From Neuroblastoma to Homeobox Genes, 1976-1992

Marshall W. Nirenberg is best known for his work on deciphering the genetic code by discovering the unique code words for the twenty major amino acids that make-up DNA, for which he won the Nobel Prize in Medicine or Physiology in 1968.

Nirenberg was the first government scientist to win the Nobel Prize. The National Library of Medicine and the Office of NIH History has amassed a collection of correspondence, laboratory administrative and research materials, and publications that documents Nirenberg's career as a researcher in biochemical genetics at the National Institutes of Health.

Dr. Nirenberg is featured in The Profiles in Science web site of the National Library of Medicine celebrates twentieth-century leaders in biomedical research and public health. Students appreciate the history, and share some of the excitement of early scientific discoveries in molecular biology. The National Library of Medicine is digitizing and making available over the World Wide Web a selection of the Marshall W. Nirenberg Papers, for use by educators and researchers.

In 2007, the Archives and Modern Manuscripts Program, History of Medicine Division completed a Finding Aid to the Marshall W. Nirenberg Papers, 1937-2003 (bulk 1957-1997). Individuals interested in conducting research in the Marshall W. Nirenberg Papers are invited to contact the National Library of Medicine.

The NLM digital materials and references provide the background for the series of six interviews conducted with Marshall W. Nirenberg, Ph.D., by Ruth Roy Harris, Ph.D., between September 20, 1995 and January 24, 1996.

The "Harris Interviews" took place in Nirenberg's laboratory on the campus of the National Institutes of Health (NIH) in Bethesda, Maryland. Harris also conducted several supplemental interviews, both by telephone and in person, with individuals either involved in the breaking of the genetic code or personally acquainted with Nirenberg: James Pittman, Joan Geiger, Philip Leder, Thomas Caskey, Sidney Udenfriend, and Perola Nirenberg. Interviews with Pittman and Geiger are now in the Marshall Nirenberg Collection at the National Library of Medicine (NLM). Notes from other interviews are held at the Office of NIH History.

A number of individuals and institutions worked on editing the interviews for clarity and content: Sarah Leavitt, Victoria Harden, Caroline Hannaway, Alan Schechter, Robert Balaban, and Alan Peterkofsky. Caroline Leake, Katrina Blair, and Mary Alvarez provided administrative and technical assistance. In 2008, Deborah Kraut edited and formatted the interviews to correspond to the NLM digital materials.

Each Section begins with the NLM digital summaries summaries and references. Additional references, when appropriate are added:

From NLM Profiles in Science:

http://profiles.nlm.nih.gov/JJ/Views/Exhibit/narrative/homeobox.html

With the success of his neuroblastoma research, Nirenberg became firmly established as a leader in the field of neurobiology during the 1970s and 1980s. He participated in major international conferences and symposia and received countless requests for advice and access to his patented cell lines from researchers at prominent universities worldwide. Nirenberg also received numerous letters from cancer patients, medical schools, and hospitals, suggesting that his work became a symbol of widespread hope that the disease could be fought on the frontlines of science. In addition to awards of merit from the National Institutes of Health and honorary degrees in science from various universities, Nirenberg also gained recognition for his contributions to cancer research and received honorary doctorates from schools of public health.

Nirenberg's research using neuroblastoma and embryonic cells brought the promise of many biomedical applications. Children's hospitals, for example, used Nirenberg's tissue cultures to study neuromuscular connections, hormonal regulation, and neuronal growth. The cultures of identical cloned cells provided an experimental alternative to the complex mixture of cell types found in the normal nervous system. The cell lines were sensitive to environmental conditions so that researchers could study the impact of various factors on cell development. As early as 1975, Nirenberg and his team used these cells to develop a technique for diagnosing and analyzing neuromuscular disorders by defining what conditions were necessary for the transmission of information from cell to cell.

In addition to neuroblastoma research, Nirenberg established a project to study the formation of neural synapses in the retinas of chickens. In the 1976 article, "Localization of Acetylcholine Receptors during Synaptogenesis in Retina," Nirenberg found that retina cells could be dissociated (separated), then reassociated, and still produce synapses. Normal neurons are nondividing and cannot produce synapses after being dissociated. Like neuroblastoma cells, retina cells provided an important model for explaining the process of synapse formation. Working with chick retina also offered a chance to apply in a new medium the knowledge he gained from working with neuroblastoma cells.

As Nirenberg's research on neuroblastoma and chick retina developed in the mid-1970s, he became interested in a different kind of problem. Intrigued by a theory developed in the 1960s by the grandfather of neurobiology, Roger Sperry, Nirenberg embarked on a new project at the Biochemical Genetics laboratory. Sperry predicted that scientists could determine the molecular basis for the location of each cell in the retina. In order to transmit a cohesive picture of the outside world it seemed clear that the cells making up the neural pathways were somehow directed to end up in a specific location. Nirenberg wanted to explain the molecular basis of this precise "molecular address" for retinal cells.

Using chick retina, Nirenberg developed a way to test Sperry's prediction that there is a molecular topographic map that exists in the retina. Nirenberg used genetically identical proteins that were cloned in the laboratory, called monoclonal antibodies, that bind to foreign molecules to fight off infection. By exposing monoclonal antibodies to antigens from different

parts of the retina, he showed that the antibody recognized a specific antigen molecule distributed in a unique pattern across the retina. This validated Sperry's prediction by demonstrating that proteins are concentrated in specific areas. These proteins are instrumental in directing cellular development in particular locations with great precision. Nirenberg published his findings in a 1981 article in the Proceedings of the National Academy of Sciences, "A Topographic Gradient of Molecules in Retina Can be Used to Identify Neuron Position." He subsequently purified the protein molecule that the antibody recognized and published the results in the 1986 essay, "Purification of a Membrane Protein Distributed in a Topographic Gradient in Chicken Retina."

While this represented an important advance in neurobiology, Nirenberg's next major research success would stem from failure. As Nirenberg explained, "we failed to clone DNA for this protein [the antigen that he purified from chick retina] and that was an important question because if we could have cloned it we could have identified the amino acid sequence of the protein. It would have given us a tool to use, a very important tool, for further studies. For some reason we were not successful in cloning it." In 1987, researchers discovered proteins in the embryo of Drosophila melanogaster, the common fruit fly, which were similar in distribution to the proteins Nirenberg mapped in chick retina. As one of the most valuable and studied organisms in the history of biological research, Drosophila presented Nirenberg with an opportunity to tie up the loose ends of his chick retina work. Nirenberg remembered, "I thought that to really understand this problem you have to go to a simpler system where you have genetics you can use. You can use genetics as a tool. Drosophila has been studied for a hundred years almost, ninety years, and there is a tremendous amount of genetic information that is known and wonderful genetic tools that can be used with Drosophila. And that's the reason I switched."

Annual reports from the Laboratory of Biochemical Genetics show that even before Nirenberg began his Drosophila work the focus of his research had gradually shifted in the 1980s toward genetic explanation for nervous system development. His work with neuroblastoma and chick retina capitalized on new laboratory tools made available by the accelerated pace of genetic technologies. In 1983, for example, Kary B. Mullis, a scientist with the Cetus Corporation in California, developed a technique that revolutionized the work of molecular biologists. Mullis developed a procedure for amplifying DNA, the polymerase chain reaction (PCR), which made it possible to read the sequence of virtually any DNA fragment. PCR has been used to detect DNA sequences, diagnose genetic diseases, carry out DNA fingerprinting, detect bacteria and viruses, and research human evolution. It even has been used to clone the DNA of an Egyptian mummy! By 1985, Nirenberg's Biochemical Genetics laboratory utilized these new techniques to develop massive collections of DNA nucleotide sequences known as DNA libraries. This allowed Nirenberg to compare the genetic composition of human cells to those of other animals. His laboratory notebooks show that this information also allowed him to begin working out the relationship between gene sequences, viruses, and neurological disease.

Nirenberg's move toward genetic explanations for nervous system function and development coincided with a growing atmosphere of excitement that pervaded the biomedical research community in the 1980s. A series of experiments demonstrated that cancer, whatever its ultimate cause, was the result of activating a family of genes called oncogenes. These genes, involved in the control of cell division, could cause cancer when modified or "over-expressed."

Early reports suggested that over-expressed oncogenes were "switched on" by biological or environmental forces, causing them to synthesize more RNA from a sequence of DNA than they normally would. This hypothesis paralleled conclusions drawn from Nirenberg's neurobiology research. Key articles such as "Synapse Formation by Neuroblastoma Hybrid Cells," published in 1983, showed that biological and environmental factors can both influence gene expression in the nervous system. Cancer researchers also believed oncogenes might control the action of other genes in a cascade until the cancer was finally initiated. This closely reflected the role of a new group of genes that caught Nirenberg's attention. Homeobox genes, discovered by Walter Gehring of the University of Basel in 1983, influence the expression of other genes important in physical development. As gene regulators, they recognize the sequences in DNA that turn genes on or off. For Nirenberg, research on homeobox genes offered a forum for answering one of the questions remaining in his work on nervous system growth: what was the relationship between genes themselves and the development of the nervous system as a whole?

After reading a paper by Michael Levine of Oxford University in 1987, Nirenberg found the perfect opportunity to bring his experience in genetics and neurobiology together. Since homeobox genes influence the process by which hereditary information is converted into physical characteristics during development, Nirenberg recognized that understanding their function could provide new avenues for research. He recalled that, "Levine found a homeobox protein that was distributed quite remarkably in some neurons in the developing embryo and not in other neurons... [Homeobox genes are] an important class of genes and to find them quite specifically distributed in specific sets of neurons was quite a remarkable observation. At that time, there were seventeen homeobox genes that were known, that had been found in Drosophila, and it was a burgeoning field of study." The relationship between homeobox genes and neural development presented an ideal opportunity for new discoveries.

Nirenberg would have to face the same risks involved with his earlier transition from genetics to neurobiology. Once again he had to branch out into unfamiliar territory. Nirenberg explained, "I had never worked with Drosophila before, but when Yongsok Kim came to my lab as a postdoctoral fellow immediately after he got his Ph.D. in Korea, I suggested to him that we look for new homeobox genes in Drosophila." By comparing the base sequences of genes with the seventeen homeobox genes already known, Kim soon discovered four new homeobox genes which he named NK-1, NK-2, NK-3, and NK-4.

In the annual report of the Laboratory of Biochemical Genetics for 1987-88, Nirenberg revealed the importance of this work. For Nirenberg, the genes provided an "experimental system" that could be used to define the relationship between specific genes and the physical development of an organism--the hope being that lessons learned from Drosophila could be applied to humans. Discovering the sequence of nucleotides in DNA allowed for the possibility of genetic therapy for diseases. A handwritten draft for a research project report from 1992 reveals that studies on the NK-2 homeobox genes enabled his team "to predict with a high degree of certainty" the relationship between genetic instructions and the development of part of the central nervous system of Drosophila. Since one of the "major goals in neurobiology" was to "understand the developmental program for the assembly of the nervous system," identifying the homeobox genes and defining their developmental role was an important advance. Nirenberg predicted that a "similar but slightly modified strategy" could be used to explain the assembly of the human nervous system.

Following the mapping of the entire genetic sequence of Drosophila in 2000, Roger Hoskins, of the Berkeley Drosophila Genome Project, confirmed Nirenberg's optimism by revealing that in a set of 289 human genes implicated in diseases, 177 are closely similar to fruit fly genes. Knowledge of homeobox genes brings the promise of understanding their role in development and may eventually prove to be beneficial in combating cancers, neurological diseases, and metabolic and immune-system disorders. At present, Dr. Nirenberg continues this project by using advanced digital scanning technology to study the genetic development of neural networks in the brains of Drosophila embryos.

The Harris Interviews – 1995 – 1996

Marshall Nirenberg (MN): When homeobox genes were discovered in the late 1980s, an explosion of information was produced by others on homeobox genes and on positional molecules in Drosophila, fruit flies. A homeobox gene is any gene in a group with a function to divide the early embryo into bands of cells with the potential to become specific organs or tissues. I thought that if I ever wanted to understand homeobox genes, I would have to switch to something like Drosophila where one could use genetics as a tool to explore function. Homeobox genes are very well and prominently expressed in the nervous system. In Drosophila, specific neurons have in some cases a specific species of homeobox genes that are expressed. For instance, some neurons express even skipped, whereas neighboring neurons do not. Only a certain number, like eight neurons per hemi-segment, express the homeobox gene even skipped.

Yongsok Kim and I decided to look for novel homeobox genes in Drosophila embryos. We studied NK-1, and we found four novel homeobox genes, NK-1 and NK-2, and they are expressed in the nervous system. When Yongsok Kim came to the lab, I had obtained a genomic DNA library from Drosophila. The homeobox, the homeodomain, is the

portion of the homeobox protein that folds in a helix conformation and is the portion of the protein that actually recognizes nucleotide sequence in DNA. So the conformation of this region of the protein is highly conserved. We used that known fact. We synthesized oligonucleotide probes to the most conserved regions of the homeobox and used that to screen the genomic DNA library. Yongsok Kim rapidly found four novel homeobox genes for Drosophila, and we also found novel homeobox genes for the mouse as well. We did a considerable amount of work on that and are working on that today.

Ruth Harris (RH): Can this be applied to humans?

MN: In human beings, the homeodomains, the amino acid sequence, 60 amino acid residues in the homeodomain, are highly conserved. We have cloned, for example, Drosophila and then looked for a mammalian counterpart, a mouse counterpart, and found it. They are very highly conserved during evolution and about two or three of the amino acids have been replaced out of the 60 from Drosophila to mammals. They are extremely highly conserved. Yongsok found four novel homeobox genes.

At the time that we did this, it took considerable courage, I must say, because this was an extraordinarily active field of research. There were 17 different homeobox genes that had been found from Drosophila studies, an outpouring of information.

The thing that really set up the field of Drosophila was that Drosophila has had almost a hundred years of intensive work by geneticists.

When Christiane Nüsslein-Volhard, an investigator from one of the Max Planck Institutes in Germany, and her colleagues in the early 1980s decided to ask the question, "Can they identify genes that are needed to form patterns, macroscopic patterns, in Drosophila larvae?" that proved to be a gold mine. In fact, this past year she and her colleagues won the Nobel Prize in Physiology or Medicine for their magnificent work.[1]

What they did was to find about 150 genes that are involved in forming patterns in the organism, and many of those genes now have been cloned. She made mutants available to the whole community. Perhaps 50 percent or two-thirds have been cloned, and most of them are gene regulators, including many homeobox genes. All of this work is very important for understanding embryonic development. It permits us to understand how patterns of tissue development can occur, such as additive segmentation.

What they used as markers were like little hairs on the belly of the larvae, which are maggots. In the last couple of years one begins to understand at the molecular level how patterns of gene expression are produced, like vertical rings going almost completely around the larvae, perhaps one or two cells in width. Or stripes going horizontally? How do you form something like that? It is really extraordinary. You have to have a repressor that represses the formation on the leading edge of this vertical stripe. The next cell expresses a protein that turns off the expression of this gene. Then, for the cells that express it, you have to have an activator for that gene that is expressed. On the other side, on the trailing edge of the thing, you have a different species of repressor that represses.

You get a nice tight line, a single line of cells or two cells in width that will express that gene. It's remarkable. You can get extraordinary patterns, and you can determine how the patterns are formed in molecular terms. Tremendously exciting information has come out of this research. Not only that, but the molecular basis for establishing the anterior-posterior axis of the embryo and the dorsal-ventral axis of the embryo is extremely important. It is beautiful work. She and her colleagues absolutely deserved the Nobel Prize.

It was clear that homeobox genes really were needed. I thought they were perfect tools for using to establish patterns of neurons, synaptic connections in neurons, and it was clear that they were needed to form synaptic connections of some kind, or at least the patterns of neurites. I felt that we could not go wrong if we studied homeobox genes, gene regulators, because they would be important for the development of the nervous system. But it was such an intensively studied field that the biggest question in my mind was, “Am I going into too highly a developed field and is the competition going to be so intense that maybe most of it is over. Is it too late to get into the field?” We jumped in anyway, and we found four new homeobox genes very rapidly. Now there are at least 70 species of homeobox genes that have been found in Drosophila and perhaps more. They all have different and important functions.

We called them NK-1 through NK-4. NK-2 proved to be extremely interesting because it was expressed in part of the central nervous system at a very early stage, just when the central nervous system was turned on. Now we know that NK-2 is the first step in neural

development for part of the nervous system. It turns on what are called proneural genes, genes that make certain helix proteins, gene regulators that are required to form little sets of equivalent cells of neuroectodermal cells that can become neuroblasts. From the set of equivalent neuroectodermal cells certain cells are selected that actually develop as neuroblasts. In the rest of the neuroectodermal cells the neural program is turned off. There is a real selection mechanism.

This has led to an extraordinary opportunity to study the early steps in the development of the nervous system. What I think is going on is that this is a mechanism for selecting cells that express the correct set of genes for gene regulators. In other words, we are selecting for cell type. We select based on the amount of gene regulators of different species—kinds of gene regulators—that are expressed by the cells, and I think that NK-2 plays a vital role in the selection process. I think that that is a very important concept that has not been explored yet and that we hope to explore.

RH: And you think that this might be applicable to mammals.

MN: Absolutely. No question in my mind. In the mouse there have been NK-2-like genes that have been found now. There is a family of NK-2-like genes, and it is known that in one case where a gene has been knocked out in the mouse, a mutation which knocks out that gene results in embryos that lack a certain portion of the nervous system. In another case one of the NK-2-like homologs is required, and if you knock out that gene, the embryos that develop lack ventricles of the heart. The entire ventricle is gone,

so it is required for the ventricle formation. They do very important things. As I say, there are five genes, and they have different distributions. They are predominantly expressed in the early nervous system. They are going to be responsible for different parts of the nervous system, just like they are in Drosophila, where they are responsible for a subset of medial neuroblasts that give rise to a subset of the neurons in the central nervous system.

RH: In looking back over almost three decades of research in the neurosciences, what do you think is the most important work that you have done in the homeobox field?

MN: We are relative newcomers to the homeobox field and to the field of Drosophila research, but I think that it is remarkable that we have discovered a gene regulator that turns on neural development in part of the nervous system of Drosophila. It is an extraordinary stroke of luck to find something like this, and it gives insight into the early events that are involved in neural differentiation. There is much that remains to be proven though. We have a great opportunity to explore this problem further.

I think that the regulation of NK-2 is and will be extremely complex. If you think of it, if you progress from anterior to posterior part of the embryo, you are starting with about 40 cells, and ultimately you have 100 cells in length. Virtually every cell is a different cell type as you go along the anterior-posterior axis of the embryo. In the neuroectoderm if you have little patches of cells that express NK-2, you initiate the neuroectodermal pathway of development, and you have bifurcation. They can either develop as

neuroblasts, or they can turn off the neural pathway of development and develop as epidermoblasts and become part of the epidermal covering of the embryo.

I think that in this selection the initial fate of the cell is determined by the position of the cells in the embryo. I think, too, that there is a mechanism available, which automatically selects the cell that expresses the right set of gene regulators to make a neuron, to make a neuroblast. In other words, you are selecting the right cells, to develop, to segregate, as neuroblasts. You are selecting for cell type. You are selecting for the internal molecular address of the cells. You are selecting for a subset of gene regulators that are the right gene regulators and also to activate NK-2 and proneural genes and also gene regulations needed to repress genes whose expressions are required to make epidermoblasts. First, you have concentration gradients in the anterior-posterior, ventral to dorsal direction, of gene regulators that determine the axis of the embryo. These, in turn, induce other gene regulators so that the set of gene regulators that are expressed is determined by the relative position of the cell in the embryo and thereby the concentrations of gene regulators to which the cells were initially exposed. It is such a simple mechanism for doing something so complicated like making a nervous system. It gives me goose bumps even to think about it! It is so beautiful.

I haven't published this yet, but I intend to. I think it is a very important idea. I think that what you end up with is a monolayer of something like 900 neuroblasts, about 450 neuroblasts on each side, and each neuroblast, almost every neuroblast, is a different cell type.

Generally, you repeat a similar pattern as you proceed from one segment to another segment. But I think that in most cases probably there are segment-specific gene regulators that are expressed so that, in effect, for almost every neuroblast that's laid down there is probably a unique cell type.

What I think is happening is that this selection process starts at the very beginning, when you generate gradients of gene regulators in opposite directions, anterior-posterior, posterior-anterior, ventral to dorsal, depending upon the position of a nucleus in the embryo. The nucleus at position A is exposed to a different level of the three primary gene regulators from, for example, say, a nucleus at position B, at two different sites. This initiates complicated induction and repression of other gene regulators. It’s a rapidly changing, dynamic state of affairs in which the net effect of this is to generate different cell types based on the position of the cells in the embryo relative to other cells.

So then you have the stripes of cells that are neuroectodermal cells that differentiate neuroectoderm. They have two possible fates: they can become neuroblasts, segregate to form a layer just inside of the epidermis, or the neural program can be turned off, and they can differentiate as epidermal cells. I think only about a quarter of the neuroectodermal cells actually segregate as neuroblasts. Seventy-five percent of the cells are turned off with respect to the neural program at differentiation and you turn on the epidermal program, and they become epidermoblasts.

I think that the selection process that I am referring to here is a means of selecting cells that express a particular gene, or set of genes, to the greatest extent because, if we are talking about the medial neuroectodermal cells, they express NK-2. NK-2 turns on proneural genes—gene regulators, lethal SKUT and AKIT—and for each little proneural gene expression one cell—in some cases it may be more than one cell—is selected to segregate as a neuroblast.

The rule is the first neuroblast down segregates, and then it remains in contact with the neuroectodermal cells in the surround. It inhibits those cells that the nerve last contacts directly by cell-cell contact. It inhibits them from developing as neuroblasts, turns off the neural pathway of development, turns on the neuroectodermal pathway of development. So the selection is to segregate first as a neuroblast. What is being selected for, I think, is the cell that has the appropriate set of gene regulators to express, for example, the most NK-2, the most proneural gene.

Adil Nazarali worked on some mammalian homeobox genes. We discovered a novel mammalian homeobox gene, HOX1.11, now called HOXA-2. The nomenclature has changed to simplify it. In Drosophila there is one cluster. One of the major problems in the homeobox genes is that there is a cluster of multiple homeobox genes in Drosophila, about 9 or 11 homeobox genes, in a relatively small region of DNA. In mammals you find this same cluster repeated four times in four different chromosomes, and the sequence of the genes has been conserved throughout evolution. Nobody understands why.[2]

First of all, the sequence of the genes has been conserved, and available evidence suggests that the genes are turned on sequentially. Not only is the sequence of the homeodomain highly conserved. Now if you compare the most closely related genes, not neighboring genes in a cluster, but the same gene in different clusters, in one of the four different clusters, they are highly related to one another. We discovered one of the genes in one of the clusters, cluster one.

We have done a lot of work with mouse homeobox genes, too. Alessandra Rovescalli from Italy has discovered six novel mouse homeobox genes that we are characterizing at the present time and trying to sequence, to clone the cDNA and genomic DNA. We are trying to find out where they are distributed, when they are expressed during development, and where they are expressed. We would like to do gene knock-out experiments to make targeted mutations, to make transgenic mice that lack those genes.

We also are using the enhancer trap method. Hsi Ping Li and his wife from Beijing have made hundreds of transgenic lines of flies by transposition of DNA using P element transposition with a marker, the β-galactosidase enzyme, that is linked to the P element so that you can stain for β-galactosidase and look at the distribution of gene expression at all stages of embryonic development. He has found some clones that are expressed with remarkable patterns in the nervous system. He has cloned some of the genes. We have found a Drosophila gene, for example, for a relatively abundant chromosomal protein that

is first ubiquitously expressed but then is found expressed only in the nervous system, both central and peripheral nervous systems, later in embryonic development.

Another gene he has found is a novel kinesin gene, which is a molecular motor that is expressed specifically in a subset of neurons in the central nervous system. It probably is involved with the transport of specific organelles like synaptic vesicles or some other organelles that are specifically of neural function from one part of the cell to another. He is trying to characterize these. He has also discovered a novel zinc finger gene regulator. This is a gene that encodes a protein that binds to DNA and regulates other genes. It is expressed only in a subset of neurons in the nervous system. That is a gold mine for work.

Kohzo Nakayama and his wife, Noriko, sequenced the NK-2 genomic DNA and cDNA when they came from Japan.[3]

Sada Asoh from Japan, Whaseon Kwon from Korea, and Mary Maral Mouradian from Lebanon also worked in my lab. Wah Kwon was another graduate student who got her Ph.D. from the University of Maryland, and she collaborated with Sada Asoh on a method for selecting DNA fragments that have functional enhancement sequences. They devised a method for selecting DNA fragments, such as mouse genomic DNA fragments, that had functional enhancer sequences, or promoter sequences. The idea behind this is that Polyoma viral DNA—this is a virus that causes tumors in hamsters—is a small circular DNA. This virus contains only 2 genes and lots of regulatory DNA and it is

known that enhancers are necessary for its expression. Enhancers are sequences that bind certain proteins that activate gene expression or repress gene expression, that is, regulate gene expression. It was found that some enhancers that normally are thought to regulate messenger RNA synthesis are required for the synthesis of viral DNA; in other words, for DNA replication.

What we did was to excise that region of the Polyoma viral DNA that is required, that contains the enhancers that activate viral DNA replication, and insert random fragments of mouse genomic DNA instead. So we made a library of mouse genomic DNA particles there. Those DNA fragments will be able to replicate if they have enhancers or promoters that act as enhancers or activators of gene expression for messenger RNA synthesis that will also have this effect on DNA replication. They will multiply when you infect cultured mammalian cells, while those viral DNAs, those Polyoma recombinants that have pieces of DNA that lack functional enhancer sequences won't replicate. So this is an enormous selection based on function, based on its ability to activate replication of viral DNA. That's what they both did. So they obtained clones, they characterized them and sequenced them, and showed that they do, in fact, act as enhancers. They identified them and found one or two that may be novel enhancers.[4] Now Sada is in Japan at the Nippon Medical School and Wah is working in Baltimore at the Johns Hopkins University School of Medicine.

Maral Mouradian is a neurologist from the NIH and is now head of a section in the Neurology Institute.[5] She wanted to learn molecular biology so she spent several years

working in our lab while she was doing clinical work here. She is a highly intelligent, dedicated individual and very talented as well. We devised a method of selecting DNA clones based on their ability to act as enhancers that activate gene expression. Others have shown that part of the viral DNA functioned as an enhancer region, and the enhancers were required for replication of DNA for viral reproduction to make new copies of the virus DNA. We cut out the enhancer region of the virus and replaced it with a library of DNA segments, random DNA segments, small segments of DNA, from the mouse genome. The rationale was that if you had an enhancer sequence that was present that would activate synthesis of new viral DNA, those viral DNA molecules would multiply when you infected mammalian cells with the viral DNA. So they would replicate and you could then purify—isolate them. If an enhancer was not present, they would not replicate. It was a highly effective selective mechanism for enhancers, and it worked very beautifully. It is a very powerful method of selection.

Dervla Mellerick was from Ireland. She worked on the distribution of NK-2 mRNA in Drosophila embryos as a function of development. She is now an assistant professor at the University of Michigan in Ann Arbor, my alma mater.

Michael] Mike Mitas from Georgia, who now is at the University of Oklahoma, became interested in triplet repeats.[6] He also worked on the cloning—looking at the regulation of a gene for a voltage-sensitive calcium channel which is expressed by neuroblastoma cells and whose expression is required for synaptic function and for stimulus secretion

coupling. The expression of this gene, we found, regulates the formation of synapses, the ability of neurons to communicate with one another.

Most recently we have collaborated with some people here at the NIH who have done nuclear magnetic resonance spectroscopy. In this connection I forgot to say also that Lan Wang, who recently was in the laboratory, has done superb work characterizing and identifying the nucleotide sequence that NK-2 protein binds to in DNA. She synthesized in *E. coli* and purified to almost essential homogeneity over 500 mg. of the NK-2 homeodomain region with some amino acids on the sides.

We then gave most of it to [James] Jim Ferretti and his colleagues, Desirée Tsao and [James] Jim Gruschus, and they determined the 3-dimensional solution structure of the NK-2 homeodomain by nuclear magnetic resonance spectroscopy. So, this has been a very productive collaboration. They showed that the NK-2 homeodomain is indeed a helix-turn-helix conformation and that binding of the homeodomain to DNA extends the DNA recognition helix to almost twice the length. It is the first time anything like that has been found.[7]

Recombinant DNA

RH: I have some other general questions to ask you. What do you think of the impact of recombinant DNA?

MN: I think it has enormous potential, and it will eventually realize its potential. But, the technology is quite difficult. It is time-consuming. We make transgenic flies, and it is much easier to make transgenic flies than it is to make transgenic mice, for example.

The surface has only been scratched with regard to use for therapeutic purposes in human beings. Much more information is needed before it will be widely used, but for those people who suffer from genetic diseases, there is no known treatment or cure for these diseases. This offers a potential means of therapy. It is extremely complicated because perhaps 2 or 3 percent of the DNA actually encodes protein: much of the rest, at least, is needed to regulate gene expression or for other purposes that are not understood. But much of it is used for regulatory purposes, and it is so complicated that you do not know the functions of much of the DNA.

The Human Genome Project is now in full swing, and in the not too distant future the entire sequence of the human genome will be determined. Also, all human genes will be identified. That will provide a remarkable outpouring of information. Already the entire genome of a microorganism has been determined. It is an extraordinary amount of information. We can start to make comparative chromosomal maps from different species by using this information.

Imagine knowing the entire instructions, which enable an entire bacterium to synthesize, to live, to reproduce and do everything else that it does! That information will be

determined for human beings in the not too distant future. But it will be a long, long time before function is determined for all the genes, and particularly for regulatory DNA.

There are the methods that are used currently for inserting genes. There are methods available to enable us to replace a defective gene with a normal working gene. Also there are methods that randomly insert genes that encode a particular protein that may be defective in the organism or the person at random sites. This is equivalent to making mutations. Nobody really knows what the effects of such random insertions are. It is difficult to do this work. It is time consuming, slow, quite difficult, but the work is in its fairly early infancy. Much more work has to be done before its final potential will be realized. But, I do not have any doubt whatsoever that these techniques eventually can be used for extraordinarily good things for people who suffer from all kinds of genetic diseases.

Of course, nobody is thinking in terms of making transgenic human beings, that is, to have those genes that are inserted reproduced and passed on to progeny. Nothing like that is being done although one does it with mice. One can do it with mice or Drosophila, for example. We have made hundreds of lines of transgenic flies. But making a transgenic mouse is a lot of work.

I think Jeremy Rifkin is totally wrong on this issue and actually harmful. [8] The scientists have been extraordinarily responsible in the use of this technology to change genomes.

Right at the beginning they had a conference. Virtually as soon as it was realized that you could do these things, they had a conference about not doing it until you could assess the possible dangers of doing it. After many years and with much work done, it was clear that there was no apparent danger, or little or no apparent danger, of doing it if you did it in the right way.

I should point out that nature does this all the time. Nature has transgenic experiments, even between different species. The viral DNA can be inserted into human DNA and cause cancers in some cases. We are subjected to all kinds of insults due to an introduction of foreign DNA into cells by carriers, which can subvert the metabolism of the cell to viral purposes. There is a regular chemical warfare going on at a microscopic level. But this is only the beginning of that type of work.

RH: What is the greatest unknown, or challenge, in biochemistry today?

MN: I still think in biochemistry that the nervous system is the biggest unknown because it is so complicated. That is why I picked it. We still don't know what memory is, although we have a much better idea of what it is. We still do not know how synaptic partners are specified, how the nervous system is assembled, or what the molecular rules are for assembling a nervous system. I think that some of the things that we see in Drosophila may also be early events in determining the position of specific neuroblasts and of selecting particular cells to develop as neuroblasts.

These will be early events in selecting cell types and in selecting those cells that express the right set of genes to be used in the mouse to make a nervous system and the selected internal molecular address for cells. Then those cells will have an external molecular address which will be cell surface proteins that will be recognized by other cells by a differential adhesiveness, which will determine the position of the cells, the class of cells, and the synaptic partners.

When I went into neurobiology, none of these things were clear. Now I understand theoretically much of the basic strategy that is used initially to construct part of the CNS [central nervous system] of Drosophila during early development. That is a tremendous advance.

When I first went into this work, I hadn't the foggiest notion of how it was done. Now I see the big picture, although the details aren't known, or anything like that, but the basic strategy I understand. I only understand it in this one organism. It has not been worked out for other organisms yet, so this is like a model system. I think that similar strategies, maybe some different strategies, will be used, but it is done in such an elegant way that it is remarkable. I don't think that people generally understand this yet. This is at the forefront of neurobiology.

But it is easy to make a nervous system: you make it using such beautiful mechanisms, generating gradients of gene regulators that generate different cell types so that the cell type that will develop is determined by the position of the cell. Then you select for the

cell that has the right set of gene regulators that make the most NK-2, the most proneural genes, and things of this sort.

You are making a really complicated computer here, and it is done in such an elegant, simple way. I think that the strategy is breathtaking. If something like that is done in Drosophila, think of what must go on in our own bodies

We are engineering marvels.

RH: Thank you, Dr. Nirenberg, for talking with me.

MN: Of course

The footnotes below will be placed in a separate digital file for linkage to this file.

[1] Christiane Nüsslein-Volhard (1942-) earned degrees in biology, physics, and chemistry in 1964 from Johann Wolfgang Goethe University, a diploma in biochemistry in 1968 from Eberhard Karls University, and, in 1973, a Ph.D. in biology and genetics from the University of Tübingen. In 1973 she joined the European Molecular Biology Laboratory in Heidelberg, Germany, where she and Eric Weischaus began research into how a newly fertilized fruit fly egg developed into a segmented embryo. In 1991 she and Weischaus received the Lasker prize, and in 1995 they shared the Nobel Prize in Physiology or Medicine with Edward B. Lewis of the California Institute of Technology on genetic development of Drosophila that could help explain birth defects in humans.

[2] Adil Nazarali, Ph.D., was with the Molecular Biological Laboratory, University of Saskatchewan College of Pharmacology and Nutrition, Saskatoon, Saskatchewan, Canada.

[3] Khozo Nakayama, Ph.D. was an associate professor in the Department of Anatomy II, School of Medicine, Shinshu University, Nagano, Japan.

[4] Their work appeared in S. Asoh, Le-Kwon, M. M. Mouradian, and M. Nirenberg, "Selection of DNA Clones with Enhancer Sequences," *Proceedings of the National Academy of Sciences* (USA), 91 (1994): 6982-86.

[5] Mary Maral Mouradian (1956-) earned a B.Sc. from the American University, Beirut, Lebanon. She was a postdoctoral fellow at the NIH from 1985 to 1990. In 1990 she became chief of the genetic pharmacology unit, National Institute of Neurological Disorders and Stroke.

[6] Michael Mitas (1959-) served in the United States Coast Guard from 1977 to 1978 and earned a B.A. in 1981 at Washington University and a Ph.D. at Emory University in 1989. He was a postdoctoral fellow at the NIH from 1989 to 1992.

[7] Lan-Hsiang Wang worked in the division of heart and vascular diseases, National Heart, Lung and Blood Institute. James A. Ferretti (1939-) earned a B.S. at San Jose State College in 1961 and a Ph.D. in biochemistry at the University of California, Berkeley, in 1965. In 1967 he worked as a research chemist at the NIH and by 2004 he was chief of the Laboratory of Biophysical Chemistry in the National Heart, Lung and Blood Institute's Division of Intramural Research. James M. Gruschus (1965-) earned a B.S. at the University of California, Berkeley, in 1986 and a Ph.D. at Cornell University in 1993. He then joined the National Heart, Lung and Blood Institute.

[8] Jeremy Rifkin (1945-) earned a B.A. from the University of Pennsylvania and an M.A. from the Fletcher School of Law and Diplomacy, Tufts University. He became head of the Foundation on Economic Trends. A political activist, he particularly campaigned against biotechnology advances.