Perspectives

Anecdotal, Historical and Critical Commentaries on Genetics

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"Joy of the Worm"

W. SHAKESPEARE, *Antony and Cleopatra*, Act V, Sc. II

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IT has been ten years since we published in GENETICS a paper describing the isolation and genetic characterization of cell lineage mutants of the nematode *Caenorhabditis elegans* (HORVITZ and SULSTON 1980). We have reviewed elsewhere what has been learned from the study of these and other mutants abnormal in the pattern of cell divisions and cell fates that characterizes *C. elegans* development (HORVITZ 1988, 1990). Here we wish to reflect upon the days of our initial experiments, and to recall our excitement, our visions and our qualms as we elucidated the nematode cell lineage and began exploring methods for its genetic analysis.

In the beginning there was SYDNEY BRENNER (see HODGKIN 1989). It was BRENNER who selected *Caenorhabditis* for the study of developmental genetics. In 1963, BRENNER wrote to MAX PERUTZ, the head of the Medical Research Council Laboratory of Molecular Biology in Cambridge, England, " . . . we propose to identify every cell in the worm and trace lineages. We shall also investigate the constancy of development and study its genetic control by looking for mutants" (cited by BRENNER 1988).

BRENNER was particularly interested in the development of the nervous system and in 1970 he hired one of us (SULSTON) to analyze the neurochemistry of *C. elegans*. SULSTON joined BRENNER's group after a postdoctoral stint with LESLIE ORGEL working on prebiotic chemistry and hoping to discover the basis of the origin of life. SULSTON used the technique of Feulgen-staining to examine nuclei within the nervous system of nematodes of different developmental stages. He noticed that the number of neurons along the ventral cord was 15 in young larvae and 57 in older animals. Now, anyone familiar with nematode biology knew nematodes were eutelic, *i.e.*, constant in cell number after hatching; as an invertebrate zoology text blithely stated (see p. 245 of BORRADAILE *et al.* 1961), ". . . in the embryo all cell division soon ceases except for that seen in the reproductive cells. Growth consists of vacuolation and extension of the cells already present." Fortunately, in one example of what has proved to be an important regular infusion of inexperts into the *C. elegans* field, SULSTON was unaware of this fact and was happy to pursue his presumably impossible observation.

SULSTON decided to try to discover how 15 cells become 57. He took advantage of the fact that ROGER FREEDMAN and SIMON PICKVANCE, two other members of BRENNER's laboratory, had found that a light microscope equipped with Nomarski differential interference contrast optics allowed individual nuclei to be observed in living *C. elegans* embryos. SULSTON placed young *C. elegans* larvae on a glass microscope slide supporting a thin pad of agar with a dab of bacteria (nematode food), which both allowed the larvae to grow and attracted them (thereby preventing them from crawling away). He dropped a coverslip over the nematodes and observed them through the microscope using Nomarski optics. Every nucleus could be seen within a living animal. So SULSTON watched an animal, waiting to see which of the 15 cells would divide to generate the 42 new ones. None of them did. Instead, ten new cells spontaneously appeared within the ventral cord. Now the inexpert was taken aback. It was one matter to discover that older nematodes have more neurons than do young nematodes, but quite another to discover that cells could be generated spontaneously, without mitosis, meiosis or any other known biological phenomenon.

So SULSTON watched some more. It proved easy to see development proceed by observing a cell and following what it did. It was much harder to study development backward in time, for example to identify the source of the spontaneously appearing ventral cord cells. One had to guess a possible origin for a cell and observe the candidate precursor cell to see if the guess was correct. The solution to the source of the ten new cells came when SULSTON realized that these cells were squeezing into the ventral cord from nearby positions out of the plane of focus; the rapid migration and distortion of the cells made it appear that at one moment there was no cell while only shortly later a cell came into view.

Watching the ten new cells proved quite interesting. In contrast to the ventral cord cells present in the newly hatched larva, these cells divided! Observing that first cell division within a living animal was truly exhilarating, not only in itself but also because it meant that in principle one could determine the whole lineage. Strikingly, each of the ten cells, as well as two other cells slightly removed from the central region of the ventral cord, divided according to the same pattern and produced six descendants, five of which were neural and one of which was non-neural. Four of the 50 neural descendants migrated out of this central region and another four died (revealing the phenomenon of programmed cell death in *C. elegans* development). These events completely accounted for the origin of the 42 postembryonic neural cells. SULSTON determined the entire development of the ventral cord over a single weekend.

How did the stereotyped division pattern expressed by all of these precursor cells relate to the specific nerve cell types generated? To answer this question required knowing precisely what type of neuron each of the descendant cells became. This in turn required waiting until Monday when JOHN WHITE returned from sailing. WHITE had been analyzing the neuroanatomy of *C. elegans*, and from the equivalent of 20,000 serial sections of each of a number of individual animals he was eventually able to describe the complete connectivity of the animal's 302-celled nervous system (WHITE *et al.* 1986). However, at this earlier time WHITE had studied primarily the ventral cord, and only WHITE knew the results of these studies. He took SULSTON's lineage data and sat down to compare the relative positions of the newly generated and preexisting cells with the positions of the seven types of motor neurons present in the adult ventral cord. WHITE soon returned with the conclusion that cells homologous in lineage history differentiated into neurons of the same type. For example, the anterior daughter of the posterior daughter of the anterior daughter of each of the precursor cells (each P.apa cell, for short) became a specific neuron type known as a dorsal AS neuron. WHITE's studies of ventral nerve cord anatomy were published together with SULSTON's studies of ventral nerve cord development (WHITE *et al.* 1976; SULSTON 1976). These observations established both the invariance and the striking relationship between lineage history and cell fate that characterize much of *C. elegans* development.

For years afterward, we were reminded of the excitement over these cell assignments by a red stain on the ceiling of the division's seminar room. WHITE had won a bet with BRENNER over a detailed aspect of the assignments and the stake, a bottle of wine, was produced at group meeting. In the absence of a corkscrew, the bottle was opened by injecting pressurized Freon from an ozone-destroying microscope duster through the cork via a hypodermic needle. The consequences were geyser-like. Sadly, the gloss of refurbishment has now obscured this moment of history.

In 1974, just prior to the shower of wine, the second of us (HORVITZ) entered the scene. A new postdoctoral fellow fresh from the molecular biology laboratory of JAMES WATSON and WALLY GILBERT, HORVITZ had been studying phage T4-induced modifications of *Escherichia coli* RNA polymerase with the belief that this training would somehow lead him into the world of neurobiology. Initially, HORVITZ was rather skeptical about the worth of watching cells divide: he wanted to do hard science, with radioactivity, gels and molecules, and he had to be convinced that what his eyes could see directly provided scientific information as reliable as what his eyes could see when they viewed the output of a scintillation counter. The thrill of directly watching development and the elegance and intriguing nature of the cell lineage diagrams that resulted—coupled with the potential for experimental intervention—soon dissuaded him of his parochial view.

HORVITZ decided to begin his foray into nematodes and behavioral systems by focusing on muscle. It was clear from the anatomy that just as the older animal had more neurons than did the younger animal, it also had more muscle cells: some of the somatic muscle cells used for locomotion and all of the vulval muscle cells used for egg laying were added after hatching. The origin of the new somatic muscles proved easy to determine, as a large blast cell (now called M) present in the newly hatched animal divided during the first larval stage to generate 18 cells located within the four longitudinal bands of muscle. However, the origin of the vulval muscles, which appeared much later, proved elusive. Again, observing development backward in time was not possible.

The two of us had different theories. HORVITZ, based on the principle from classical embryology that muscle derives from the mesoderm, suggested that somehow the M cell descendants generate the vulval muscles, despite the fact that these cells were located quite far from the vulva and did not look like blast

cells. SULSTON, by contrast, noted that the vulval muscles differentiated in the region of the gonad from cells very similar to gonadal cells in morphology, and suggested that the somatic gonad would prove to be the source of the vulval muscles. So one day a race began, with each of us starting on a different track. SULSTON followed the gonadal cell lineages while HORVITZ stared at the 18 M cell descendants. SULSTON's gonadal lineage proliferated, generating more and more potential vulval muscle precursor cells, while the M descendants did nothing. Two of HORVITZ's 18 cells withdrew their candidacies, differentiating into non-muscle cells known as coelomocytes. Then, two of the 16 remaining descendants of M, one on each side of the animal, started to move. They migrated from the posterior region of the animal to positions directly flanking the developing gonad, which was still being observed by SULSTON. These two cells grew and then divided, each generating eight descendants, which then differentiated into four vulval and four uterine muscles.

Classical embryology had won, and the race to the vulval muscles was soon followed by the elucidation of the complete postembryonic cell lineages of *C. elegans* (SULSTON and HORVITZ 1977; KIMBLE and HIRSH 1979). But although classical notions of development had in this case led to the correct prediction, their absolute generality failed later, as the study of the embryonic lineage (SULSTON *et al.* 1983) revealed that a number of cells transgress their presumptive embryonic developmental boundaries. For example, certain neurons derive from embryonic mesoderm and certain muscles derive from embryonic ectoderm. Generalities often have exceptions in biology.

By determining the *C. elegans* postembryonic cell lineages, we had described many of the problems of developmental biology at the level of single cells. The issue now became how to proceed from description to mechanism. We discussed two general approaches. First, there were the classical methods of experimental embryology, in which various bits and pieces of developing organisms were removed and/or transplanted. With this direction in mind, JOHN WHITE began pursuing a conceptually similar approach based upon modern technology, namely a laser microbeam. WHITE, who received his basic training in physics, is a tinkerer; he loves to design and implement new technologies and he is extraordinarily good at doing so. One of his most recent contributions is the confocal microscope (WHITE, AMOS and FORDHAM 1987). WHITE designed a system that allowed him to focus a laser beam inside an animal being viewed with Nomarski optics, and he found he could kill single cells in living animals in this way. (WHITE also found he could destroy expensive microscope objectives, as HORVITZ discovered after returning from lunch one day to continue some cell lineage studies; his microscope no longer worked, and the reason proved to be that WHITE had borrowed, and melted, the objective.) The technique of laser microsurgery could be used to define the functions of individual cells, either in the mature organism or during development (WHITE and HORVITZ 1979; SULSTON and WHITE 1980). Such experiments have helped reveal that cell interactions play a major role during *C. elegans* development, and that the invariance of normal development to a significant degree reflects the invariance of cell interactions.

The second possible approach toward the analysis of cell lineage was genetics. BRENNER (1974) had already established *C. elegans* as a genetic system. The disadvantage, and the advantage, of using genetics for the study of cell lineage was that it was entirely exploratory. In our pessimistic moments we feared that it would be impossible to isolate cell lineage mutants: any mutation that perturbed one cell division might well perturb so many divisions as to lead to an uninterpretable lethality (at least at that point, prior to the elucidation of the embryonic cell lineage). Furthermore, we suspected that even if we found mutants abnormal in specific postembryonic cell divisions, very few would be interesting. After all, one could easily imagine that leaky mutations in any housekeeping gene would cause a defect in the set of cells most sensitive to decreases in the activity of that gene. How could such mutations be distinguished from those in important developmental control genes?

With these concerns in mind, the rationale we offered in our first discussion of *C. elegans* cell lineage mutants, at an MRC joint worm-fly group meeting in March of 1976, was that mutations offered a useful complement to the laser: each could destroy the functions of particular cells, and the pleiotropies that resulted using the two methodologies were likely to be very different. Nonetheless, what we really hoped was that mutations that perturbed cell lineage would lead us to interesting genes. Unlike the laser, which could reveal the developmental functions of cells but go no further, mutations in principle could lead to an understanding of the genes and molecules that specified development.

The problem was how to begin. Because we did not even know if cell lineage mutants could exist, we hardly could know what phenotypes to seek. As described in the GENETICS paper published ten years ago this month (HORVITZ and SULSTON 1980), we divided the problem into two parts: first deciding what methods to use to identify mutants defective in cell lineage (the direct observation of lineages in living animals would be prohibitively slow) and then deciding what mutant phenotypes to examine using these methods. Our methods were based on the idea that animals abnormal in the number of cells of a particular type might well be abnormal in cell lineage. So we looked at fixed and stained mutant strains by techniques that

allowed the visualization of individual cells, such as Feulgen-staining (which could be used to observe nuclei in the ventral nerve cord and the vulva) and formaldehyde-induced fluorescence (which revealed dopaminergic neurons). Only after seeing anatomical abnormalities did we use Nomarski optics to determine if these abnormalities reflected cell lineage defects. These methods require the establishment of mutant strains, as opposed to allowing the direct screening of living individuals. We did not have the confidence to attempt the more powerful approach of examining the cellular anatomy of living individuals using Nomarski optics, and it was not until later that ED HEDGECOCK proved that this method could be highly efficient and successful (HEDGECOCK and THOMSON 1982).

Our choice of mutant phenotypes to be examined was dictated by our desire to have some logical rationale but not to limit ourselves given our ignorance. For this reason, we examined mutants with behavioral or morphological abnormalities that we suspected might be consequences of cell lineage defects, and we also examined at random worms derived from mutagenized parents or grandparents. For example, we thought that defects in the behavior of egg laying could reflect defects in postembryonic cell lineages. One of the major consequences of the postembryonic cell divisions is sexual maturation. Not surprisingly, the young larva and the adult face many of the same biological challenges and need many of the same cells. However, only the adult must reproduce. For the *C. elegans* hermaphrodite, this means that only the adult needs the cells necessary for egg laying and copulation, namely the cells of the vulva, the vulval and uterine muscles, and the neurons that innervate these muscles. We knew that egg laying is not essential for either viability or fertility, so that homozygous egg-laying defective strains could be established. (Animals that cannot lay eggs are nonetheless fertile because the *C. elegans* hermaphrodite is internally self-fertilizing, and fertilized eggs can develop and hatch *in utero*.) It was very easy to recognize mutants defective in egg laying, either as animals severely bloated with retained eggs or as "bags of worms" formed when internally hatched larvae consume the body of their mother-father but remain (transiently) trapped within its cuticle. We hoped that among mutants defective in the vulval cells, the sex muscles or the sex neurons would be some that were abnormal in the lineages that generate these cells.

We also examined mutants with other behavioral or morphological abnormalities that we thought might be caused by defects in cell lineage. For example, BRENNER (1974) had isolated many mutants with locomotory defects, and it seemed likely that some of these would have defects in the postembryonic lineages that generate the ventral cord motor neurons. Similarly, BRENNER had also isolated, but not described in print, three mutants with abnormal ventral growths. He had suspected these growths to be supernumerary vulva-like structures, and we confirmed his suspicion by determining the cell lineages of these mutants and finding that extra cells underwent vulval cell division patterns.

Finally, because we really had no idea what mutant phenotypes to anticipate, we screened at random the F_1 and F_2 progeny of mutagenized hermaphrodites. We placed single animals on Petri plates and used the anatomical techniques described above to examine some of the progeny while saving the unfixed, unstained and living siblings to establish mutant strains. This clonal mutant hunt allowed us to isolate cell lineage mutants that were sterile as homozygotes and thus could not have been isolated in either of our other two screens.

These initial experiments led to the identification of 24 cell lineage mutants that defined 14 genes. Three simple attributes of these mutants were very exciting to us. First and foremost, cell lineage mutants existed. Second, many of these mutants could be established as homozygous viable strains. Third, some appeared to be null mutants in which the activities of particular genes were completely eliminated. These observations proved that we could indeed find mutants abnormal in specific cell lineages and established that different genes function in different cell lineages, suggesting that it could be possible to define the set of genes that specifically controls any particular cell division.

A fourth and completely unexpected feature that intrigued us was that most of our cell lineage mutants could be considered to be homeotic at the level of single cells. Specifically, the abnormal phenotypes of most of these mutants result from transformations in cell fates, with particular cells expressing not their own fates (as recognized by patterns of cell division and by the types of descendant cells generated) but rather fates normally expressed by other cells. Genes defined by such mutants seemed likely to function in specifying cell fates and seemed to be excellent candidates for playing important roles in controlling development.

Now, ten years after we described the first *C. elegans* cell lineage mutants, the numbers of *C. elegans* cell lineage mutants, genes and researchers continue to increase dramatically. Specific cell lineage genes have been shown to have specific and distinct functions in generating cellular diversity during development. Although confusing at first, genes with broadly pleiotropic effects have proved easier to understand than genes specific for single cell divisions. For example, we were able to conclude that *lin-17* acts to make certain sister cells different from each other because mutations in this gene cause a variety of blast cells to

produce identical instead of different daughters (STERNBERG and HORVITZ 1988), and that *lin-14* and the other heterochronic genes control developmental timing because they perturb the relative time of expression of multiple cell lineages as well as of other developmental events (AMBROS and HORVITZ 1984). Similarly, other genes act to make certain daughter cells different from their mother cells (CHALFIE, HORVITZ and SULSTON 1981), while still others play fundamental roles in controlling cell-cell interactions (GREENWALD, STERNBERG and HORVITZ 1983; KENYON 1986; PRIESS, SCHNABEL and SCHNABEL 1987; AUSTIN and KIMBLE 1987).

The study of the genetic control of vulval development has identified a network of about 50 interacting genes that regulate the three rounds of cell division that constitute the vulval cell lineages. These genes control the generation of the six potential vulval precursor cells, the actions of two distinct pathways of intercellular signalling that determine which of three alternative fates will be expressed by each vulval precursor cell, and the expression of the fates of these cells once these fates have been determined (FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG and HORVITZ 1989; R. HORVITZ, P. STERNBERG, I. GREENWALD and colleagues, work in progress).

The molecular characterization of *C. elegans* cell lineage genes is progressing at an ever increasing rate, in part as a consequence of the availability of a nearly complete physical map of the *C. elegans* genome (COULSON *et al.* 1988). The physical map allows a gene to be cloned simply by knowing its position on the genetic map and using previously cloned DNA from the region to rescue the mutant phenotype in germline transformation experiments (FIRE 1986; WAY and CHALFIE 1988). Some cell lineage genes encode familiar types of proteins, and the presumed functions of these genes in controlling such processes as cell-cell interactions and gene expression can readily account for their roles in specifying cell lineage. For example, the gene *unc-86*, identified in our first genetic study, causes certain daughter cells to express characteristics different from those of their mother cells (CHALFIE, HORVITZ and SULSTON 1981). *unc-86* also controls the differentiation of certain nondividing cells (DESAI *et al.* 1988). Together, these observations indicate that *unc-86* regulates the expression of novel cell-type-specific traits. The molecular analysis of *unc-86* revealed a mechanistic basis for its action: *unc-86* encodes a protein with a homeodomain and extended similarity to a variety of mammalian transcription factors (FINNEY, RUVKUN and HORVITZ 1988). Thus, *unc-86* presumably acts by controlling the transcription of cell-type-specific genes, which in turn causes the expression of cell-type-specific characteristics. The class of transcription factors defined by *unc-86*, known as the POU proteins (for *p*ituitary, *o*ctamer-binding and *unc*-86) (HERR *et al.* 1988), seems likely to regulate development not only in *C. elegans* but in many other organisms as well.

Other genes involved in the generation of cellular diversity during development also have proved to have molecular structures that are interpretable in the context of their mutant phenotypes. For example, the gene *lin-12* was discovered on the basis of its effects on the vulval cell lineages and found to control the fates not only of the vulval cells but also of many other cell types with fates regulated by cell-cell interactions (GREENWALD, STERNBERG and HORVITZ 1983; FERGUSON and HORVITZ 1985). Thus, *lin-12* seemed likely to function in intercellular signalling. The DNA sequence of *lin-12* showed that this gene encodes a transmembrane protein in the same family as the LDL receptor and the *Drosophila Notch* protein (GREENWALD 1985; YOCHEM, WESTON and GREENWALD 1988), and genetic mosaic analysis indicated that the *lin-12* protein is probably the receptor in a system of inductive signalling (SEYDOUX and GREENWALD 1989).

Not surprisingly, some cell lineage genes encode proteins that are novel in sequence (*e.g.*, RUVKUN *et al.* 1989; KIM and HORVITZ 1990). These genes promise to reveal new types of proteins that play regulatory roles in development.

The initial goals of BRENNER—to identify every cell in the worm, to trace lineages and investigate the constancy of development, and to discover mutants—have certainly been fulfilled. Furthermore, we now know a lot about genes that can mutate to perturb the *C. elegans* cell lineage. Studies of *C. elegans* cell lineage genes have revealed that neither the most pessimistic nor the most simplistic view of the genetic control of cell lineage is valid: on the one hand, every gene that affects a particular cell division is not required for all other cell divisions; on the other hand, the hypothesis of one gene, one cell division clearly is untenable. Rather, each cell division appears to be controlled by a number of genes, and many of these genes control other cell divisions as well. How specific combinations of these genes interact to cause the expression of distinct cell fates remains to be elucidated. Today's dream is to identify every gene that controls the worm's cell lineage, and to determine at a molecular level how these genes specify the development of *C. elegans*.

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