Perspectives

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One Lucky XX Male: Isolation of the First *Caenorhabditis elegans* Sex-Determination Mutants

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THIS article marks the 25th anniversary of a paper reporting the first sex-determination mutants to be found in the nematode *Caenorhabditis elegans* (Hodgkin and Brenner 1977). The isolation of these mutants initiated an extensive analysis of nematode sex determination and dosage compensation, carried out by a number of laboratories over the subsequent decades. As a result, the process of sex determination is now one of the most thoroughly understood parts of *C. elegans* development, in both genetic and molecular terms. It has also proved to have interesting repercussions on the study of sex determination in other organisms.

Significant research on sex determination in *C. elegans* was published many years earlier, by Victor Nigon (1949, 1951). Nigon was consistently a man ahead of his time: he carried out important work on *C. elegans*, long before it became fashionable, and then moved on to study vertebrate hematopoiesis, again well ahead of the field. In both cases, he perceived the great potential of these experimental systems for the analysis of biological problems.

For *C. elegans*, Nigon's careful cytological observations led him to define the karyotype of the two natural sexes, which are the self-fertilizing hermaphrodite (essentially a modified female) and the male (Nigon 1949). He found that hermaphrodites have six pairs of similarly sized chromosomes: five autosomal pairs and two X chromosomes (XX, or 2A;2X). Males have the same set of five autosomal pairs, but only one X chromosome (XO, or 2A;1X). Males can, therefore, arise spontaneously, as a result of the rare meiotic loss of an X chromosome. They can mate with and cross-fertilize hermaphrodites, resulting in the production of equal numbers of male and hermaphrodite cross-progeny. Nigon also generated and investigated tetraploid versions of the worm (Nigon 1951). He deduced that 4A;4X animals were hermaphrodites and that 4A;2X animals were male. This suggested that sex was determined by the ratio of X chromosomes to autosomes, as later confirmed and extended in work by Madl and Herman (1979), rather than by the absolute number of X chromosomes. The system therefore resembled Drosophila, in which sex is also determined by an X:A ratio. Classic work by Bridges (1925), establishing the ratio mechanism, led him to propose a balance theory for sex determination, in which sex is determined polygenically by the balance between feminizing factors located on the X chromosome and masculinizing factors located on the autosomes. With hindsight, such an interpretation looks misleading. The real control of sexual phenotype, in all the systems that we understand, is exerted by a small number of key genes, which act as master regulators for sex. In the case of *C. elegans*, their power is especially striking.

The article we published in 1977 had its origins in the earlier investigations of Sydney Brenner, which established *C. elegans* as a promising and versatile genetic system (Brenner 1974). A major part of his initial exploration of the genetics of the worm involved extensive mutant screens, mainly using ethylmethane sulfonate (EMS) as a mutagen. He searched in particular for mutants with abnormal behavioral phenotypes, but also noted and retained hundreds of mutants with many other morphological or developmental abnormalities. In April 1970, Brenner observed that one of the abnormal hermaphrodites that he had picked from his 37th EMS mutagenesis experiment produced a self-progeny brood containing about 25% males, instead of the normal 100% hermaphrodites. He guessed that the males might be homozygous for a recessive mutation that transformed XX animals from hermaphrodite into male. There was some precedent for such mutations from other organisms, particularly the *tra* mutant of *Drosophila melanogaster* (Sturtevant 1945). Consistent with this guess, he found that the males were abnormal, in that they did not mate successfully. Also, two-thirds of their hermaphrodite sisters produced 25% males again in the next generation, as expected for a Mendelian recessive. As a result, the mutation could be propagated and the population grown to a point where it

could be frozen in liquid nitrogen, awaiting a time when it might be revived and properly analyzed. One of the major advantages of *C. elegans* genetics is the ability to freeze away such oddities and (with luck) remember them down the line.

That took a few years. When I joined Sydney's lab as a graduate student the following year, I was (and remain) deeply interested in how genes encode behavior. The behavioral differences between hermaphrodites and males of *C. elegans* in the adult stage are profound. Among other things, adult males are sex obsessed and hermaphrodites are wholly uninterested; mating is a very one-sided business. Mating is also fairly complicated, perhaps the most elaborate part of the worm's behavioral box of tricks. So I decided to explore the basis of the difference between male and hermaphrodite, by both genetic and anatomical means. Serial section electron micrographs quickly revealed that the male nervous system is decidedly more complicated than that of the hermaphrodite, and detailed reconstruction of the male neuroanatomy looked like a daunting prospect. Genetics was more fun. I began to search for and study mutants with abnormal mating behavior or aberrant male development.

After a while, Sydney informed me of the possible sexual transformation mutant that he had isolated, and I persuaded him to thaw it out. As advertised, the revived population contained a mixture of hermaphrodites and males, and some of the hermaphrodites produced more males in their self-progeny, so I could keep the mutant line going. The males, which we presumed to be sexually transformed XX animals, looked remarkably similar to normal XO males, and they certainly tried to mate with hermaphrodites, although with no apparent success. It seemed worth testing their incompetence more stringently, and I therefore tried setting up crosses under especially favorable conditions. This involved putting lots of males together with hermaphrodite partners homozygous for a recessive uncoordinated mutation called *unc-17*. The uncoordinated phenotype—severe coiling—meant that the hermaphrodites could not escape the attention of males by swimming off to other parts of the crossing plate, and it also meant that any cross-progeny would be immediately apparent, because they would be able to move normally and grow faster.

One of these favorable crosses was successful! A few days after setting up a series of cross-plates, I observed wild-type hermaphrodites moving around on one of them. But I saw no males at all, apart from the aging mutant males that had been added initially. This observation immediately showed that the mutant male parent must have had two X chromosomes, rather than one, like a normal XO male. So a single mutation could completely transform the sexual fate of a worm, from fertile hermaphrodite to fertile male.

We found out subsequently that the result was fortunate, because this first mutation, *e440*, is one of the weaker alleles of *tra-1* (as we came to call the gene concerned). Only about 1 in 30 *e440* XX males ever sires progeny, and these rare individuals are fertile only as young adults, producing no more than about a dozen cross-progeny. Nevertheless, it was enough to make the point, and enough to re-engage Sydney's attention. At the time, the ability to make fertile XX males was technically valuable, because it permitted complementation tests between many sex-linked mutations. Most of the mutations on the X chromosome that had been isolated caused hemizygous males to be too deformed or uncoordinated to be able to mate with hermaphrodites, making crosses impossible. So there were several sets of independently isolated sex-linked *unc* mutants that looked similar in phenotype and had similar map locations on the X chromosome, but there was no way of carrying out a complementation test to decide whether or not they were allelic or to construct double mutants for the purposes of mapping and gene interaction studies. However, all but a few of these *unc* mutations are recessive, and *tra-1; unc/+* XX heterozygous males therefore move normally and can be used in crosses. The *tra-1* mutation provided a handy solution for the problems of dealing with severe sex-linked mutants, as Sydney noted in the discussion of his 1974 article (Brenner 1974).

As well as being useful, the *tra* mutations were obviously relevant to the developmental genetics of males and to sex determination in general. Alerted to the existence and usefulness of this mutant class, Sydney and I searched for and found further *tra* mutants. Among these was a stronger allele of *tra-1*, which produced XX males of greater potency, but to our surprise, most of them defined a different gene, which we called *tra-2*. The *tra-2* mutants also transformed XX animals from hermaphrodites into males, but the transformation was less complete. The *tra-2* XX animals had slightly abnormal male anatomy, did not exhibit mating behavior, and were never capable of cross-fertilizing hermaphrodites. The different phenotypes of *tra-1* and *tra-2* mutants were exciting, because they raised the possibility of genetically dissecting mating behavior. It might be possible to define the mutant defects in terms of neuroanatomy and thereby work out which bits of neuronal circuitry did what, during mating.

Finally, there was a third gene, *tra-3*, defined by a remarkable mutant referred to in Sydney's notebooks as "bizarre pseudomale picked by David Baillie." This was an autosomal recessive mutation, like *tra-1* and *tra-2*, but it differed from them in showing a striking maternal effect. XX animals homozygous for *tra-3* developed as perfectly normal hermaphrodites, if derived from a heterozygous *tra-3/+* hermaphrodite mother. But in the next generation, all of the XX self-progeny were transformed into masculinized animals.

The three *tra* genes were mapped and found to be located on different autosomes. We carried out a standard genetic characterization; for example, performing

tests with sex-linked markers to prove that the *tra-1* mutants carried and transmitted two X chromosomes. For both *tra-1* and *tra-2*, there were noticeable differences between the phenotypes associated with some of the different alleles. The existence of allelic series for the two genes was useful, because it allowed us to infer that the strongest masculinizing effect might be due to a complete loss of function in the gene, and therefore that the normal role of the *tra* genes was to *prevent* male development in XX animals. At the time, this interpretation did not seem as obvious as it does now. Later research on both *tra-1* and *tra-2* confirmed the idea by the isolation of dominant mutations with the opposite effect, which can transform XO animals from male into hermaphrodite or female states (Hodgkin 1983a; Kuwabara 1996).

The *tra-3* gene was defined by only a single allele, causing incomplete and variable masculinization, and we gave it less attention than the other two genes. Subsequently, however, more *tra-3* alleles were obtained and proved very useful in working out the detailed genetics of the sex-determination pathway. In the 1980s, moreover, the original *tra-3* mutation came to play an important role both in the analysis of nonsense suppression and in the development of methods for transforming *C. elegans* (Kimble *et al.* 1982; Fire 1986).

We also included observations on what proved to be the first dosage compensation mutant in *C. elegans*. Again, this came out of the cornucopia of mutants preserved from Sydney's initial EMS screens. Two independent mutants had been picked for their dumpy phenotype (short and fat) and were grown as hermaphrodite populations, which were uniformly dumpy. But after a while, the culture plates were found to be swarming with males of normal size and shape, along with unchanged dumpy hermaphrodites. Genetic tests showed that these males were still homozygous for the mutation, called *dpy-21*, despite their wild-type phenotype. The mutation was, therefore, sex limited in its expression, in contrast to the vast majority of *C. elegans* mutations. But was the dumpy phenotype a consequence of being a hermaphrodite or a consequence of having two X chromosomes rather than one? The *tra* mutations made it easy to carry out a test, by making a double mutant. The XX *tra-1; dpy-21* animals were both male and dumpy, which meant that the dumpiness was a consequence of X chromosome dosage. Subsequent work on *dpy-21* and a related gene, *dpy-26*, led to the suggestion that the normal function of these genes lies in dosage compensation (Hodgkin 1983b; Wood *et al.* 1985; Meyer and Casson 1986). Their products are required to downregulate sex-linked gene expression in the sex with two X chromosomes, thereby equalizing expression between the XX and XO sexes. The molecular machinery involved is now understood in some detail (Meyer 2000).

The mechanism of dosage compensation (downregulation in the XX sex) contrasted with the mechanism in Drosophila (upregulation in the single X sex). However, the basic genetics of sex determination appeared not so different: in both fly and worm, primary sex determination depends on the ratio of X chromosomes to autosomes, and in both cases single-gene mutations causing profound masculinization of XX animals could be identified. Partly for this reason, we called our first masculinization mutants *tra*, in reminiscence of the Drosophila *tra* gene. The naming also paid homage to the great geneticist Alfred Sturtevant, who isolated the first Drosophila *tra* mutant. And for quite a while, there was the possibility that there might be deep evolutionary conservation of sex-determination mechanisms. Much subsequent research on both systems resulted in increasingly detailed genetic and then molecular models for the regulatory pathways involved in sex. But the more we learned, the more differences could be seen between the two pathways (Hodgkin 1990; Cline and Meyer 1996). It became clear that sex-determination mechanisms evolve rapidly and involve much less conservation than other aspects of development. Ultimately, it was established that *tra* in Drosophila encodes an RNA-binding protein, which works by regulating alternative splicing in target genes (Nagoshi *et al.* 1988), whereas *tra-1* in *C. elegans* encodes a zinc-finger transcription factor (Zarkower and Hodgkin 1992). So *tra* in flies and *tra-1* in worms are analogous but not homologous.

Now, in a further twist to the tale, it appears that there may be conservation between sex in flies and sex in worms after all, but it lies in the genes downstream. Both *tra* in Drosophila and *tra-1* in *C. elegans* regulate genes that share a similar motif, encoding a distinctive DNA-binding sequence. The Drosophila gene is *dsx,* and the nematode gene is *mab-3,* and the motif has been named the DM domain, after these two genes. The mechanism of regulation is different in the two species, but the DM products are sufficiently similar that a *mab-3* mutant worm can be partly rescued by introducing a transgene expressing DSX-M, the male-specific product of *dsx* (Raymond *et al.* 1998). DM domain factors also play important roles in vertebrate sex determination (Zarkower 2001). This class of transcription factor may represent the one kernel of conservation in the evolution of sex.

At the time of our original work, there was little inkling of the great advances in molecular genetics that would allow us to clone and sequence these genes and get down to the biochemical realities. Then as now, however, forward genetic analysis was clearly an effective way to initiate the investigation of any biological problem. The satisfaction of arriving at strong biological conclusions from simple genetic observations remains as great as ever.

Note added in proof: With fortunate timing, this article coincides with the award of the Nobel Prize in Physiology or Medicine for 2002 to Sydney Brenner, Robert Horvitz, and John Sulston. In addition to

their many other discoveries, all three laureates have made major contributions to the understanding of sex determination and sexual differentiation in *C. elegans*.

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