tra-3(e1767)/tra-3(+); fog-2(q71); xol-1(y9)*
males, *tra-3(e1767); fog-2(q71); xol-1(y9)*

Strain 9 (CB5590): hermaphrodites, *her-1(e1518) sdc-3(y52); xol-1(y9)*
males, *her-1(e1518) sdc-3(y52)/her-1(+) sdc-3(y52); xol-1(y9)*

Strain 10 (CB5666): females, *her-1(e1518); sdc-2(y15)*
males, *her-1(e1518)/her-1(+); sdc-2(y15)*

Strain 11 (CB4628): females, *tra-2(e1095); fem-1(e1927); xol-1(y9)*
males, *tra-2(e1095); fem-1(e1927)/ fem-1(+); xol-1(y9)*

Strain 12 (CB4722): females, *tra-2(e1095); fem-2(e2105); xol-1(y9)*
males, *tra-2(e1095); fem-2(e2105)/ fem-2(+); xol-1(y9)*

Strain 13 (CB4692): females, *tra-2(e1095); fem-3(e1965); xol-1(y9)*
males, *tra-2(e1095); fem-3(e1965)/ fem-3(+); xol-1(y9)*

Strain 14 (CB5638): hermaphrodites, *sup-34(e2227)/ sup-34(+); tra-3(e1107); xol-1(y9)*
males, *tra-3(e1107); xol-1(y9)*

Strain 15 (CB5035): females, *tra-1(e1834)*

eDp25(tra-1(e1575e1816)
males, *tra-1(e1834)*

Strain 16 (CB4706): females, *tra-2(e1095); fem-1(1927); xol-1(y9); eEx14[fem-1(+)]*
males, *tra-2(e1095); fem-1(1927); xol-1(y9)*

Strain 17 (CB5362): *tra-2(ar221); xol-1(y9)*

Strain 18 (CB3674): *dpy-26(n199); her-1(e1561)*

Strain 19 (CB 3911): hermaphrodites, *dpy-27(rh18) 4A;3X*
males, *dpy-17(rh18) 4A;2X*

Strain 20: see Sigurdson *et al.* (1986)

Strain 21 (CB6121): females, *eT5(III;X); fog-2(q71)*
males, *eT5(III;X)/eT1(III;V); fog-2(q71)/eT1(fog-2(q71) V;III)*

RESULTS

Sex determination by the autosomal gene *tra-1*: The gene *tra-1* encodes a zinc-finger transcription factor, TRA-1A, which is related to the CI protein in Drosophila and GLI proteins in mammals (Zarkower and Hodgkin 1992). TRA-1A plays an irreplaceable role in regulating all aspects of sexual differentiation in *C. elegans*. For at least some genes, TRA-1A acts as a transcriptional repressor, and it is possible that its major function is to prevent expression of male-specific genes, either globally or in specific cells. Consequently, null mutations of *tra-1* result in the transformation of *XX* animals into males, and gain-of-function mutations result in feminization of *XO* animals. The first artificial sex-determination system to be reported for *C. elegans* (Hodgkin 1983a) was based on control by *tra-1*, exploiting the isolation of point mutations that cause either complete masculinization of *XX* animals or complete feminization of *XO* animals. Null mutations of *tra-1* are recessive and cause both *XX* and *XO* animals to develop as low-fertility males, whereas *gf* mutations are dominant and cause both *XX* and *XO* to develop as females (Hodgkin 1987a). Consequently, a stable male/female strain can be constructed:

Strain 1: Females, *tra-1(gf)/tra-1(null)*

Males, *tra-1(null)/tra-1(null)*

Both females and males can be either *XX* or *XO*. However, in the absence of other factors, strains of this type tend to stabilize as pure *XX*. This occurs for several reasons: the *XO* females are less fertile than the *XX* females, the *XO* males are not more fertile than the *XX* males, and *XO* × *XO* crosses are intrinsically less productive because of the production of inviable nullo-X zygotes.

Two variants of this basic strain have been constructed to demonstrate the complete epistasis of *tra-1* over mutations in the upstream gene *tra-2*. Most loss-of-function mutations in *tra-2*, including null mutations, result in the transformation of *XX* animals into abnormal males with fully masculinized gonads but incompletely transformed genitalia. Consequently, these *tra-2 XX* males are unable to mate and are often described as pseudomales. One unusual non-null *tra-2* allele has been isolated, *q276*, which results when homozygous in the development of more completely transformed *XX* males (T. B. Schedl, personal communication). These are frequently able to mate and sire progeny, although less frequently than *tra-2(q276) XO* males. Construction of double mutants of *q276* with *gf* (feminizing) mutations of *tra-1* resulted in fertile female development of both *XX* and *XO*, showing that the masculinization caused by *q276* can be completely overridden by *tra-1* activity. A stable strain of the following type was constructed and kindly made available by T. B. Schedl:

Strain 2: Females, *tra-2(q276); tra-1(gf)/+*

Males, *tra-2(q276)*

This strain grows with greater fertility than strain 1 because *tra-2(q276) XX* males are more fertile than *tra-1(null) XX* males. *tra-1(q276) XO* males are still more fertile, and therefore strain 2 tends to grow as a mixture of *XX* and *XO* animals, because this greater *XO* male fertility outweighs the lower female or occasional hermaphrodite fertility of the *tra-1(gf)/+ XO* animals.

Gain-of-function mutations in *tra-2*, which cause either partial or complete feminization of *XO* animals, have also been isolated. The *gf* mutations first isolated behave as hypermorphs, probably by reducing translational repression of *tra-2*. The strongest of these, *e2020* (Doniach 1986; Okkema and Kimble 1991), results in a deletion within the 3′ untranslated region of TRA-2A mRNA; this mutation causes *XX* animals to develop as females, while *XO* animals usually develop as fertile but slightly feminized males. Under starvation conditions, the feminization of the *XO* animals is much stronger, so that they develop as sterile intersexes with abnormal gonads and essentially female tails. Homozygous double mutants of *e2020* with *tra-1(null)* alleles were constructed and found to develop as males, irrespective of nutritional state. To test this further, a strain of *tra-2(e2020); tra-1(null)/+ XX* females and *tra-2(e2020); tra-1(null) XX* males was constructed and several lines were propagated for at least five generations. Testcrosses confirmed the genotype of both types of males and females. This strain (CB5172) is not stable, however, because spontaneous *tra-2(e2020); tra-1(null)/+ XO* animals eventually arise. These develop into high-fertility males, and in successive generations the population rapidly converts to a pure *tra-2(e2020)* culture of *XX* females and *XO* males. To make a stable strain, a *xol-1* mutation was introduced, which results in the death of all *XO* animals but does not affect *XX* animals:

Strain 3: Females, *tra-2(e2020); tra-1(null)/+; xol-1 XX*

Males, *tra-2(e2020); tra-1(null); xol-1 XX*

This strain was propagated for >10 generations, 50% female and 50% male, without change in properties. The males in the population have the striking property that when outcrossed to any normal hermaphrodite, they invariably sire 100% female progeny, despite their own male phenotype. This is because the *e2020* mutation is dominant and all progeny are necessarily *tra-2(e2020)/+; tra-1(null)/+ XX*.

A related stable strain was also constructed using the *eg* mutation *e2531* (Hodgkin and Albertson 1995; Kuwabara 1996a). The *e2531* mutation results in a missense change in an extracellular domain of the receptor TRA-2A, which probably prevents binding to the inhibitory ligand HER-1A, and as a result *XO* animals carrying one or two doses of *e2531* are completely transformed into fertile hermaphrodites. The *e2531* mutation was originally isolated *in cis* with the *gf* mutation *e2046*, and *e2046e2531* mutants therefore develop as females, irrespective of X chromosome dosage. A null mutation of *tra-1* is fully epistatic to *tra-2(e2046e2531)*, despite the presence of two different dominant feminizing mutations in the *tra-2* gene (Hodgkin and Albertson 1995):

Strain 4: Females, *tra-2(e2046e2531); tra-1(null)/tra-1(+)*

Males, *tra-2(e2046e2531); tra-1(null)/tra-1(null)*

In the four strains above, an active *tra-1* gene [either *tra-1(+)* or *tra-1(gf)*] acts as a dominant feminizing factor, so the sex-determination system resembles that found in birds, with heterogametic *ZW* females and homogametic *ZZ* males. However, the Z and W chromosomes (derived from *LGIII*, the location of *tra-1*) are isomorphous.

Sex determination by the autosomal gene *tra-2*: The most important product of the *tra-2* gene is a transcript encoding the membrane protein, TRA-2A, which is believed to act as a receptor for the masculinizing HER-1A ligand. Null mutations of *tra-2* result in male development of *XX* animals, but the *XX* males are abnormal and mating defective, as a result of the bypass effect described in the Introduction. If the bypass is eliminated by also mutating *xol-1*, then the double-mutant *tra-2; xol-1 XX* males are able to mate. This allowed Miller *et al.* (1988) to construct a stable strain in which *tra-2* acts as a dominant feminizing factor:

Strain 5: Females, *tra-2(gf)/tra-2(null); xol-1*

Males, *tra-2(null); xol-1*

In this strain, *xol-1* acts as an enhancer of the masculinization caused by *tra-2*, such that *tra-2(null); xol-1 XX* animals are fertile mating males. The presence of the *xol-1* mutation also means that all *XO* animals are inviable, so this is an obligate *XX* strain. A variant on this strain was constructed using *lf* mutations only:

Strain 6: Females, *tra-2(null)/tra-2(+); fog-2; xol-1*

Males, *tra-2(null)/tra-2(null); fog-2; xol-1*

This strain, like strain 5, grows as a stable male/female population. The *fog-2* mutation results in transformation of hermaphrodites into females because the gametes normally destined to develop as sperm develop as oocytes instead, but *fog-2* does not affect differentiation of sperm within the testis of a male (Schedl and Kimble 1988).

These various strains, which are useful for strain construction, complementation of sex-linked genes, and other purposes, provide convenient sources of *XX* mating males. *XX* males of different genotypes differ markedly in fertility, and their fertility has a major effect on how well the different artificial strains grow. A systematic comparison of *XX* male fertility was carried out by testing males under standard conditions (Table 2). The data show that wild-type *XO* males are more fertile than any of the *XX* males, as might be expected. Both *tra-2(q276) XX* and *tra-2(e1095); xol-1(y9) XX* males are much more fertile than any *tra-1 XX* male, with maximal male fertility seen with the latter. However, combining *tra-2(q276)* and *xol-1(y9)* does not lead to any further increase in male fertility, probably because the *q276* mutation is a complex allele with both masculinizing and feminizing effects.

A different *tra-2*-determined strain, in which all viable animals are *XO*, was constructed using the dosage compensation mutant *dpy-26(n199)*, which results in the death of almost all *XX* animals but does not affect *XO* animals (Hodgkin 1983b; Plenefisch *et al.* 1989):

Strain 7: Females, *tra-2(gf,eg)/+; dpy-26 XO*

Males, *tra-2(+)/tra-2(+); dpy-26 XO*

Like other obligate *XO* strains, this strain does not grow with great fecundity, but is stable. In all *tra-2*-dependent strains, a dominant feminizing factor is provided by an active *tra-2* gene on *LGII*; hence, this behaves as a W chromosome.

Maternal sex determination by the autosomal gene *tra-3*: The *tra-3* gene encodes a calpain protease, which can act to cleave TRA-2A and thereby to enhance its activity (Sokol and Kuwabara 2000). It may also increase *tra-2* activity by modulating translational repression of TRA-2A (Goodwin *et al.* 1997). Null mutations of *tra-3* resemble weak mutations of *tra-2* in their effect, causing *XX* animals to develop into infertile males or intersexes (Hodgkin and Brenner 1977). *XO* animals are unaffected and develop as fertile males. In addition, the gene exhibits a strong maternal rescue effect: *tra-3* homozygous *XX* progeny generated from *tra-3/+* heterozygous mothers develop as normal fertile hermaphrodites because they have inherited sufficient *tra-3* product (either mRNA or protein) to sustain normal development. In the next generation, however, these rescued hermaphrodites produce self-progeny broods consisting entirely of *XX* pseudomales.

The maternal rescue effect made it possible to use a

TABLE 2

Male fertility measurements

Genotype	Fraction fertile	No. assayed	Mean progeny sired	Range
Wild type *XO*	11/11	11	1881	213–2871
tra-1(e1099) XX	10/46	10	43	2–262
tra-1(e1781) XX	3/36	3	27	15–63
tra-2(q276) XX	12/30	4	408	260–728
tra-2(e1095); xol-1(y9) XX	9/9	3	1202	956–1412
tra-2(q276); xol-1(y9) XX	13/20	0	NA	NA
tra-2(ar221); xol-1(y9) XX	10/20	0	NA	NA

Tests and lifetime mating assays were carried out as in Hodgkin and Doniach (1997). The No. assayed column gives the number of fertile males for which complete lifetime fertilities were measured. For comparison, the table also includes data previously published in Hodgkin (1987a; *tra-1* alleles, lines 2 and 3) and Hodgkin and Doniach (1997; wild type, line 1). NA, not applicable.

tra-3 mutation to construct a stable male/female strain in which sex is determined by maternal genotype. Populations of this strain consist of males and two kinds of female, thelygenic (producing entirely female progeny, abbreviated T) and arrhenogenic (producing entirely male progeny, abbreviated A):

Strain 8: T females, *tra-3(null)/tra-3(+); fog-2; xol-1*

A females, *tra-3(null)/tra-3(null); fog-2; xol-1*

Males, *tra-3(null)/tra-3(null); fog-2; xol-1*

Crosses between T females and males produce all-female broods consisting of equal numbers of T females and A females. Crosses between A females and males produce only males. Consequently, the strain is stable and grows as a mixture of 25% T females, 25% A females, and 50% males. Only a few of the males are capable of siring progeny because of the relatively weak effect of *tra-3* mutations even when enhanced by *xol-1*, so this strain grows very poorly.

Strain 8 provides an example of maternal sex determination comparable to that naturally observed in the carrion fly *C. rufifacies* (Schutt and Nothiger 2000).

Sex determination by the autosomal gene *her-1*: The *her-1* gene encodes a small secreted protein, HER-1A, which is believed to be an extracellular ligand for the transmembrane receptor protein TRA-2A (Kuwabara *et al.* 1992; Perry *et al.* 1993). HER-1A mRNA is produced only in *XO* animals, as a result of transcriptional regulation of *her-1* by SDC-2 (Dawes *et al.* 1999). Loss-of-function mutations of *her-1* are recessive and result in transformation of *XO* animals into fertile hermaphrodites (Hodgkin 1980). Gain-of-function mutations of *her-1* are dominant and cause partial masculinization of *XX* animals (Trent *et al.* 1988). More extreme masculinization is seen in homozygous *her-1(gf); xol-1 XX* animals, some of which develop into mating males. However, heterozygous *her-1(gf)/+; xol-1 XX* males are unable to mate successfully, which precludes use of *her-1(gf)* as a simple dominant masculinizing factor.

Instead, to construct a strain in which *her-1(+)* could act as a dominant masculinizing factor, an unusual *sdc-3* mutation, *sdc-3(y52)*, was used, which results in masculinization of *XX* animals but does not affect dosage compensation, unlike most *sdc-3* alleles. *XX* animals of genotype *sdc-3(y52); xol-1* develop into mating males (Delong *et al.* 1993) As expected from the standard model for sex determination, a *her-1(null)* mutation was fully epistatic to *sdc-3(y52)*. This allowed construction of a male/hermaphrodite strain:

Strain 9: Hermaphrodites, *her-1(null) sdc-3(y52)/ her-1(null) sdc-3(y52); xol-1*

Males, *her-1(null) sdc-3(y52)/her-1(+) sdc-3(y52); xol-1*

This strain was cultured successfully as a mixture of hermaphrodites and males for six generations and also preserved as a male/hermaphrodite frozen stock. Further propagation, without deliberate picking of males, resulted in reversion to a pure hermaphrodite population, with loss of *her-1(+)*, as expected. A stable male/female *XX* strain could presumably be constructed by introducing *fog-2*, as in strain 10 (below).

A male/female *XO* strain in which sex is controlled by *her-1* was assembled by making use of a different *sdc* mutation, a strong allele of *sdc-2*. This results in the death and masculinization of all *XX* progeny. *XO* animals are not affected by *sdc-2*, and *her-1(null); sdc-2 XO* animals develop into fertile hermaphrodites. Incorporation of *fog-2* permitted the establishment of a male/female strain:

Strain 10: Females, *her-1(lf) fog-2; sdc-2 XO*

Males, *her-1(lf) fog-2/her-1(+) fog-2; sdc-2 XO*

In strains 9 and 10, *her-1(+)* behaves as a dominant masculinizing factor, and the presence of a single active

her-1 gene is sufficient to cause completely male development. This therefore corresponds to a single-locus, dominant masculinizing system such as a dominant Y, prevalent throughout the animal kingdom.

Sex determination by *fem-1*: The three *fem* genes, *fem-1*, *fem-2*, and *fem-3*, encode cytoplasmic proteins believed to act as second messengers connecting modulation of TRA-2A activity at the cell membrane to regulation of TRA-1A activity in the nucleus.

Various protein-protein interactions between these five proteins have been discovered, but the full details of their roles and regulation in soma and germline are far from clear. Moreover, in addition to their roles as regulators of *tra-1* activity, the FEM proteins are also required for spermatogenesis. FEM-1 contains ankyrin repeats and has mammalian homologs; FEM-2 is a protein phosphatase, while FEM-3 lacks obvious homologs in taxa other than nematodes (Spence *et al.* 1990; Ahringer *et al.* 1992; Pilgrim *et al.* 1995).

All three *fem* genes exhibit similar genetic properties: loss-of-function mutations result in fertile female development of both *XX* and *XO* individuals, but only if *fem* activity is absent from both maternal parent and zygote (Doniach and Hodgkin 1984; Hodgkin 1986). If *fem(+)* activity is present in the mother, incomplete feminization occurs even if the zygote is homozygous *fem(null)*. The residual male development occurs because *fem(+)* products can be inherited from the mother via ooplasm.

Strong mutations of either *fem-1* or *fem-3* are epistatic to masculinizing mutations of *tra-2* at all temperatures, allowing construction of:

Strain 11: Females, *tra-2(null); fem-1(null); xol-1*

Males, *tra-2(null); fem-1(null)/fem-1(+); xol-1*

A *fem-1(+)* allele is never present in the females of this strain, so the maternal rescue effect does not interfere. Strain 11 is stable and grows well. In this strain, *LGIV*, containing the dominant masculinizing activity of *fem-1(+)*, behaves like a mammalian *Y* chromosome, so the strain mimics *XX* female/*XY* male patterns of sex determination.

Sex determination by *fem-2*: An analogous strain was constructed for *fem-2*. All mutations of *fem-2* are temperature sensitive, probably as a result of intrinsic temperature sensitivity in the whole pathway such that *fem-2(+)* activity is essential only at higher growth temperatures (Hodgkin 1986). At a temperature of 25°, *fem-2(null)* mutations result in complete feminization of *XX* and *XO*, as in mutations in *fem-1* and *fem-2*. As a result, it was possible to construct a male/female strain dependent on *fem-2*:

Strain 12: Females, *tra-2(null); fem-2(null); xol-1*

Males, *tra-2(null); fem-2(null)/fem-2(+); xol-1*

This strain was propagated successfully at 25°, but on shifting to a lower temperature, all animals become partly or wholly masculinized, and the strain dies out. This effect should allow selection for modifiers that increase feminization by *fem-2(null)* at lower temperatures, but no success occurred in several shift-down experiments of this type.

In contrast to strains 1–4, in which *LGIII* behaved as a sex chromosome carrying a dominant feminizing element and therefore as a W or Z chromosome, in strain 12 *LGIII* carries (at a different location) a dominant masculinizing element and therefore behaves as a Y or an X chromosome.

Sex determination by *fem-3*: A strain comparable to strains 11 and 12 was constructed using *fem-3* instead of *fem-1*:

Strain 13: Females, *tra-2(null); fem-3(null); xol-1*

Males, *tra-2(null); fem-3(null)/fem-3(+); xol-1*

In this strain, *LGIV* carries either a dominant masculinizing *fem-3(+)* allele or a recessive feminizing *fem-3(null)* allele and therefore behaves as a Y or an X chromosome.

Sex determination by a suppressor tRNA: The strains described in preceding sections all utilize one or more of the seven major sex-determining genes to control sexual phenotype. Additional genes can be artificially recruited into the network of interacting genes, and thereby given the status of sex-determining factors, by making use of suppression effects. An example is provided:

Strain 14: Hermaphrodites, *sup-34/+; tra-3(am); xol-1*

Males, +/+; *tra-3(am); xol-1*

The amber nonsense mutation used in this strain, *e1107*, is efficiently suppressed by a single dose of *sup-34*, which encodes an amber-suppressing Trp tRNA (Kondo *et al.* 1990). In the absence of *sup-34*, the *tra-3; xol-1* animals develop as low-fertility males. The strain grows as a sustained mixture of hermaphrodites and males because *sup-34/sup-34* homozygotes are much less fertile than *sup-34*/+ heterozygotes. An obligate female/male strain could be constructed by introducing a *fog-2* mutation, as in strain 8.

In this strain, the presence or absence of a tRNA mutation determines the difference between hermaphrodite and male development, and the mutation behaves as a dominant feminizing element. The tRNA gene in question is located on *LGI*, which therefore behaves as a sex chromosome although it does not carry any of the major sex-determination genes discussed above.

Sex determination by a fragment chromosome: Experiments investigating the properties of a small chromosomal inversion mutation, *eC2*, which includes a *tra-1(gf)* mutation, led to the generation of a derived free duplication, *eDp25* (Zarkower *et al.* 1994). This consists of two fused right ends of *LGIII* (from *dpy-18* to the right telomere) and also carries a *tra-1(gf)* mutation, which feminizes both *XX* and *XO* animals. A male/female strain based on this duplication was constructed:

Strain 15: Females, *tra-1(lf); eDp25(tra-1(gf))*

Males, *tra-1(lf)*

This strain is comparable to strain 1, being *tra-1* based, but in this case the functional copy of *tra-1* is located on a small chromosomal fragment, so the determining factor is episomal rather than chromosomal. Fragment chromosomes are frequently lost at meiosis in *C. elegans*, especially ones such as *eDp25*, which tend to pair with themselves and therefore lack a partner to disjoin from. Consequently, the sex ratio in strain 15 is extremely male biased, in contrast to most of the preceding strains. Six fertilized females from strain 15 produced a total of 143 females and 639 males (82% male). This potentially creates a situation selecting for modifiers that would shift the ratio toward equality, but in fact the mating efficiency of *tra-1(lf)* males is so low that such selection is unlikely to operate. However, possible modification of this type has been observed with a different strain, as described below (strain 16).

Sex determination by an extrachromosomal element: Further variations on the sex-determination system can be achieved by making use of artificial transgene arrays that carry normal or mutant versions of cloned sex-determination genes. This was demonstrated by making use of arrays carrying a wild-type copy of *fem-1*; these arrays are able to rescue both the somatic and germline phenotypes of *fem-1(null)* mutants (Spence *et al.* 1990). Strain 11 (above) was modified by introducing such an array to replace the chromosomal copy of *fem-1(+)* with an extrachromosomal array:

Strain 16: Females, *tra-2(null); fem-1(null); xol-1*

Males, *tra-2(null); fem-1(null); xol-1; eEx[fem-1(+)]*

This strain was propagated successfully for >50 generations as a male/female culture. Two features of construction and propagation of strain 16 are noteworthy. First, during early generations of propagation one unusual self-fertile animal was observed; this was picked and used to establish a line of mixed hermaphrodites and females. In this derived line (strain CB4707), the properties of *eEx14* appear to have been modified so that it is active in the germline but not in the soma. Consequently, the *tra-2; fem-1; xol-1; eEx14* animals develop as somatic females, but are able to produce enough sperm for substantial self-fertility. The CB4707 line is no longer dependent on mating for propagation, so there is strong selection for this kind of modification of array expression. However, the effect is surprising in that most transgenic arrays are expressed much better in somatic tissues than in the germline, rather than vice versa, as in this case. In later generations of propagation of strain 16, no self-fertile animals appeared, suggesting that *eEx14* had stabilized in its properties.

Second, the sex ratio in populations of strain 16 is determined by the efficiency with which the masculinizing array is transmitted and expressed, rather than by the meiotic segregation of chromosomes (as in most of the other strains discussed so far). In strain 11, for example, the observed sex ratio in total progeny from 11 single pair matings was 1708 females:1766 males. This ratio is not significantly different from the 1:1 ratio expected for normal meiotic segregation of chromosome IV, which bears the sex-determining locus in strain 11. Extrachromosomal arrays, however, are not reliably handled by the meiotic machinery, and therefore ratios very different from 1:1 can be generated in principle. Populations that start at a sex ratio different from 50:50 are under selection to move to a more equal sex ratio, according to the argument ascribed to Fisher (1930). In strain 16, a small shift in the sex ratio produced from single pair crosses, from 56% female (133/237) at an early generation to 49% (508/1042) at a later generation, was indeed observed during long-term propagation. However, neither of these ratios is significantly different from 50%. It is nevertheless likely that a strongly biased starting sex ratio could be created by using an extrachromosomal array with different transmission properties, which could be examined over time, thereby allowing a direct test of the Fisher principle.

Sex determination by temperature: Temperature-sensitive mutations have been obtained for most, although not all, of the seven major sex-determination genes. These permit construction of strains in which sex is determined by temperature, not by chromosomal determinants. Two contrasting strains, of which the first exhibits male development only at high temperature and the second exhibits male development only at low temperature, illustrate this possibility:

Strain 17: Hermaphrodites, *tra-2(ar221); xol-1 XX* (15°)

Males, *tra-2(ar221); xol-1 XX* (25°)

Several different temperature-sensitive mutations of *tra-2* have been isolated during work on this gene (Klass *et al.* 1976; Hodgkin and Albertson 1995). The *ar221* mutation (isolated and generously made available by J. Hubbard) is more extreme in its range of variation than those previously reported, such that *tra-2* is almost inactive at the high temperature and substantially active at the low temperature. Animals of strain 17 grown at 25° (after shifting the parent hermaphrodites to this temperature at late L4 stage) are invariably male in phenotype and exhibit mating behavior. Of 25 individuals examined in detail by Nomarski microscopy, all had well-formed male gonads with abundant spermatogenesis and no signs of oogenesis. Most had fully developed male tails, but 5/25 exhibited some defects, such as stunted copulatory spicules. In mating tests, half (10/20) were capable of siring progeny (Table 2). Animals of strain 17 grown at 15° were usually self-fertile hermaphrodites, although most exhibited signs of mascu-

linization such as an Egl (egg-laying defective) phenotype and a truncated tail spike. Increased masculinization was seen when the strain underwent partial starvation. Strain 17 (CB5362) is potentially useful for genomic and biochemical studies because populations can be grown in bulk at low temperature, synchronized, and then shifted to high temperature. The resulting population will consist of pure phenotypic *XX* males, at any desired stage of development, which can be used as starting material for extraction of protein or RNA. These males will not be completely identical to wild-type *XO* males (which cannot easily be prepared in pure populations), but differences may be minimal and irrelevant for many purposes.

The contrasting strain, which is male at low temperature and hermaphrodite at high temperature, was made possible by a temperature-sensitive mutation in the gene *her-1*:

Strain 18: Hermaphrodites, *her-1(e1561); dpy-26(n199) XO* (25°)

Males, *her-1(e1561); dpy-26(n199) XO* (15°)

The *dpy-26* mutation results in *XX* inviability, so the only surviving animals are *XO* in karyotype, as in some previous strains (strains 7 and 10). At low temperature, the *her-1* gene has sufficient activity to direct male development, while at high temperature, the *her-1* gene is inactive and consequently animals develop as hermaphrodites. Approximately 90% of animals grown at low temperature are male, with 10% intersexes; 100% of animals grown at high temperature are hermaphrodite in phenotype, although of low fertility like *XO* hermaphrodites of other genotypes.

Sex determination by a 3X/2X mechanism: As a result of X chromosome overexpression, mutations in the dosage compensation gene *dpy-27* result in lethality to diploid *XX* animals (Plenefisch *et al.* 1989). Similarly, tetraploid *4A; 4X* animals mutant for *dpy-27* are also inviable. However, *dpy-27 4A; 3X* hermaphrodites are viable and non-Dpy, presumably because of the lesser overexpression from the X chromosome (Hodgkin 1987b). As a result, a tetraploid strain homozygous for *dpy-27(rh18)* can be maintained. Because the X chromosome is present in three copies, self-progeny consist of a mixture of *4X* (inviable), *3X* (hermaphrodite), and *2X* (male) individuals:

Strain 19: Hermaphrodites, *dpy-27(rh18) 4A; 3X*

Males, *dpy-27(rh18) 4A; 2X*

This strain grows well as an obligate tetraploid population. Complete broods for eight hermaphrodites were counted: average self-progeny consisted of 92 zygotes, of which 23% were inviable, 47% were hermaphrodite, and 30% were male. Occasional intersexes (<1%) were also seen. In this strain, the X chromosome dose is still sex determining, but the distinction is now between *3X* (hermaphrodite) and *2X* (male), rather than between *2X* (hermaphrodite) and *XO* (male).

Sex determination by neo-X and neo-Y chromosomes: Most of the previous strains have a karyotype related to that of wild-type strains of *C. elegans*. Chromosomal rearrangements can be used to create distinctly different sex karyotypes, however. This was first done by Sigurdson *et al.* (1986), who used *mnT12* (a fusion between the right end of *LGX* and the left end of *LGIV*) to generate a neo-*X* neo-*X* hermaphrodite, neo-*X* neo-*Y* male strain:

Strain 20: Hermaphrodites, *mnT12(X;IV)/mnT12(X;IV)*

Males, *mnT12(X;IV)/+ IV*

In this strain, the normal *XX* hermaphrodite/*XO* male system is converted into an *XX* hermaphrodite/*XY* male system, with a normal *LGIV* behaving as a neo-*Y*. However, the neo-*Y* does not itself have male-determining properties.

Isolation of mutants defective in telomere maintenance has allowed the generation of many other X autosome fusions in *C. elegans* (Ahmed and Hodgkin 2000 and our unpublished results). All of these are like *mnT12* in being homozygous viable and could be used to generate corresponding neo-*X*/neo-*Y* systems. One of them, *eT5* (a fusion between the left end of *LGX* and the right end of *LGIII*), was combined with *fog-2* and an autosomal translocation, *eT1*, to create a male/female strain of a more complicated kind, with multiple *X* and *Y* chromosomes, such as occur in many insect species. The resulting strain has an arrangement resembling that found in the beetle *Cyrsylus volkameriae* (Virkki 1968), with $X_1X_2X_1X_2$ females and $X_1X_2Y_1Y_2$ males:

Strain 21: Females, *eT5(X;III)/eT5(X;III); fog-2 V/fog-2 V*

Males, *eT5(X;III)/eT1(III;V); fog-2 V/eT1 (fog-2 V;III)*

The translocation *eT1* is a reciprocal translocation of the right arm of *LGIII* and the right arm of *LGV* (Rosenbluth and Baillie 1981). In strain 21, *eT5* and a normal *LGV* behave as neo-X_1 and neo-X_2 chromosomes, and the reciprocal parts of *eT1* behave as neo-Y_1 and neo-Y_2 chromosomes. The strain propagates as a male/female strain because of the presence of the *fog-2* mutation. In the males of this strain, the *eT1* reciprocal translocation acts as an efficient recombination suppressor for the right arm of *LGIII* and the left arm of *LGV*. Therefore, all genes in these regions are effectively insulated from recombination with their homologs, and they are also transmitted solely in and by males. They will never be exposed to selection in a female individual. This sets up a situation for the long-term accumulation of male-biased mutations in these regions, and perhaps for the development of further chromosomal dimorphism. In principle, it may be feasible to propagate this or comparable strains for long enough to see if such

effects occur because populations of *C. elegans* will go through at least 100 generations a year, if maintained with adequate nutrition.

The promiscuous chromosome fusions made possible by *mrt-2* and related mutants, together with existing translocations and rearrangements, should also allow creation of yet further variations in sex karyotype. Construction of variant strains has been far from exhausted by the present study.

DISCUSSION

The strains described here demonstrate the considerable variety of sex-determination systems that can be artificially created for *C. elegans*. In most of these strains, a single factor assumes the major role for determining sex, but this factor can be a transcription factor (strains 1, 2, 3, 4, and 15), a transmembrane receptor (strains 5, 6, 7, and 17), a calpain-related protease (strain 8), an extracellular protein (strains 9, 10, and 18), a conserved cytoplasmic protein (strains 11, and 16), a phosphatase (strain 12), a novel cytoplasmic protein (strain 13), or a tRNA (strain 14). It follows that in animals with unknown sex-determination mechanisms, more or less any kind of molecule might be the primary determinant.

Moreover, the genes encoding these critical factors are scattered across the genome, so any one of autosomes *II*, *III*, *IV*, or *V* can easily be turned into a sex chromosome. The only one of the five autosomes that does not naturally carry a major sex-determination gene is *LGI*, and even this chromosome can be recruited into playing the sex-determining role by making use of the amber suppressor *sup-34* (strain 14). An alternative route to recruiting *LGI* would be to fuse *X* and *I* or to integrate the *fem-1(+)* extrachromosomal transgene used in strain 16 into *LG1*. Gross chromosomal rearrangements or chromosome fusions can also be used to create karyotypically diverse stable strains, as in strains 19–21. All the strains described in this article have different sex chromosome constitutions and different life history parameters from the natural *C. elegans*, and this will impose different selective pressures on these strains as compared to the ancestral race. Since the strains are stable, it should be possible to grow them indefinitely, creating multiple possible scenarios for *in vitro* chromosome evolution. The 3-day generation time of *C. elegans* means that cultivation for hundreds of generations is feasible. On the other hand, the amount of variation available for selection *in vitro* is limited, so it is hard to predict whether interesting changes would arise if these were mainly dependent on *de novo* mutation.

In some of these strains, the sex-determination system has been altered so that the dosage compensation apparatus is no longer necessary. The compensation machinery might therefore be expected to undergo change or loss during prolonged cultivation of such strains. Dosage compensation mechanisms have probably arisen and decayed multiple times during animal evolution as a result of comparable changes in the sex-determination system.

The construction of the assorted strains described in this article involved the creation of many double- and triple-mutant combinations that have not been created previously. None of them exhibited a phenotype unpredicted from the standard genetic model for sex determination, and our analyses provide strong support for the correctness of the model and for an essentially hierarchical organization. The pathway, however, does remain far from fully described or understood, particularly in molecular details.

The natural *C. elegans* system has several properties that may have favored these artificial manipulations of sex determination. First, the existence of a self-fertile *XX* hermaphrodite sex means that both *XX* and *XO* germ cells must be able to naturally undergo spermatogenesis. In other animals, sex-transformed *XX* males are usually defective in spermatogenesis. Oogenesis seems to be less critically dependent on correct sex karyotype, so that, for example, *XO* female mice are fertile (Ashworth *et al.* 1991). Second, the seven major sex-specific regulatory genes affect both somatic and germline phenotypes in parallel ways, so any one of these seven major genes can be made to act as a switch. Third, these genes are largely or completely specific to sex determination, so mutants do not exhibit pleiotropic phenotypes. Fourth, the sex-determination genes are organized in a relatively simple hierarchy, so that epistasis in double-mutant combinations is usually complete. Fifth, nematode chromosomes are holocentric, so that fragment chromosomes and extrachromosomal arrays are transmitted fairly stably through meioses. Similarly, chromosomal fusions are stable.

The malleability of *C. elegans* with respect to sex determination is not a unique property of this species. Some animal species appear to be comparably flexible and have the potential to adopt several different sex-determination systems. Different races of housefly (*Musca domestica*) exhibit a variety of sex-determination schemes (Schutt and Nothiger 2000). Similar variability is encountered in certain mammalian species such as wood lemmings (Liu *et al.* 2001). These systems are poorly understood at present, so it is difficult to speculate about the factors underlying their malleability or about whether they share any of these contributory factors with *C. elegans*.

The strains reported here provide mimics of many but by no means all of the different sex-determination systems encountered in the biological world. Four important categories that have not been imitated in the present study are control by nutrition, control by social factors, control by endosymbionts, and control by ploidy (as in Hymenoptera). The first two may well become possible by means of further manipulation. In the case

of nutritional control, some of the existing *C. elegans* sex-determination mutants exhibit striking changes in phenotype under starvation conditions, suggesting that they might be used in this way. For example, the hypermorphic *tra-2* mutation *e2020* has little effect on *XO* animals when they are well fed, so they develop into fertile males; but under starvation conditions, the *XO* animals are extensively feminized in the nongonadal soma (Doniach 1986). Combination with other starvation-sensitive mutants might allow complete transformation from fertile male to fertile female, depending on food supply.

Environmental sex determination by social factors is a related and potentially overlapping effect, because population crowding can affect both nutrition and social signals. In the case of various parasitic nematodes, some of which tend to develop into males at high population density and females at low population density, it seems more likely that the relevant factor is social rather than nutritional (Triantaphyllou 1973). Peckol *et al.* (2001) recently demonstrated that the regulation of some genes in *C. elegans* is dependent on social factors. They found that expression of an *srd-1* reporter transgene is very sensitive to population density, probably detected by means of dauer pheromone, and modulated even at population densities well below those that trigger dauer larva formation. If the pheromone-sensitive regulatory region in *srd-1* were identified and cloned into an appropriate sex-determination gene construct, it would be possible to create crowding-based sex determination in *C. elegans*.

Endosymbiotic bacteria, which are known to affect sex determination in many arthropods (Bandi *et al.* 2001), are absent from the Caenorhabditis species. However, they exist and are important in related nematodes such as the filarial genera Brugia and Wuchereria (Bandi *et al.* 1998). If it becomes possible to introduce such bacteria (Wolbachia species) into *C. elegans*, conceivably by ooplasm injection, then further modification might be feasible, leading to an endosymbiont-based sex-determination system.

Haplo-diploid sex determination may be impossible to re-create in *C. elegans*, because haploids of this species are unable to complete embryogenesis (Schierenberg and Wood 1985). Their inviability is somewhat puzzling in that chromosomal balance should be identical between a normal diploid (*AA;XX*) and a haploid (*A;X*). Furthermore, no recessive lethals are segregating, and no imprinting appears to occur in *C. elegans* (Haack and Hodgkin 1991). One possible explanation is that excess dosage compensation occurs, resulting in insufficient X chromosome expression and therefore in lethality. Another possibility is that the nuclear volume of a haploid *C. elegans* cell is simply too small to sustain normal gene regulation and expression. Nuclear volume seems to be determined largely by DNA content, and the 50-Mb DNA content of a haploid *C. elegans* nucleus would be smaller than any known animal genome. If this is the critical factor, perhaps it might be possible to rescue haploid development by adding extraneous transgenic DNA, but unless the problem of haploid inviability can be solved, this realm of the sex-determination universe will remain inaccessible to *C. elegans*.

Other limitations are also apparent from the work so far. For example, it appears to be much easier to create usable temperature-sensitive mutations in some genes, such as *tra-2* or *fem-1*, than in others, such as *tra-1*. One can imagine a variety of possible explanations for this difference, but whatever the explanation, it has potential evolutionary consequences for transitions between chromosomal and environmental sex determination. It appears likely that it is much easier to put some genes under environmental control than others, so those genes would be more likely to evolve into the critical factor or factors.

Part of the enduring importance and power of classical mutational analysis is that it can be regarded as accelerated evolution and that it explores the envelope of possible past and future change in any biological system, thereby revealing potential courses for natural evolutionary events. Overall, this survey demonstrates that a few mutational changes can radically change the nature of sex determination in a single species in many different ways. By extension, any change that can be achieved by simple genetic manipulations in the laboratory must surely be even easier to achieve in nature, often in ways that we may not have imagined yet. The principle known as Orgel's Second Rule is apposite here: "Natural selection is cleverer than Leslie Orgel" (F. H. C. Crick, personal communication). Transitions between different sexual systems are evidently not so difficult. The more challenging questions are to determine the past course of evolution for any of these sex-determination mechanisms and to identify what selective forces drive the transitions in the real world.

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