Caenorhabditis elegans: The Cell Lineage and Beyond (Nobel Lecture)**

John E. Sulston*[a]

Thank you so very much for inviting me to be here. It gives me a mingled sense of humility at how much I owe to others, and of joy that the collective work on the worm has been recognised in this way.

Among the first of my many mentors was my PhD supervisor, Colin Reese, who was developing nonaqueous methods for oligonucleotide synthesis (see, for example, ref. [1]). Colin passed me on to my postdoctoral supervisor Leslie Orgel at the Salk Institute, to work on prebiotic chemistry. The plan was to see to what extent we could copy RNA chains without enzymes. The products were obtained in low yield, and the challenge was to work out the sequences that had been produced. We used cutting with different ribonucleases, and chromatography to separate the fragments (see, for example, ref. [2]).

My introduction to Caenorhabditis elegans came in 1969 with my move, at Leslie's suggestion, to Sydney Brenner's group at the Medical Research Council (MRC) Laboratory of Molecular Biology (Figure 1). Sydney was reputed to be setting up a group to work on the nervous system of a nematode, though at that point nobody knew much about it.[3]
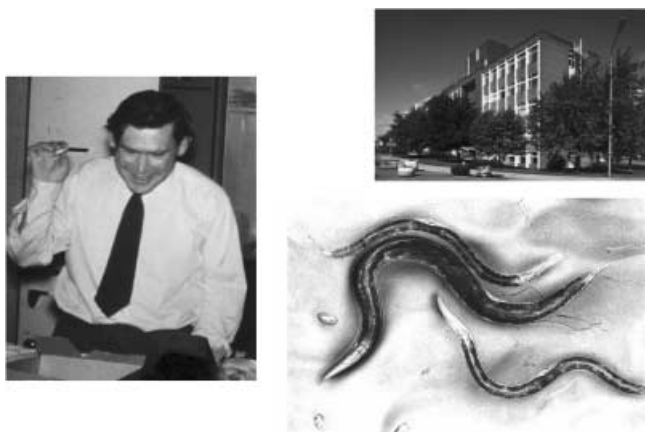

Figure 1. Sydney Brenner, MRC Laboratory of Molecular Biology and worms.

Sydney had already collected numerous mutations that affected the behaviour of the worms,[4] and one of my first

projects was to investigate the level of glutamate decarboxylase in these mutants, for γ-amino butyrate (GABA) was believed to function as an inhibitory transmitter. I continued to work on GABA for a while, with a view to finding mutations that affected its production and the behaviour of the worms, but didn't find anything useful—entirely my fault, since plenty of such mutations were found later on.

We also had a look at the DNA content of the worm genome by using the newly developed technique of renaturation analysis,[5, 6] and came up with an estimate of 20 times that of Escherichia coli.[7] At that time we were not certain of the ploidy of the cells, so even such a simple thing as the comparison of estimates from annealing analysis and chemical analysis was of interest. We counted the stable RNA genes, and attempted to estimate the proportion of the genome that was transcribed. This also led me into a brief collaboration with Gerry Rubin, then a research student with Andrew Travers; we demonstrated linkage between the 5S and larger ribosomal genes of yeast.[8]

Meanwhile I played with the technique of formaldehyde-induced fluorescence (FIF; reviewed in ref. [9]) of catecholamines, purely because I knew about it from working with Steve Kuffler and Ed Furshpan when they had run a summer school at the Salk Institute. After some fiddling, necessary on account of the small size of the nematode neurons, I was able to get nice results (Figure 2). The fluorescence spectra indicated that the transmitter was dopamine. Marilyn Dew and I mutagenised some worms and looked for mutants, and found several genes that were required, some for the dopamine to be visible at all, others for loading it into vesicles.[10] We tried to find a function for the dopaminergic cells, reasoning from their appearance in electron micrographs that they would be involved in mechano-

[a] Dr. J. E. Sulston
The Welcome Trust Sanger Institute Genome Campus
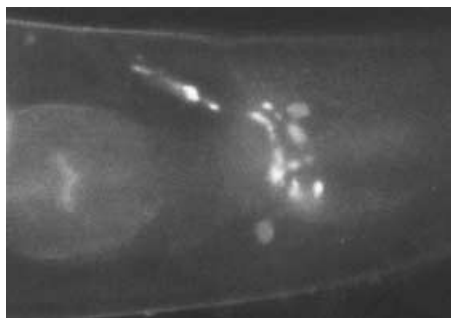Hinxton, Cambridge CB10 1SA (UK)
Fax: (+44) 1223-494919
E-mail: jes@sanger.ac.uk

Figure 2. Formaldehyde-induced fluorescence of dopamine in the nerve ring and retrovesicular ganglion of the worm.

Figure 3. At hatching the ventral cord (VC) has 15 cells; a few hours later there are 57. The figure shows 4′,6-diamidino-2-phenylindole (DAPI)-stained specimens in the first and second larval stages of development (L1 and L2). RVG, retrovesicular ganglion.

sensation. Our search was unsuccessful, but it did reveal the first of another series of mutants—the *mecs*—that were insensitive to gentle touch and were adopted by Marty Chalfie in his research programme.[11, 12]

But another aspect of the FIF had now diverted me. There were half a dozen FIF-positive neurons in the head of the newly hatched larva, but curiously an extra pair appeared later on. It was curious because the review literature at that time indicated that the complement of neurons is complete at hatching. However, when I started to stain worms by the Feulgen technique in order to discover which were the catecholamine-containing ones, it became clear that numerous neurons in the ventral nerve cord were formed some time after hatching (Figure 3). Perusal of the primary literature showed that
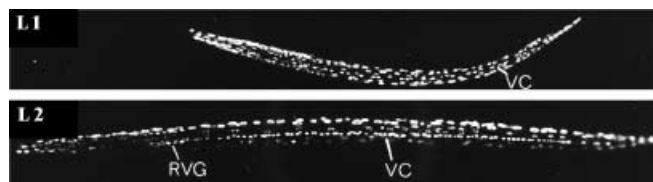

Dr. John E. Sulston obtained a B.A. degree in organic chemistry at the University of Cambridge (UK) in 1963, where he remained to carry out research on oligonucleotide synthesis in the laboratory of Professor Colin Reese and earned a Ph.D in 1966. He then studied prebiotic chemistry as a postdoctoral fellow in the laboratory of Professor Leslie Orgel at the Salk Institute for Biological Sciences, San Diego (CA, USA). In 1969 he joined Professor Sydney Brenner's group at the Medical Research Council Laboratory of Molecular Biology, where he began his research on C. elegans. *His research has revealed important information about programmed cell death both in* C. elegans *and in humans. Dr. Sulston was also involved in mapping the genome of* C. elegans *and in the human genome project. In 1986 he was elected to the Royal Society (UK). In 1992 he became Director of the Sanger Centre, Cambridge (UK). In the same year he became an honorary fellow of Pembroke College, Cambridge (UK) and received an honorary ScD from Trinity College, Dublin (Ireland).*

this observation was not actually novel,[13] but it sufficed to get us started in earnest on the cell lineage.

The significance was that although Sydney had wanted from the beginning of the project to follow cell lineages, it was presumed that the most interesting development happened in the egg. So this was the only stage that had been studied, but it was proving very difficult to see anything. This meant, however, that there were already Nomarski differential interference contrast (DIC) microscopes in the laboratory. DIC provides an image of a thin optical section of a specimen, with the rate of variation in refractivity from point to point, in a given direction, represented by intensity. To the observer the effect is as though the refractivity were modelled in relief and then shadowed in the given direction: a powerful input to the eye. Since there is no preparation necessary, the specimen can remain alive. Unlike phase contrast microscopy, the objective and condenser work at full aperture in DIC, so interference from regions above and below the focal plane is minimised.

Thus I started looking at the larvae. At first this seemed even more difficult than the egg, because all attempts to hold the larvae down for viewing killed them or at least stopped them from developing. Then I realised that I shouldn't hold them down but let them go free, crawling between the top of a thin but perfectly flat layer of agar and the cover slip. By scraping a very thin coating of bacteria onto the centre of the cover slip I got them to stay in the area, browsing gently along at a pace that allowed me to watch and draw the cells. Seeing my first cell division was an exciting moment, because it implied that determining the larval lineage was possible (Figure 4).

And indeed so it proved. The first area that I looked at seriously was the ventral nerve cord (Figure 5) because John White was studying it and we were curious to find out how the various classes of cells were derived. John had joined the lab at the same time as me.[14] His initial role was to work on automation of the reconstruction of the neuroanatomy of the worm from serial section electron micrographs skilfully produced by Nichol Thomson (Figure 6). As it turned out, the computers of the time were not powerful enough to allow full automation, but, characteristically undaunted, John recruited Eileen Southgate to assist him and performed the reconstruction in short order by hand.[15]

So, if I could determine the cell lineages, John could correlate the division pattern with the fates of the cells. The answer was immediate: the cells were formed from a stereotyped sublineage
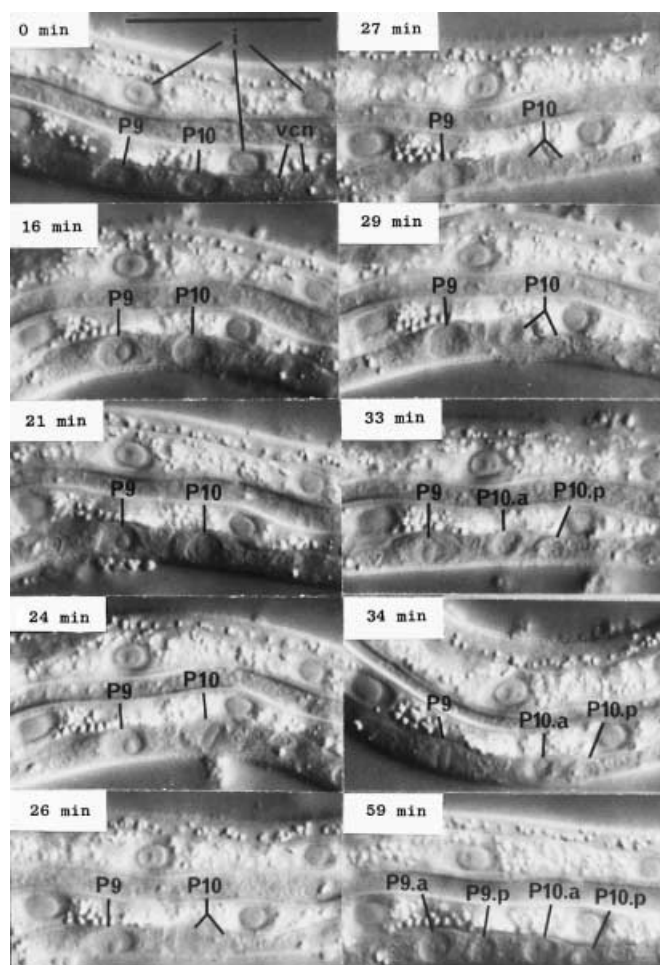
Figure 4. *The first division of two of the ventral cord neuroblasts viewed by Nomarski optics in a living animal (reproduced, with permission from Elsevier Science, from J. E. Sulston, H. R. Horvitz, Dev. Biol. **1977**, 56, 110–156*

Figure 5. *Drawings recording neuroblast divisions over a two-hour period in the anterior ventral cord and retrovesicular ganglion, 1974.*

Figure 6. *Nichol Thomson (left) and John White (right), MRC Laboratory of Molecular Biology.*

repeated along the length of the cord, and each class of neuron arose from a particular branch of the sublineage (Figure 7; ref. [16]).

At the same time, it was obvious that certain cells died and disappeared, in a completely invariant but sex-specific pattern. Such a cell would become more refractive over a period of half an hour, until it resembled a raised disc in the DIC image; then it would rapidly shrink and gradually disappear (Figure 8). Programmed cell death was already known from other systems,[17, 18] but now in the worm it was both visible and predictable.

At this point Bob Horvitz joined the group (Figure 9a). He was accustomed to collecting information in a very different way— through carefully designed biochemistry, radioactive labels and so forth—and was at first bemused by the idea of sitting and watching cells divide.[19] But in a very short time he became a tremendous enthusiast, and it was he who urged that we continue to determine the entire larval lineage of the hermaphrodite.[20] By now Judith Kimble (Figure 9b) was in the group as my postdoctoral researcher, but while in David Hirsh's lab she had already determined the gonadal cell lineages.[21] After Bob returned to the US, I went on to complete the male cell lineage,
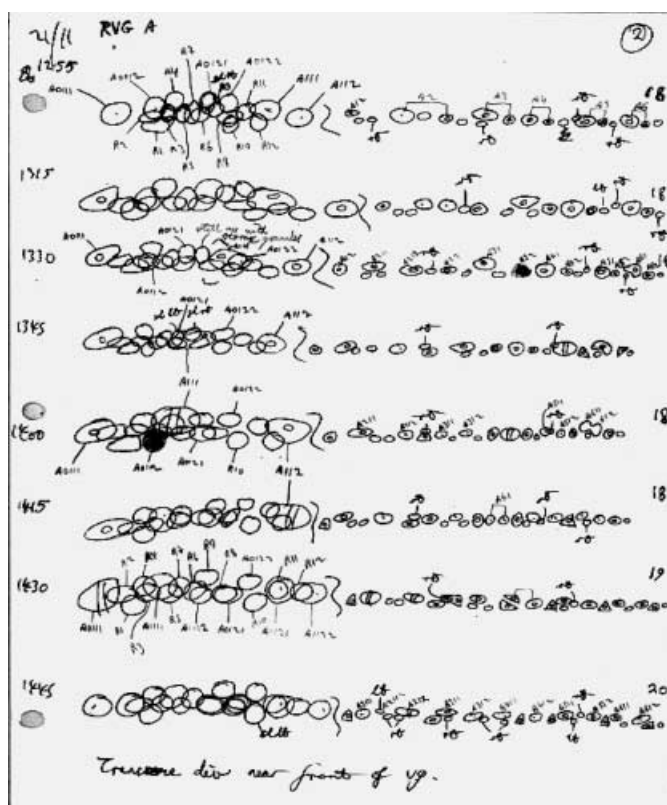
which fed into the neuroanatomy being determined by Donna Albertson (Figure 9c) and the genetics of male determination being studied by Jonathan Hodgkin (Figure 9d).[22, 23] So now we had all the larval lineages worked out.

Cell assignments were a fine thing, but the aim was to find out how they, and the cell divisions that preceded them, worked. A direct approach to learning about interactions between the cells, and about their functions, would be to kill them selectively. John White devised a system with a pulsed laser beam that projected through the objective of the microscope.[24, 25] It worked wonderfully well, because the large aperture of DIC optics meant that the beam reached high intensity only in the cell of interest. In most cases killing a cell simply removed that cell, and any progeny, from the eventual structure of the animal and so
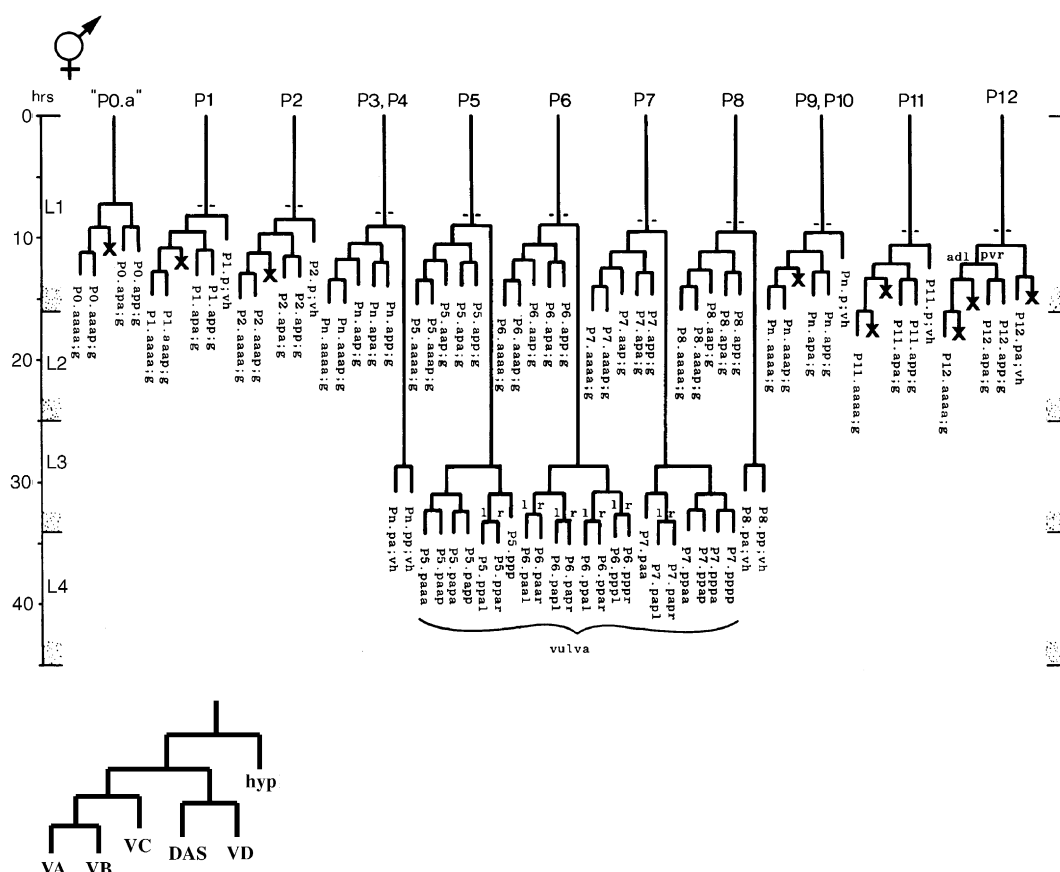
Figure 7. Cell lineages of the ventral cord. Between 6 and 15 h after hatching each blast cell goes through a stereotyped set of divisions (with small variations at the ends of the cord), and in most places the fates of the progeny are correlated with their positions on the lineage as shown in the insert. Variations at the end of the cord include a defined pattern of programmed cell death, shown by Xs in the main picture (reproduced, with permission from Elsevier Science, from J. E. Sulston, H. R. Horvitz, Dev. Biol. **1977**, 56, 110–156.
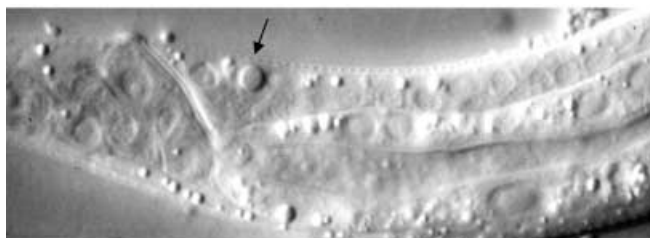

Figure 8. The arrow points to a programmed cell death as it passes through its peak refractivity; posterior end of the cord, Nomarski optics.

allowed us to discern its function. For example, killing the two HSN neurons that lie on either side of the gonad (HSN stands for hermaphrodite specific neuron; later found to undergo programmed cell death in the male) blocked egg laying. But in certain cases a killed cell was replaced by another (Figure 10), and so we were able to map out the regulatory possibilities in development.[26]

Among the cell interactions revealed by the laser experiments were two cases of cell death by murder.[22] In each of the cell pairs concerned, prior removal of the engulfing cell allowed survival of the cell programmed to die. We speculated that the majority of

cell deaths were suicides, that is, that engulfment was not a necessary trigger, and this subsequently proved to be the case.

But of course the real aim was to go further and discover the genes that control the programme. We began to hunt for mutations, but it was not easy to decide what to look for. Nevertheless we gradually accumulated a few mutations that affected the cell lineage, some by analysis of the existing collection, others isolated by predicting phenotypes (for example Bob Horvitz's continued foray into egg-laying defects[27]), and others by looking at specimens taken at random from mutagenised stocks.[28, 29] The animals were examined by DNA staining, FIF and Nomarski optics to determine whether the lineage was altered. The possibilities were endless, but by now the news of the cell lineage had been passed around the community and many groups were engaged in mutation and laser experiments to study different functions and body parts of the worm. Sharing information in this way has always been a feature of the worm community, and was greatly fostered by Bob Edgar's initiative in starting the newsletter known as the Worm Breeders' Gazette (Figure 11). The newsletter continues to appear, but in recent years has been hugely extended by electronic resources.[30]

As for genes involved in programmed cell death, the deoxyribonuclease nuc-1 gene product was known from an early stage as a result of the DNA stain screens, but it was only

Figure 9. *Top left: Bob Horvitz. Top right: Judith Kimble. Bottom left: Donna Albertson. Bottom right: Jonathan Hodgkin. Photographs from the 1970s,* MRC Laboratory of Molecular Biology.
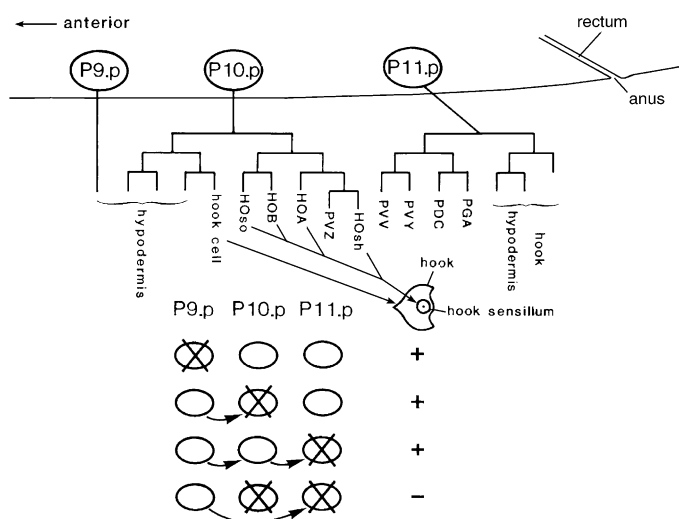

Figure 11. *Bob Edgar (recent photograph) with the first issue of the Worm Breeder's Gazette.*
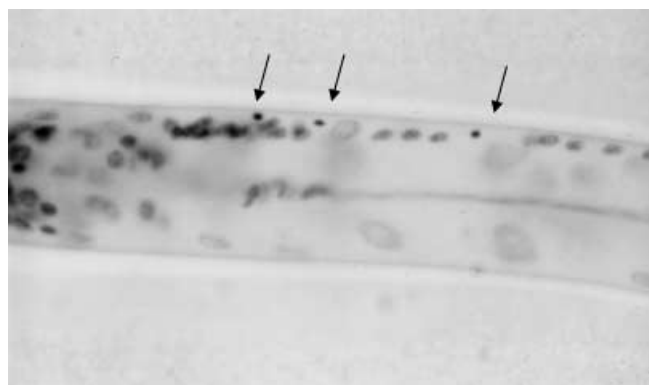
Figure 12. *Three cell deaths (arrows) at the anterior end of the cord, revealed in a* nuc-1 *mutant by persistence of their DNA in small clumps. Feulgen stain.*

Figure 10. *An example of fate regulation in the male tail. In the group of three precursor cells, any one can be killed without causing a phenotypic defect, because of replacement regulation. Killing two cells results in a defect because further proliferation does not occur.*

difficult and screened live mutagenised worms by DIC optics. By this time we knew that the normal fate of a dying cell is to be phagocytosed by its neighbours.[31] Ed found a number of mutations in two genes, *ced-1* and *ced-2*, required for the phagocytosis; with the loss of activity of either gene the dead cells persisted as prominent refractive objects, providing an obvious phenotype (Figure 13; ref. [32]). Those deaths that had

a scavenging enzyme and did not have to do with initiating cell death (Figure 12). The first step into the pathway itself was taken by Ed Hedgecock, who did what the rest of us thought was too
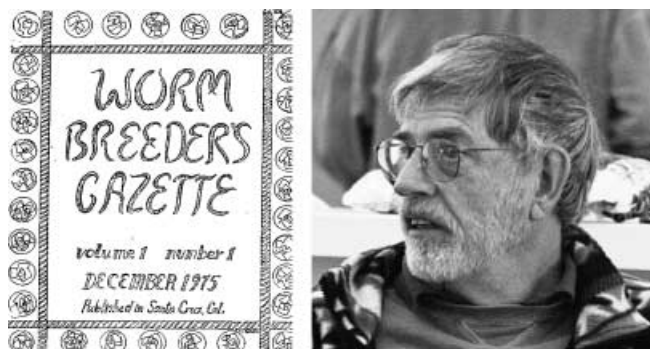

Figure 13. *Ed Hedgecock, MRC Laboratory of Molecular Biology, 1970s, with a* ced-1 *mutant. The arrowhead points to one of several persistent cell corpses visible in the mutant (reproduced, with permission from American Association for the Advancement of Science, from E. M. Hedgecock, J. E. Sulston, J. N. Thomson,* Science **1983**, 220, 1277 – 1279.

previously been identified as murders failed to occur in these mutants, confirming their special character. Subsequently Hilary Ellis used the same procedure of DIC screening to find revertants of CED-1 mutants, and so discovered *ced-3*, the first gene known to actually control cell death.[33] This was the first of many,[34] and that story is taken up in Bob Horvitz's lecture.

One further type of cell death was discovered by Marty Chalfie in the course of his programme on *mec*s (Figure 14). These abnormal deaths are caused by mutations in the *deg* genes that result in overactivity of an ion channel in a certain class of cells.[35] The phenotypes of these mutants are unaffected by loss of *ced-1* and *ced-2*.
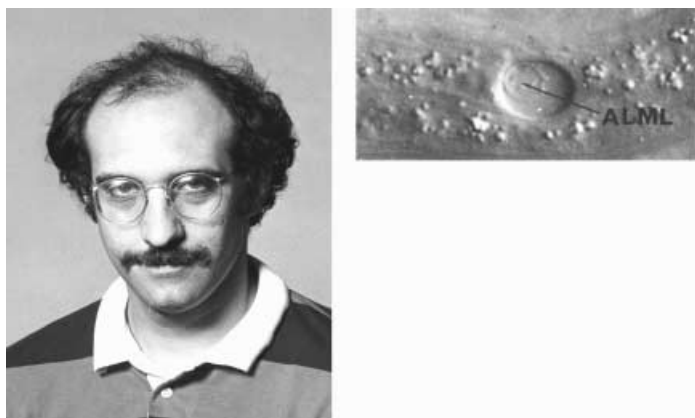

Figure 14. Marty Chalfie, MRC Laboratory of Molecular Biology, 1970s, with a cell dying in a dominant mec-4 *mutant (reproduced, with permission from Elsevier Science, from M. Chalfie, J. E. Sulston,* Dev. Biol. ***1981****, 82, 358 – 370.*

Meanwhile, only limited progress had been made on the egg. A century previously the early lineages had been worked out by observation of fixed and DNA-stained specimens (see, for example, ref. [36]; reviewed in ref. [37]), but now, to go further, living specimens would have to be used. Roger Freedman and Simon Pickvance had tried recording with DIC optics on 16-mm film, as had T. Kaminuma in Tokyo, and G. von Ehrenstein and E. Schierenberg in Göttingen.[38] The cells were visible, but retrieval of the information about cell divisions, especially when the division axis was vertical, proved impossible after the first few
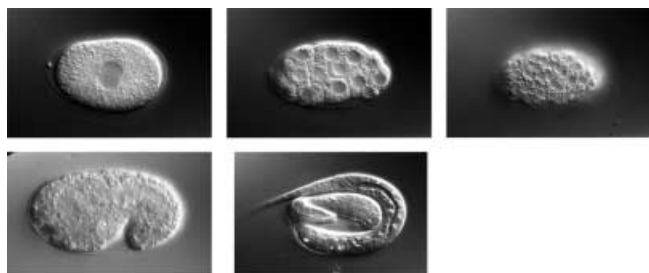

Figure 15. The egg, seen by Nomarski optics, from pronuclear fusion to hatching.

rounds (Figure 15). So, little by little I started looking by eye and drawing as I had for the larvae. At first it was hard, but I had the time to persist, and soon the structures became clearer in my mind. The lineage is almost invariant, so I was able to return to each stage and each group of cells and became familiar with them. One gadget that helped a lot was a traditional cross hair made from gossamer, which provided a point of reference in the image without degrading it at all—much better than an engraved plate. Over the course of a year and a half it was finally done. We had the entire story of the worm's cells from fertilised egg to adult (Figure 16; ref. [39]). Once again, comparison with the anatomy determined from Nichol Thomson's E/M sections was crucial, but between us we were able to assign all the cells to their eventual roles (Figure 17).[40, 41]

Later John White built a device—the 4D microscope—that records on optical discs and allows much better retrieval, and there are now hopes of fully automating the observation of the lineage by means of histones linked to the green fluorescent protein.[42]

At this point, we discussed our findings mainly in observational terms because there was little else we could do. For example, we were intrigued by cells that changed their function during development. Thus the DD motor neurons innervate ventral muscle in the youngest (L1) larva, but then rearrange their connections to innervate dorsal muscle in the next stage (L2), by which time new ventral innervation has been provided from postembryonically generated neurons.[43] In mutants that have no postembryonic cell division, the DDs rearrange regardless, and the mutant becomes abruptly uncoordinated at L2. Clearly this was telling us something about development, but what?

Again, there was always a lot of discussion around the lab about autonomous versus nonautonomous determination of cell fate—rather reminiscent of the politically charged debated about nature-versus-nurture for human development that we hear so much of now. Classical developmental biology, it seemed to me, was too often concerned with setting up an experiment to settle an issue once and for all. I felt that it was no use going on arguing about whether this or that view of development was correct, but that we had to go out and find the genes. It also seemed likely that everyone would in the end be proved correct: life doesn't follow a particular neat set of rules but uses all sorts of mechanisms.

But how to find the genes? Did we need to find all of them or just the subset that would become obvious by mutational analysis, genetics and selective cloning of those parts of the genome thus revealed? Later, as we embarked on the genome map, I was told by one enthusiastic *Drosophila* researcher in the laboratory: "Don't worry about doing this, in five years time it will all be over". He was inspired by the recent discovery of the homeobox (reviewed in ref. [44]), and indeed that was a wonderful breakthrough, but would it be the whole story? I suspected not, that life would be too complicated to solve by classical genetics alone, and that we would indeed have to find all the genes before we were done.

At this point my account diverges from the Nobel citation that the three of us have received. Bob continued along the path that
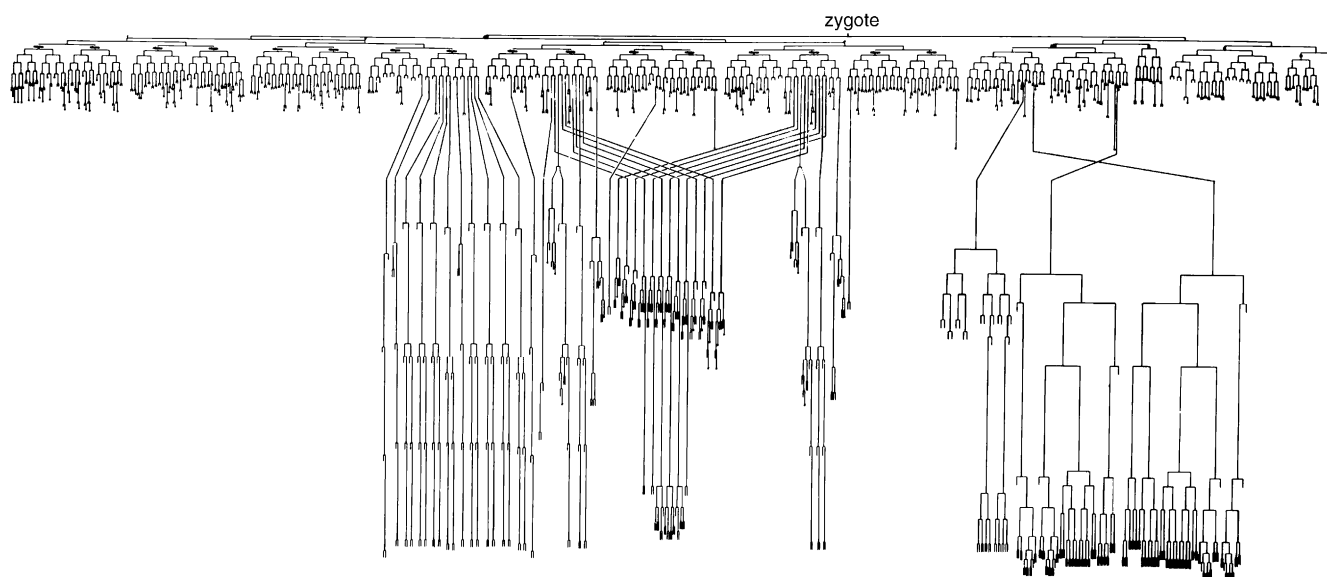
Figure 16. *The complete cell lineage of the hermaphrodite, from fertilised egg to adult.*

PARTS LIST FOR THE NEMATODE *C. ELEGANS*

AB	P₀ a		Embryonic founder cell
ADAL	AB plapaaaapp		Ring interneurons
ADAR	AB prapaaaapp		
ADEL	AB plapaaaapa		Anterior deirids, sensory receptors
ADER	AB prapaaaapa		in lateral alae, contain dopamine
ADEshL	AB arppaaaa		Anterior deirid sheath cells
ADEshR	AB arpppaaa		"
ADEso	H2 aa	L&R	Anterior deirid socket
ADFL	AB alpppppaa		Amphid neurons, dual ciliated sensory
ADFR	AB praaappaa		endings, probably chemosensory, enter
			ring via commissure from ventral ganglion,
			take up FITC
ADLL	AB alpppppaad		Amphid neurons, dual ciliated sensory
ADLR	AB praaapaad		endings, probably chemosensory, project
			directly to ring, take up FITC
AFDL	AB alpppapav		Amphid finger cells, associated with
AFDR	AB praaaapav		amphid sheath
AIAL	AB plppaappa		Amphid interneurons
AIAR	AB prppaappa		"
AIBL	AB plaapappa		Amphid interneurons
AIBR	AB praap...		"

Figure 17. *The beginning of the complete list of cells, as it appeared in the first worm book (reproduced, with permission from Cold Spring Harbor Laboratory Press, from W. B. Wood,* Embryology in *The Nematode Caenorhabditis elegans (Ed.: W. B. Wood and the community of C. elegans researchers),* **1988,** *pp. 215–241; permission conveyed through Copyright Clearance Center, Inc.*

brought us all to Stockholm, whilst I began a different though parallel project. My excuse for including a brief description of it here is: first that it has contributed to the scientific success of the cited enterprise, and second that it led to some philosophical conclusions on which I would like to end.

As the lineage work came to an end, I was uncertain what to do next. Many people thought that, given my knowledge of the egg, I should analyse embryonic mutants. But there were already several groups engaged in this work (reviewed in refs. [45–47]), and I didn't think I could add much to their efforts. But also there was another problem: there were already too many mutants available for the rate at which the genes could be isolated by the relatively new art of molecular cloning. Finding them in the 100-million base pair genome was immensely time consuming. When Bob Horvitz was leaving for MIT I asked him what he planned to do about it. "Heavy duty molecular biology" was his pained but realistic reply. It niggled me that each person should be wasting so much time on this business with gels and filters, groping around in the immensity of the genome for the few thousand bases of interest to them, and my thoughts came to a head during a seminar by Matt Scott about his heroic mapping of the *antennapaedia* region in *Drosophila*. Surely it should be possible to do it all in parallel for much less proportionate effort? Total sequencing was at that time impossible to contemplate, but large-scale mapping seemed feasible.

I came back full of enthusiasm for this concept, started to learn from Jon Karn the molecular skills that I'd missed while closeted

with the microscope, and discussed approaches to character-ising the clones with him and Sydney. I started making clone libraries in λ. The crucial moment was the decision by Alan Coulson to join me, following the retirement of his boss Fred Sanger. We switched from λ clones to cosmids, and Alan got the fingerprinting to work efficiently enough to put through thousands of clones. Rodger Staden started us off with a simple software package for manual reading of the gels and matching, but we soon exceeded its capacity. So I learned to write Fortran programmes and developed an electronic assembly system. Frank Mallett and the workshop built a scanner for the films and we wrote a semi-automatic package to read out the positions of the bands.

It was going well, and we had a map covering the genome in a few hundred segments, but the remaining gaps were proving intractable.[48] Fortunately Bob Waterston visited on sabbatical at the very time that David Burke and Georges Carle were developing the yeast artificial chromosome (YAC) cloning system in Maynard Olson's laboratory.[49] Bob speculated that YACs might clone segments that cosmids could not. He returned home and quickly constructed the first YAC library for the worm, and we saw that they filled the gaps. Yuji Kohara came on sabbatical, and that summer we all worked on the long series of hybridisations that knitted the map together at last.[50] And Bob joined Alan and me in what has become a 20-year collaboration (Figure 18).
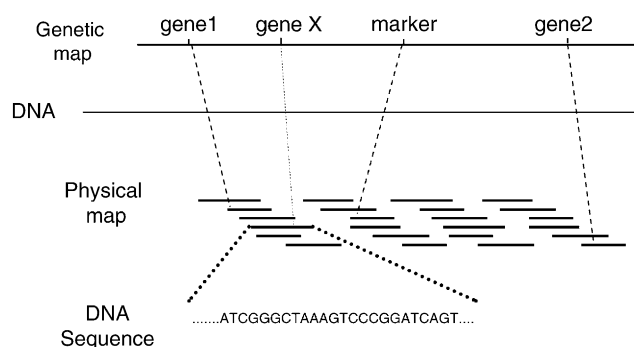

Figure 19. The principles of a genome map. The intractably long DNA molecule of the genome is converted into an overlapping series of clones, immortalised in frozen bacteria or yeast cells and issued to researchers when needed. To maximise benefit from this physical map it is essential to accumulate publicly information from previous gene locations, so that the hunter of gene X knows where to look from using the aligned genetic map as a guide.

Figure 18. The architects of the genome map and sequence. From left to right: Bob Waterson, Alan Coulson, Fred Sanger.

The map was by now proving its worth in gene finding, and was the most complete for any complex genome. Andy Fire's introduction of efficient transformation meant that the cosmids could be tested directly for rescue of mutants, simply by injecting them in turn into the gonad.[51] One important thing we were learning was to ensure that the information remained open. This was standard practice in the worm community but was especially important for the genome project. The project would only be useful if everyone cooperated in placing their genetic data on the physical map, so that the genetic and physical maps would become more and more closely aligned. Only thus would gene hunting actually be facilitated (Figure 19). So this was not a matter of open data for its own sake, but was simply the way to make the map useful,

andto that end we devised simple rules so that researchers could contribute and yet still be credited for their individual accomplishments.

So when our two laboratories moved on to sequencing—first the worm[52] and then joining in with the much larger group for the human genome[53]—it was natural for us to bring the principle of free data release along. It was the pragmatic way to proceed in what was recognised as the growing field of genomics, and in due course it became formalised in the Bermuda statement: the result of a meeting of the major human sequencing groups at Bermuda in 1996.

The human genome project was well on course for finishing in 2005, or very likely earlier, given the Wellcome Trust's commitment to it in the UK. In 1998 the worm sequence was published, with some gaps but essentially complete, the first animal sequence to be brought to this level. But also in 1998 came a claim that a private enterprise would sequence the human faster and cheaper, outrun the public consortium, and release the data. Apart from technical doubts, we thought it financially implausible that an industrial sponsor would release data freely. As it turned out, we were correct, but there followed a hectic three years of science politics to ensure that the human genome remained in the public domain.

That's now over, mercifully. But, in closing, I'd like to highlight the key reason for making a fuss about free release of genome data. The fact is that proprietary databases don't work for such basic and broadly needed information as the sequence of the human genome (Figure 20). Not only do such databases create a class distinction between rich and poor researchers, but worse
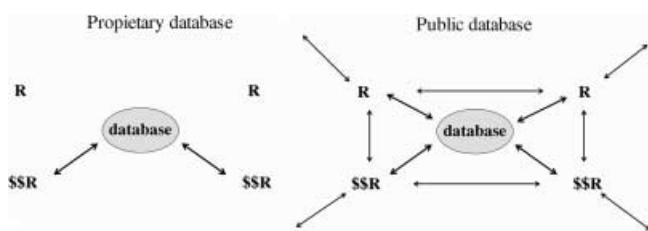
Figure 20. *Contrast in communications for users of proprietary and public databases. A proprietary database can be accessed only by fee-paying researchers ($$R), who must agree not to redistribute data in order to protect its confidentiality. A public database can be accessed by all, and everyone can communicate freely with one another.*

they inhibit communication between researchers in general. In order to protect the market value of a proprietary database, the owner must prohibit redistribution of the contents—otherwise the information would quickly leak out and be widely known. But often—and bioinformatics is a case in point—it's not possible to disseminate results without disseminating the underlying data at the same time. Attempts to get around this lead to fearful tangles and it's far better to fund and distribute the information in the public domain from the start.

Additionally, this dispute was a microcosm of the debate about how scientific research should be financed. At the moment more and more funding is coming with profitable applications in mind. Whilst worthy in themselves, applications shouldn't be the only way to drive basic research. Short-term goals are too limited to control exploration of the natural world, and anyway not everything we discover should be exploited. Furthermore, many of the most important potential applications—for example, the neglected diseases such as tuberculosis and malaria, found mainly in the poorer parts of the world—cannot be researched through funding for profit: there is no market to repay the investment.[54] It seems obvious that we have to revert to a greater measure of public funding and stop imagining that somehow the free market will solve everything on its own.

This lecture has taken a meandering route and ended up far from its starting point. To loop back, I think the important purpose of science is to explore, discover and understand. I'm glad if I've been able to contribute a little to that process, and hugely grateful to all my colleagues, both here and elsewhere, for their achievements and for the fun I've had. I hope that we can apply our ever-increasing knowledge wisely, for the good of all.

[1] H. P. M. Fromageot, C. B. Reese, J. E. Sulston, *Tetrahedron* **1968**, *24*, 3533 – 3540.

[2] J. E. Sulston, R. Lohrmann, L. E. Orgel, H. T. Miles, *Proc. Nat. Acad. Sci. U.S.A.* **1968**, *60*, 409 – 415.

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