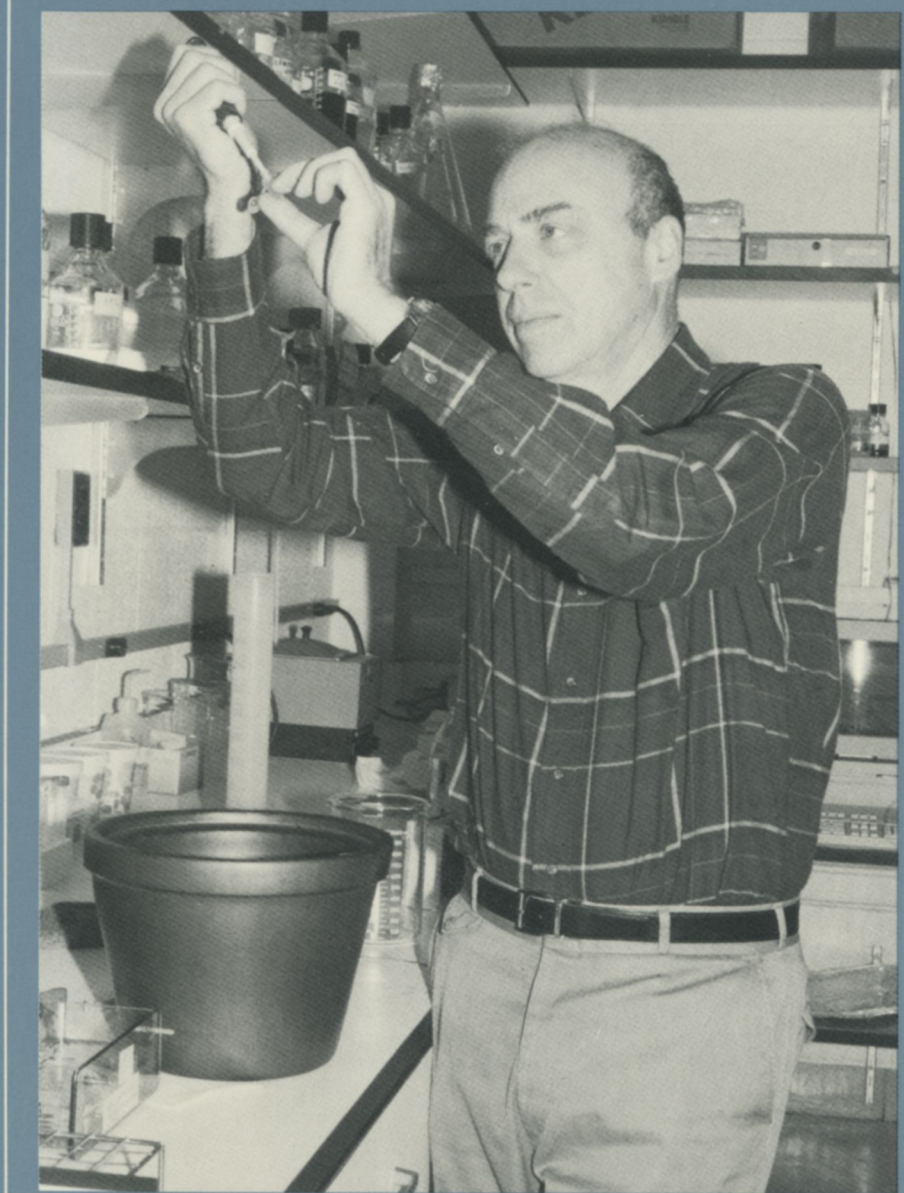

One Scientist's Journey

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An essay about basic research from
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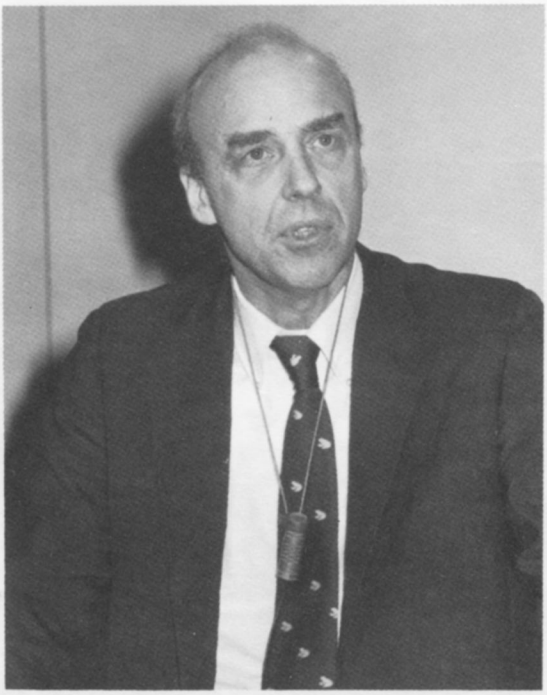
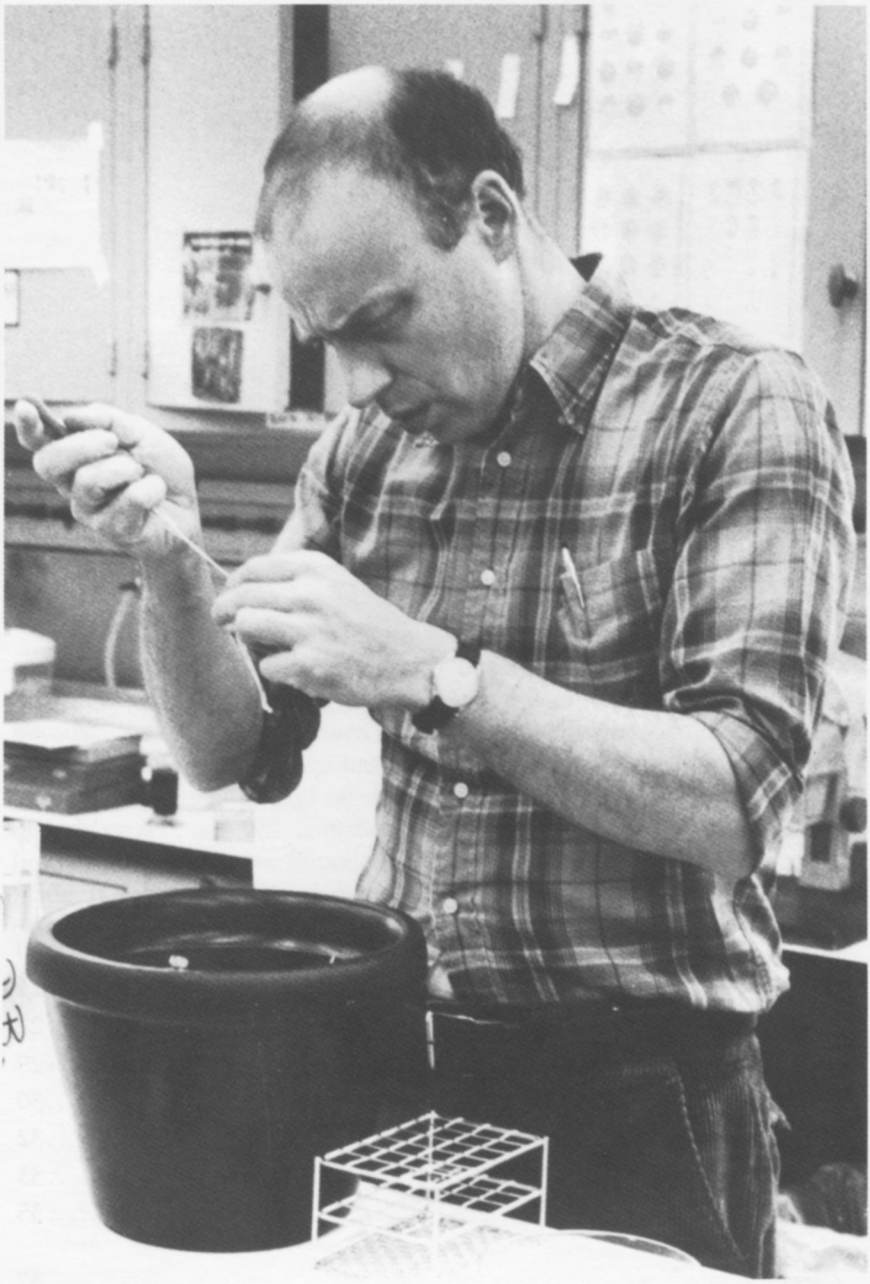
PREFACE

Donald D. Brown is one of many well-known biologists whose career coincided with, indeed helped launch, the explosive and fascinating growth of molecular biology. But Dr. Brown's career is also interesting because of the way it illustrates the process by which research is driven. For over thirty years, Brown has pursued the same general question: how do genes turn on and off during development to give rise to the orderly growth and differentiation of cells and tissues? This question lies at the root of every direction he has chosen, every discovery he has made. It forms the unifying theme of his career—and of the following essay.



Contents



Working in the lab, early 1970s

Ex ovo omnia.
Everything from an egg.
William Harvey
De Generatione Animalium, 1651

Biological development is at once commonplace and mysterious. From the union of sperm and egg there emerges a new organism, similar to its parents yet different in countless ways.

For hundreds of years, scientists curious about life's beginnings could explore only the easily seen manifestation—the embryo itself. They learned how to poke and prod individual cells of frog or chick embryos; with fine needles, they destroyed certain cells, or they transplanted bits of tissue from one embryo to another, and then watched what happened.

When molecular techniques came along, some 35 years ago, the genes—known to play a central role in development—suddenly became accessible. A new kind of embryologist emerged, one well versed in techniques of gene cloning, recombination, and engineered mutation. One of these embryologists was Donald D. Brown. In the late 1950s, Brown began an effort to rephrase the essential questions of development in terms of the genes. It was not an easy task; it had only been in 1953 that the double-helix structure of DNA—shown conclusively just a few years before to be the material of the gene—was discovered.

Choosing a Path

Donald Brown remembers vividly learning about the double helix for the first time, "as though it were yesterday." Only 22 years old, he was in his second year of medical school at the University of Chicago, where he had enrolled after spending three years as a pre-med major at Dartmouth College. Brown was not a keen medical student. He said later he never found medicine particularly interesting and he didn't feel he was much good at it. He much preferred doing experiments on bacteriophages (bacteria-infecting viruses) in the University's biochemistry department.

At the time, the young medical student was leaning seriously toward a career in research. He was also beginning to think about how embryology might fit into such a career. "I thought that [studying embryology] would be a wonderful way to put together my interests in research with a little bit of medicine, and get off the beaten path," he said years later. He had taken no courses in embryology in medical school nor attended any lectures on the topic; the only thing he knew about embryology was that "it was a field so primitive that no modern research was being done in it. And yet it had this huge, incredible problem—how an egg develops into a multicelled organism."

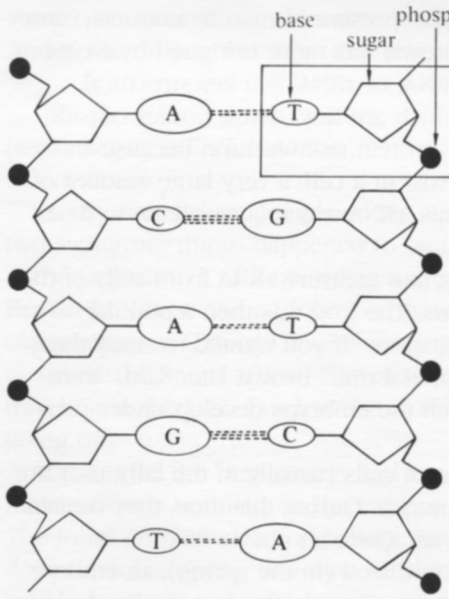
Brown was aware that the only way he could begin to tackle this problem was first to get "some really good training" in biochemistry. (At the time, there was no discipline called 'molecular biology.') By the time he left the University, in 1956, he had earned not one degree, but two—a doctorate of medicine and a master's degree in biochemistry. He spent the following year as an intern in New Orleans; when he left, he was never to practice medicine again.

The next several years were unsettling for Brown. Not sure exactly what to study, or where, he began his odyssey in Bethesda, Maryland, as a research associate at the National Institutes of Health (NIH). It was at NIH that he did independent research for the first time. NIH was, he remembers, "a marvelous place to work," but the research he was doing, on schizophrenia, didn't appeal to him. More attracted by work on bacterial gene function that the French biologist Jacques Monod was doing, he arranged to spend a year at the Pasteur Institute in Paris. (Accompanying him was his wife, Linda, whom he had met and married in New Orleans, and their first child, Deborah; two more children would be born later.) Brown found his Paris sojourn a rich, stimulating experience. Even though he wasn't, as he puts it, "in the fast lane" at the Pasteur Institute, he was excited by the discoveries being made there. Only five years later, those discoveries would win for Monod (with his colleagues François Jacob and André Lwoff) a share in a Nobel Prize.

Returning to the United States in 1961, Brown went directly to Baltimore, Maryland, to the Carnegie Institution's Department of Embryology. He had learned about the Department before leaving for Paris, while visiting The Johns Hopkins University nearby. Though no one at Carnegie was investigating biochemical aspects of embryology (the staff members were all classical or experimental embryologists), Brown had immediately warmed to the Department. He felt it was a place where he could do the sorts of experiments he wanted to do, "without anyone looking over my shoulder." He joined the Department as a research associate—the first of a new flock of embryologists that would slowly displace the old order.

Searching for a Model System

When Brown joined the Department, he still had not figured out how to explore, on the molecular level, the great problem of embryology—how a multicelled organism arises from a single cell. He assumed, like



Two strands of a DNA molecule. **The bases of opposite strands are connected by hydrogen bonds (dashed lines)—A always opposite T, C opposite G. (A stands for adenine, T for thymine, C for cytosine, and G for guanine.) When the strands separate, the order of bases determines the sequence of the transcribed, single-stranded RNA. In RNA, thymine is replaced by uracil, and the sugar is slightly different.**

other scientists at the time, that every cell of a multicellular organism contains a full array of that organism's genes, and that the genes are turned on and off (rather like light switches) depending on the type cell they are in. In a muscle cell, only muscle-specific genes are expressed; in a skin cell, a totally different array of genes are on. Brown had no idea how to explore the mechanisms behind the switches; he didn't know how to approach the question of differential control. Even Jacques Monod in Paris hadn't been encouraging. When Brown told him of his intentions to study embryology, Monod had said it would be a waste of time, that embryology was much too complicated.

The cells of higher organisms (the eukaryotes) are indeed complicated. The DNA in eukaryotic cells, present in extraordinary quantities, is intertwined with many different kinds of protein, forming a tangled nuclear complex called chromatin. With the tools available in 1961, these chromatin-complexed genes were inaccessible. The only genes that were accessible were the simple genes of bacteria—the prokaryotes. But because prokaryotes begin and end their lives as single cells, their genes were not particularly useful for understanding cell and tissue differentiation. Besides, no one knew if prokaryotic genes were anything like eukaryotic genes.

What most scientists interested in eukaryotic genes studied instead was protein—a gene's ultimate product. By charting the gradients of proteins in embryos, these scientists could determine—roughly—which genes operated where. Brown, impatient to move forward, found this method unsatisfactory. He was keenly aware that proteins are not the direct products of genes. A gene first makes RNA, a single-stranded, mirror-image copy of DNA, in a process called transcription.

Brown didn't know a lot about RNA (neither did any one else at the time), but he did know that it came in several varieties, each having a different function. One of those varieties was believed to carry DNA's

protein-making instructions; this type, present in minute amounts, came to be known as messenger RNA. Brown was more intrigued by a type called ribosomal RNA. Ribosomal RNA, or rRNA, is a key structural ingredient of a cell's ribosomes, which are tiny, dense bodies outside the nucleus that play a key role in protein manufacture. Because an enormous number of ribosomes exist in a cell, a very large amount of rRNA is also present. Because of this, rRNA was relatively easy to find and extract.

Brown began an effort to extract and measure rRNA from cells of the common leopard frog, *Rana pipiens*. The frog was then a popular organism for biological experimentation. "If you wanted to study the biochemistry of early development, as I did," Brown later said, "then frogs were perfect. You could watch the embryos develop under your eyes."

When a female frog produces germ cells (usually in the fall), they are stored for several months in her ovaries. During this time, they remain as single cells, and are called oocytes. Once an oocyte matures it becomes an egg. When the egg is fertilized (in the spring), an embryo develops. An embryo undergoes striking metabolic changes, hatching in about six days to become a swimming larva.

To conduct his experiments, Brown needed to have a constant supply of frogs and frog embryos. He decided to raise his own. He had heard that all one needed to grow frogs was tap water, since frog embryos are nutritionally independent; that is, they themselves can manufacture all the proteins and carbohydrates they need from nutrients originally in the egg. Opting for distilled water (as any trained biochemist would), he soon ran into a serious problem: all of the embryos stopped growing when they reached 9 millimeters in length, one or two days after hatching. Shortly afterwards, they died. Brown was baffled. Might the embryos require some key inorganic ingredient missing in the distilled water, he wondered? He did some experiments. The answer, it turned out, was yes; the embryos needed magnesium. When he added magnesium, development proceeded normally. (Later, he found that trace amounts of other inorganic ions, such as potassium, calcium, and sodium, also present in tap water, were needed by frog embryos.)

Brown was astounded that by 1961 no one had figured out the frog embryo's nutritional requirements. Once he established the critical need for magnesium (a "rather mundane" first discovery, he says), he resumed his goal: to study rRNA. He picked up where he had left off, extracting and measuring levels of rRNA and ribosomes in frog embryos at various stages of development. It didn't take him long to discover that the stage at which ribosomes begin rapidly to accumulate in the embryos correlated exactly with the stage of magnesium deficiency.

This was puzzling. He had earlier determined that frog embryos begin making protein soon after they are fertilized. How, then, do newly fertilized embryos make protein when they don't begin making essential parts of the protein-making machinery, the ribosomes, until seven or eight days later? He could only conclude that the young embryos must be making protein using ribosomes originally made (and stored) in the maternal egg. It was only when these ribosomes are

worn out—one or two days after hatching—that new ones are needed. And it was only at this stage that magnesium is required.

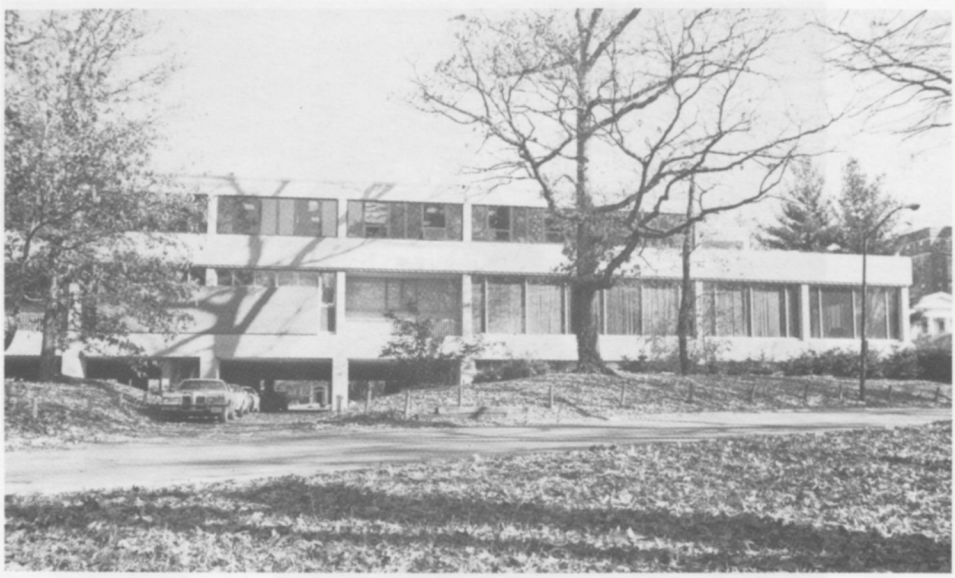
Beginning of a Collaboration

Shortly after Brown completed his magnesium-deficiency experiments, two significant things happened to him. He was offered a staff position at the Department of Embryology (which he accepted), and he met a British biologist named John Gurdon. Gurdon had been doing experiments at Oxford University using an organism Brown had never heard of: the South African clawed toad, *Xenopus laevis*. In 1962, while traveling in the U.S., Gurdon visited the Department to see what was going on.

At the time, the Department had just moved from a building on the campus of The Johns Hopkins Medical School, in downtown Baltimore, to a new, modern building on a corner of the main campus of The Johns Hopkins University. Everything was in disarray, and Brown and Gurdon managed to exchange barely more than a few words.

It wasn't until several months later that Brown thought again about that meeting, while reading a paper written by a group of Oxford scientists. The paper described

Brown and John Gurdon in 1985.



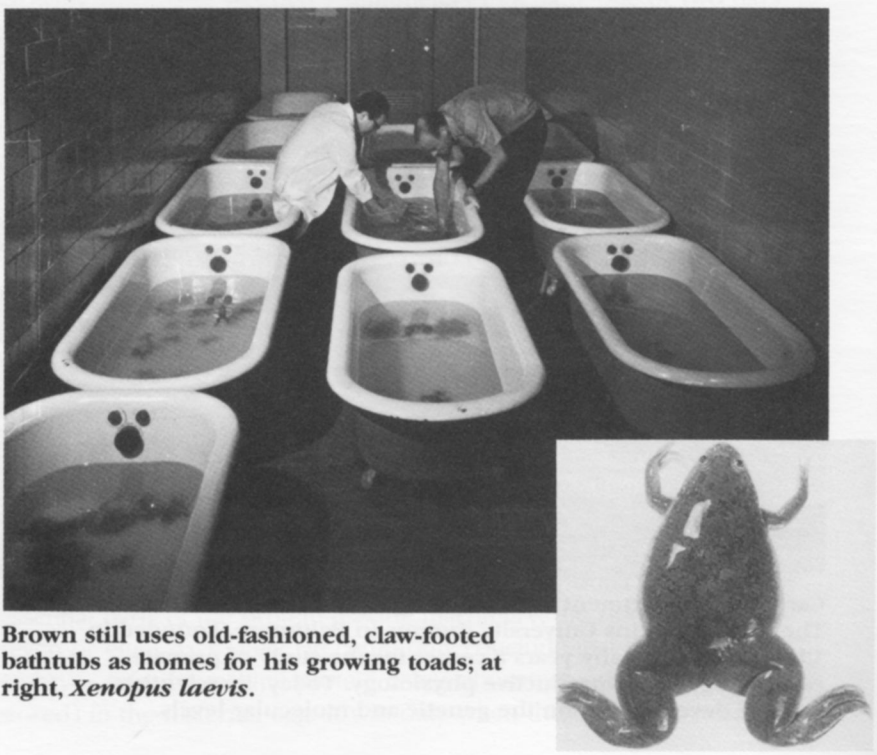
Carnegie's Department of Embryology is located on a corner of The Johns Hopkins University campus in Baltimore. Founded in 1914, it was for many years a center for the study of primate embryology and reproductive physiology. Today, its scientists explore development on the genetic and molecular levels.

mutant *Xenopus* embryos whose nuclei were missing two small nobs of material called nucleoli that, in normal cells, are present on each of two identical chromosomes. (*Xenopus* cells contain eighteen different chromosome pairs; the nucleoli appear on just one pair.) The British scientists had found that the anucleolate mutant embryos died two or three days after being hatched—exactly the stage at which Brown's magnesium-deprived embryos had died.

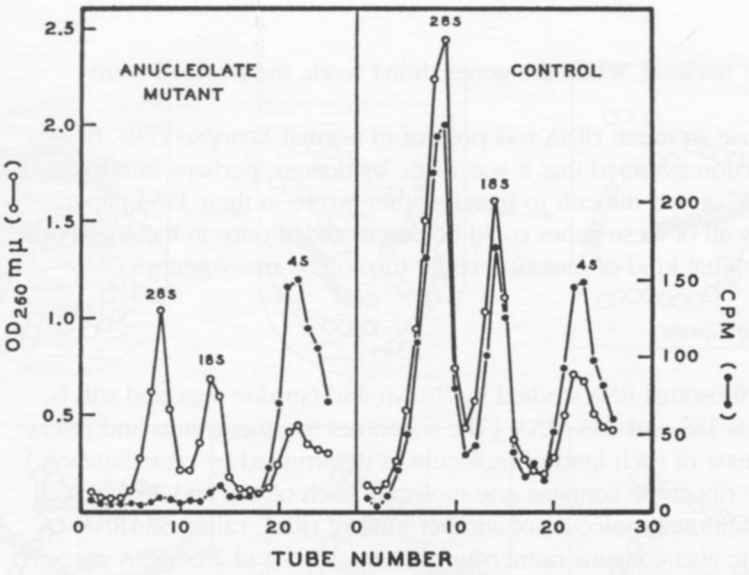
Brown was all but sure the two observations were related, that, in fact, the *Xenopus* mutants had died a "ribosome-less death." He had only just become aware, from experiments done elsewhere, that nucleoli were associated in some unknown way with rRNA. He wanted to pursue the matter, to study the correlation between rRNA and nucleoli, using the *Xenopus* anucleolate mutants. But where was he to get them?

John Gurdon was the only person he knew who was familiar with *Xenopus*. Brown quickly fired off a letter. Receiving no immediate reply (unknown to him, Gurdon was still traveling), Brown started doing some sleuthing on his own; as it turned out, a biological supplier near Baltimore sold the toads. Brown repeated the biochemical experiments he had done previously with the leopard frog, and got the same results. In the process, he found *Xenopus* to be much easier to use. He began raising them in old, claw-footed bathtubs, retrieved from a local junkyard.

Meanwhile, Gurdon, having finally received Brown's letter, wrote back, agreeing to supply anucleolate embryos for Brown's experiments. Soon, a steady supply of *Xenopus* embryos was arriving from overseas. Gurdon, in England, raised the anucleolate embryos by mating two



Brown still uses old-fashioned, claw-footed bathtubs as homes for his growing toads; at right, *Xenopus laevis*.



Results of Brown and Gurdon's experiments comparing RNA synthesis of anucleolate mutant *Xenopus* embryos (left), and normal, control embryos (right). Both genotypes were incubated for 20 hours in a radioactive solution and then allowed to develop another 48 hours to the tadpole stage. The RNA was extracted and separated by mass (the lower the tube number, the higher the mass). Lines with closed circles, representing radioactive RNA, designate newly made ribosomes (CPM = counts per minute); lines with open circles designate the total amount of ribosomes, including those inherited from the oocyte. (OD_{260}mμ is a measure of optical density.) In the anucleolate embryos, it is clear that no new ribosomes have been made; only inherited ribosomes are present. (The small amount of new RNA made by the anucleolate mutant is either transfer RNA, labeled "4S" RNA in figure, or mRNA. These RNAs are synthesized from other genes that are not affected by the anucleolate mutation.)

parents whose cells each contained one nucleolus. (Each parent carried the mutation in just one chromosome.) This mating, in accordance with Mendelian genetics, yielded offspring of which one-quarter contained two nucleoli per cell, one-half contained one, and one-quarter had none. Gurdon rushed the growing embryos to Heathrow airport, where they were loaded onto a Baltimore-bound airplane. Because the anucleolate embryos survived for only a few days, time was critical. But there were always problems with the airlines. "It was hell," Brown recalls, "like being on call all night at the hospital." Once back in his lab, Brown incubated the embryos in a radioactive solution, which served to label the newly made rRNA. He then extracted and measured the rRNA—both old and new—in each.

After six months, Brown and Gurdon finished their experiments. They established that the amount of rRNA inherited from the egg was the same in all the embryos. However, the amount of *newly* manufactured rRNA was not. In embryo cells having one or two nucleoli, new rRNA was actively being synthesized. But in the cells with no nucleoli, new rRNA was entirely absent. (See figure, above.) This could only mean that the rRNA genes were found in association with the nucleoli—and only

with the nucleoli. When the genes didn't work, the nucleoli were absent.

Because so much rRNA was present in normal *Xenopus* cells, Brown and Gurdon assumed that it was made by dozens, perhaps hundreds, of genes. What was difficult to imagine, they wrote in their 1964 paper, was how all of these genes could be deactivated at once in the anucleolate mutant. What kind of mutation could turn off so many genes?

Isolating Genes

The ribosomal RNA studied by Brown and Gurdon was, and still is, known as 18S and 28S rRNA. (The S denotes Svedberg units and refers to the mass of each kind of molecule as determined by centrifugation.) A single ribosome contains one molecule each of 18S and 28S, as well as an additional molecule of another kind of rRNA, called 5S rRNA. (A ribosome also contains numerous proteins.) 18S and 28S rRNA are synthesized at the same time during normal *Xenopus* development. In fact, these two molecules are transcribed together, as one long precursor molecule, which then degrades into two smaller segments. (In their experiments, Brown and Gurdon had found that the anucleolate mutants not only made no 18S and 28S rRNA, they made no precursor as well.)

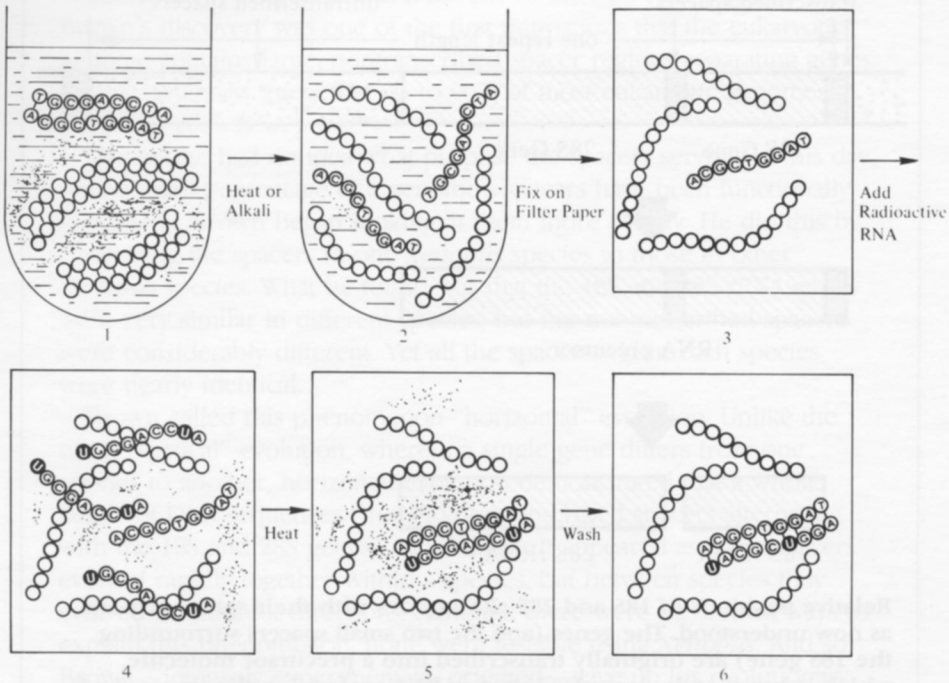
In 1964, Brown was aware of 5S rRNA's role in ribosome manufacture, but he wasn't sure how it was regulated. He suspected that its synthesis was closely coordinated with that of 18S and 28S rRNA, since a ribosome needs all three kinds of rRNA to function properly. At the time, however, he paid scant attention to it. He was too deeply involved with 18S and 28S rRNA.

In 1965, a year after Brown and Gurdon published their paper about the anucleolate embryos, a pair of scientists working in Scotland—Max Birnstiel and Hugh Wallace—discovered why the 18S and 28S rRNA genes were inactive in the mutant embryos. These genes failed to work, the two found, not because of a mutation in the genes, but because the genes were gone—they were deleted entirely from the genome. (The genome is an organism's full array of genetic material.)

In making their surprising discovery, Birnstiel and Wallace had used a newly developed technique called nucleic acid hybridization.* Hybridization takes advantage of RNA's similarity to DNA. It involves separating the double strands of DNA (by the application of heat), and then introducing single radioactive RNAs into a solution containing the DNA strands. The RNA seeks out and binds to its complementary DNA; it can then be located by virtue of its radioactivity. Birnstiel and Wallace were the first to use this now common technique to study the ribosomal genes of *Xenopus*.

Brown was pleased to have an answer to his and Gurdon's question. But he was more excited by the potential of the hybridization technique.

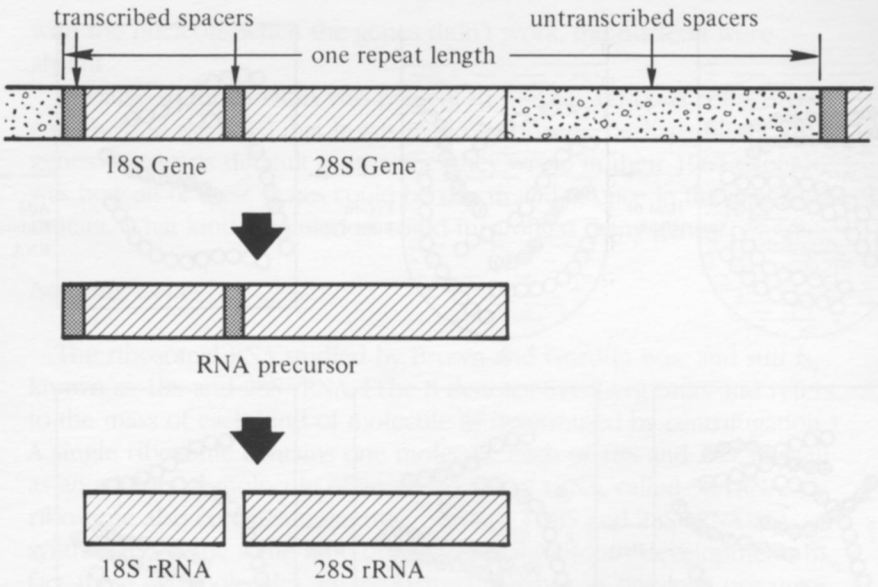
*This technique had been partially developed by a group of Carnegie biologists, including Roy Britten, working in Washington, D.C., at Carnegie's predominantly physics-oriented Department of Terrestrial Magnetism.



Hybridization assay. **In hybridization technology, one strand of a gene is paired with its RNA product on the basis of complementarity. As illustrated in the simplified diagram above, a sample of double-stranded DNA (1) is separated by treatment with heat or alkali (2). (The gene under assay has lettered nucleotides.) The separated strands are fixed on filter paper (3). The filter paper is incubated in a solution of radioactive RNA molecules made by, and therefore complementary to, the genes under assay (4). The RNA molecules diffuse over the paper and come in contact with their complementary strands of DNA, forming DNA-RNA hybrids (5). The remaining, unbound RNA is washed away (6). The amount of the bound RNA that is left, as measured by radioactivity, indicates how much of the DNA is the gene. Using this technique, Brown and colleagues were able to determine how many copies of the 18S and 28S rRNA genes were present in each *Xenopus* cell.**

He realized immediately that it heralded a new era in genetic research, that it could be used as an assay, or test, for the isolation (or purification—the word more often used by scientists) of known genes. Just as an RNA/DNA hybrid could be located in the genome by virtue of its radioactivity, so too could it be separated from that genome. Birnstiel and his colleagues succeeded in purifying small amounts of 18S and 28S rRNA genes shortly thereafter, as did Brown. The 18S and 28S were the very first eukaryotic genes to be isolated.

Purifying genes in 1967 was not as easy as it seemed. Brown and Birnstiel were successful only because the 18S and 28S rRNA genes happen to have two critical characteristics: (1) they exist in many copies in *Xenopus* cells (most genes exist in a single copy), and (2) they are much denser than most other DNA in the cell. This made them easier to separate (by centrifugation) from the total DNA.



Relative positions of 18S and 28S rRNA genes with their spacer regions, as now understood. The genes (and the two small spacers surrounding the 18S gene) are originally transcribed into a precursor molecule, which subsequently degrades into two RNA molecules; the transcribed spacers are eliminated.

Spacers. Once Brown had purified the DNA containing 18S and 28S rRNA genes, he set out to analyze, or characterize, the genes structurally. Helping him in this effort was a team of local collaborators that included his technician, Mrs. Eddie Jordan (who remains with him to this day), and a small, constantly changing group of young scientists called postdoctoral fellows. Supported by NIH or by private foundations, fellows typically work at the Department for two or three years before moving elsewhere to pursue their independent careers. Occasionally, also, Brown is joined by graduate students from the nearby Johns Hopkins University, where he began teaching part-time in 1964.

From his hybridization experiments, Brown had derived a rough estimate of the numbers of 18S and 28S rRNA genes present in each *Xenopus* cell. There were, as he had suspected, hundreds of both kinds. Clustered at each nucleolus were some 450 18S genes and an equal number of 28S genes. Since body cells have two sets of all chromosomes (and two nucleoli), each normal *Xenopus* cell thus contains about 900 copies of each type of gene.

As he continued his experiments, Brown found something very curious. Not all of the DNA in the purified rRNA genes hybridized to the gene's RNA product. Whole stretches of it, in fact, seemed to consist of nucleotides differing completely from the RNA. He concluded that these stretches were not transcribed. Brown called these nontranscribed stretches "spacers," for they appeared, in each gene cluster, to fall in between the two genes, creating an alternating pattern—first an 18S gene, then a spacer region, then a 28S gene.

The concept of spacers was a novelty in the mid-1960s; in fact, Brown's discovery was one of the first intimations that the eukaryotic genome contains large, nontranscribed spacer regions separating genes. (Today, scientists know that up to 90% of most eukaryotic genomes consist of such DNA.)

Though he had no idea what purpose the spacers served (to this day, only a small percentage of a genome's spacers have been functionally identified), Brown began looking at them more closely. He did this by comparing the spacers in one *Xenopus* species to those in other *Xenopus* species. What he found was that the 18S and 28S rRNA genes were very similar in different species, but the nontranscribed spacers were considerably different. Yet all the spacers within each species were nearly identical.

Brown called this phenomenon "horizontal" evolution. Unlike the usual "vertical" evolution, wherein a single gene differs from one species to another, horizontal evolution demonstrated that a whole family of DNA sequences (the 450 nontranscribed spacers alternating with the 18S and 28S genes) could differ. It appeared as if the spacers evolved rapidly together within a species, but between species they evolved in different directions. Although there were theoretical ways to explain this phenomenon, none were testable using *Xenopus*. And so Brown—intensely experimentally oriented—gave up his evolutionary ponderings on spacers. (He says that after a "last gasp" in 1972, at which time he came up with a totally wrong theory about the evolution of the immune system, he gave up theorizing about evolution entirely.)

Meanwhile, he was becoming increasingly preoccupied with another, related project going on in his lab—a project directly in line with his major interest: the control of gene expression.

Evolution of an Insight: Amplification

The project had its origin in an international meeting about the nucleolus that Brown attended in late 1965 in Montevideo, Uruguay. Brown had traveled to Uruguay to give a talk about his work with the rRNA genes. When finished, he sat in on a lecture about amphibian oocytes presented by Oscar Miller, a biologist from the Oak Ridge National Laboratory in Tennessee.

Oocytes are very young egg cells that have not yet matured. Because a *Xenopus* oocyte has four instead of two sets of chromosomes (because of the way the cell divides during meiosis), Brown assumed it would contain four, not two, nucleoli. And so, when Miller projected a slide of the nucleus of a *Xenopus* oocyte onto the screen at the front of the room, Brown suddenly sat up straighter. For there, clustered around the edges of the nucleus, were not four but hundreds of nucleoli. Brown was dumbfounded. "When Miller explained that each one of those nucleoli had some chromosomal material in it," he recalls, "I knew immediately why: the ribosomal RNA genes had to be amplified."

What he meant by amplified was that the genes were increased in number; instead of the expected 1800 copies of each gene (450×4), it appeared that the oocyte contained millions of rRNA genes. It was a

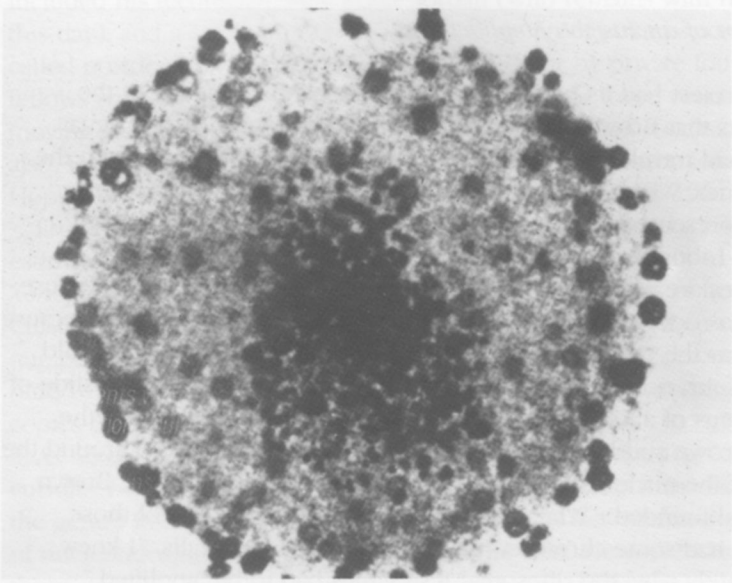
heretical idea, since, at the time, scientists assumed that all cells of an organism contained the same number of genes. But Brown didn't see how any other hypothesis could fit. Once he returned to Baltimore, he enlisted the help of Igor Dawid, a fellow staff member, and his technician Eddie Jordan. In what Brown calls a "heroic effort," Mrs. Jordon isolated, by hand, over 10,000 individual oocyte nuclei for use in the experiments.

Using a hybridization assay, Brown and Dawid found that the 18S and 28S rRNA genes and spacers were indeed present in great excess in those oocyte nuclei. As many as three-and-one-half million rRNA genes were churning out the ribosomal RNA products in each nucleus. This was a 2000-fold increase over the numbers of 18S and 28S genes present in other *Xenopus* cells.

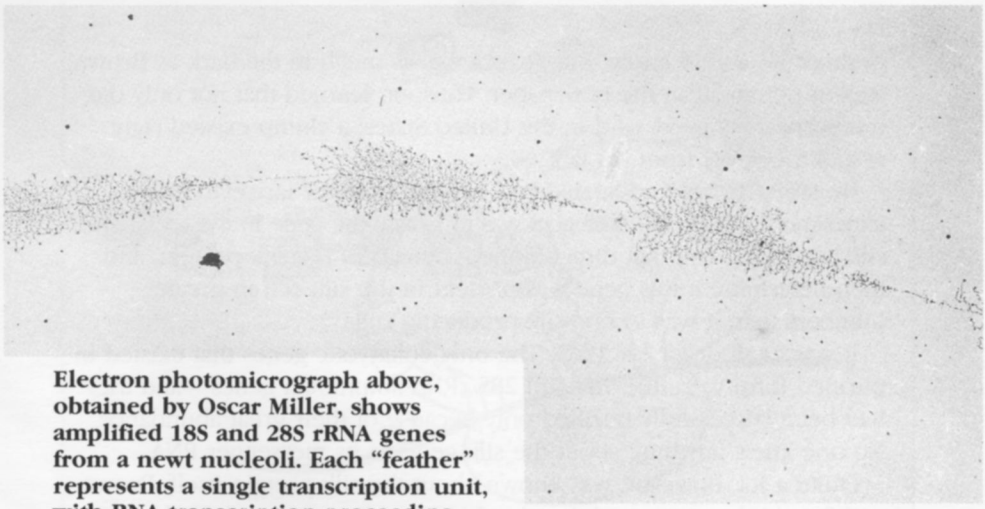
An oocyte expresses its millions of amplified genes only until the cell undergoes meiosis. At that time, the nucleus breaks down, all of the nucleoli disappear, and the extra rRNA genes become dormant. Two nucleoli reappear later in embryonic development, once rRNA synthesis resumes. For the rest of the animal's life, the number of nucleoli remains at two, and the extra rRNA genes never turn on again.

The phenomenon of gene amplification, which was independently discovered by Joseph Gall at Yale University (who was also present at Miller's talk), took most biologists by surprise. All cells of an organism, it appeared, do *not* carry the same numbers of genes, as was assumed. In *Xenopus*, and, as it turned out, in other amphibian oocytes, there is an actual increase in the number of one kind of gene.

Brown too was surprised that genes could be amplified. But his surprise had come earlier, while attending the meeting in Uruguay. It



What Brown saw for the first time at the Uruguay meeting: a *Xenopus* oocyte nucleus, containing hundreds of dark-stained nucleoli.



Electron photomicrograph above, obtained by Oscar Miller, shows amplified 18S and 28S rRNA genes from a newt nucleoli. Each "feather" represents a single transcription unit, with RNA transcription proceeding from left to right. Separating the genes are nontranscribed spacers.

was when he saw Miller's slide that he realized amplification was occurring. The results of his experiments merely bore out his intuition. He says he often works by intuition, because of the way he learns: "I consider myself an extremely slow learner," he explains. "I have a very hard time understanding anything. But once I do, I have tremendous use of it; I have good ability with assimilated information."

Brown had also realized—when he saw Miller's slide—exactly what purpose amplification served. It was the solution to the toad's problem of how to make massive amounts of two substances (18S and 28S rRNA) in a minimum amount of time.

Gene amplification was the first of several gene control mechanisms that Brown would discover in the years ahead. All of them solved the same problem—the demand for quantity—but in quite different ways.

Mulberry Trees in Baltimore. Brown was curious about how amplification worked; how, he wondered, were amphibian oocytes able to amplify some of their genes and not others? But he was even more intrigued by another tantalizing question. Could other genes, besides rRNA genes, undergo amplification?

rRNA genes are "housekeeping" genes; they are found in the cells of virtually all higher organisms, and their final product is not protein, but ribosomal RNA, a structural part of the protein-manufacturing machinery. To determine whether amplification was a control mechanism operating in specialized, protein-producing cells (such as skin or blood cells), it was necessary to find a gene that was turned on in only one cell type and that made its protein in exaggerated quantities. The cell that Brown had his eye on was the highly specialized silk-producing cell in the silkworm, *Bombyx mori.*

The first step was to culture *Bombyx* in the laboratory. Brown knew that silkworms ate mulberry leaves, but where was he to find mulberry trees in Baltimore? At the time, a young Japanese postdoctoral fellow, Yoshiaki Suzuki, was working in Brown's lab. Brown consulted him;

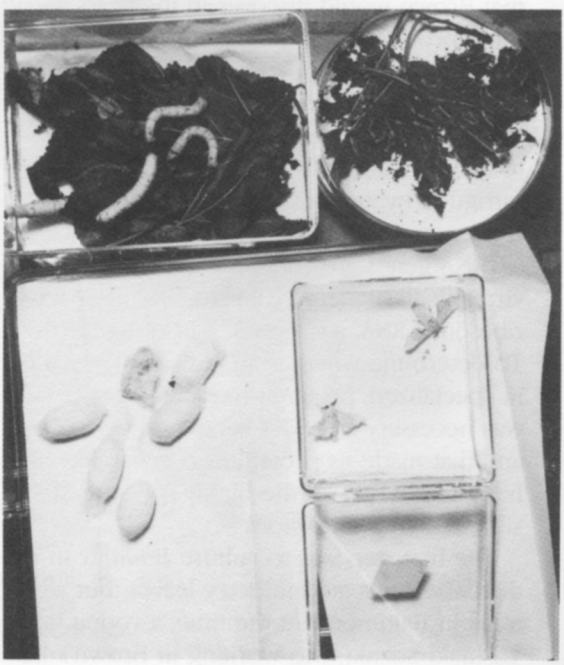
perhaps he would know. But Suzuki was as much in the dark as Brown. Brown put an ad in the newspaper. He soon learned that not only did mulberry trees grow wild in the United States, a clump existed right across the street from his lab!

By 1969, Brown and Suzuki had enough *Bombyx* larvae to begin experiments. Their ultimate goal was to isolate the gene in the specialized silk-producing cell that directed the synthesis of the silk protein, and then determine if this gene was present in the silk cell in greater numbers than it was in non-silk-producing cells.

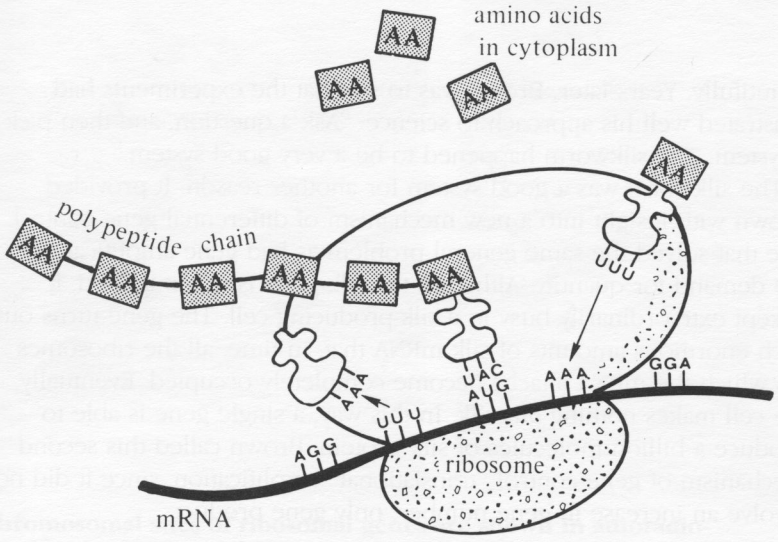
It was a tall order for 1969. The only eukaryotic genes that existed in purified form were the 18S and 28S rRNA amphibian genes—and they had been successfully purified only because of their great abundance. No one knew anything about the silk gene or its messenger RNA.

Quite a lot, however, was known about the silk protein itself. Brown and Suzuki thus reasoned that the thing to do was to work backwards, to look for the messenger RNA by virtue of qualities predicted by the protein. "Our idea," says Brown, "was to prove we had the right messenger RNA—not by showing it made silk, but by showing it had the right sequences characteristic of the silk protein." They knew that the silk protein molecule was very large. They also knew that it consisted mostly of just three amino acids, in a regular repeating pattern.

By this time, scientists knew which sequences of mRNA nucleotides corresponded to the approximately twenty amino acids that make up polypeptide chains. Thus, by knowing the silk protein's amino acid order, Brown and Suzuki were able to predict the nucleotide composition and size of the messenger RNA. Like the protein it made, they reasoned, the messenger RNA should be large—even larger than the largest



Silkworm collage. **At top left, silkworm larvae feed on mulberry leaves. When a larva is mature, after five or six weeks, it proceeds to spin strands of continuous silk, forming a full cocoon (bottom left) in three days. In ten days, a moth (bottom right) emerges; the sexes mate almost immediately, eggs are laid, and the cycle starts again.**



Model for protein synthesis. **A polypeptide chain of amino acids is assembled at the site of the ribosomes, where individual molecules of transfer RNA, each one carrying a single amino acid, interact with the mRNA, which bears DNA's message in a triplet code: three nucleotides code for one amino acid. As each tRNA donates its amino acid to the growing polypeptide chain, it moves away into the cytoplasm to pick up another amino acid. (Note: drawing not to scale.)**

ribosomal RNA. Pinpointing the largest mRNA they could find in the cell, they proceeded to isolate it.

The experiments were complicated. Working with the messenger RNA was cumbersome. First it had to be separated from the other RNAs; then it had to be made radioactive; third, its sequences had to be determined, in order to make sure that it was the correct message. RNA sequencing methods were crude, and the experiments took two years. Finally, by 1971, they were able to show that their suspect had all the features they had predicted for an mRNA encoding the silk protein. It was the first eukaryotic messenger RNA of known function to be isolated in sufficient quantities for biochemical analysis.

Shortly thereafter, Brown and Suzuki, joined by postdoctoral fellow L. Patrick Gage, began the hybridization experiments. Soon, they had an answer to their original question. The silk gene, it turned out, was not amplified. In every kind of cell of *Bombyx* they tested, there was but one gene copy that made silk. Here was the first good evidence that in a highly specialized cell, one that produces one kind of protein and no other, the differential expression of one gene, and not its gain or loss, was what accounted for specialization.* *Bombyx* had served its purpose

*In recent years, researchers have discovered that amplification is, indeed, a valid gene control mechanism for some protein genes in specialized cells. The first evidence for this came in 1980, when Allan Spradling, a colleague of Brown's whose lab is down the hall, discovered that eggshell genes in the fruit fly amplify under certain conditions.

beautifully. Years later, Brown was to say that the experiments had illustrated well his approach to science: "Ask a question, and then pick a system. The silkworm happened to be a very good system."

The silkworm was a good system for another reason. It provided Brown with insight into a new mechanism of differential gene control, one that solved the same general problem as had gene amplification: the demand for quantity. Although the silk gene is not amplified, it is kept extraordinarily busy in a silk-producing cell. The gene turns out such enormous amounts of silk mRNA that, in time, all the ribosomes (to which the mRNA attach) become completely occupied. Eventually, the cell makes nothing *but* silk. In this way, a single gene is able to produce a billion molecules of silk protein. Brown called this second mechanism of gene control "translational" amplification, since it did not involve an increase in gene number, only gene product.

Enter the 5S rRNA Gene

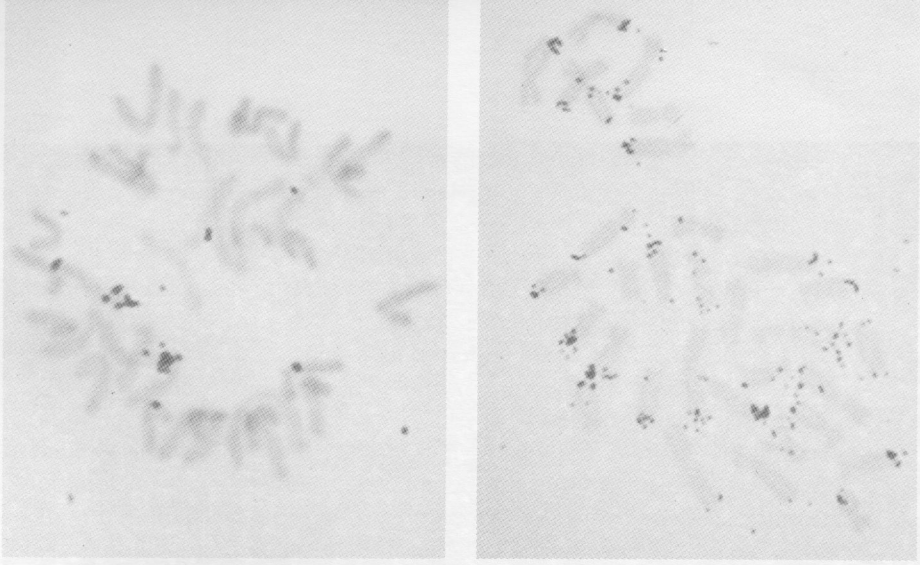
Brown and Suzuki continued to work on the silk gene and its expression for several years. But while Suzuki still studies the silk system at his lab in Japan, Brown returned, with full intensity, to the rRNA genes of *Xenopus*.

A few years before, Brown's attention had begun to shift away from the 18S and 28S rRNA genes toward the smaller, 120-nucleotide-long 5S rRNA genes. In 1968, he had determined that the 5S rRNA genes are not amplified in the oocyte. All cells contain the same number—about 20,000 copies. He then found that the 5S rRNA genes are located not in the nucleoli (as are the 18S and 28S genes) but in clusters at the ends of each chromosome. This was perplexing. Why were all the genes that made ribosome components not located in the same place in the genome?

In bacteria, the 5S rRNA genes sit right next to the 18S and 28S rRNA genes on the chromosome. In fact, the three genes are transcribed together. This scheme made sense to Brown. He had expected to find the same thing in *Xenopus*. When he didn't, he began to suspect that the 5S genes responded to a mechanism of gene control quite different from amplification.

In 1971, he figured out a way to purify the 5S genes. (Purifying the 5S genes turned out to be more difficult than purifying the 18S and 28S genes.) He and his colleagues then began to work out the genes' structure. They found that the 5S genes, like the 18S and 28S genes, were separated from one another by spacer regions. Unlike the 18S and 28S genes, however, the 5S genes did not make an intermediate precursor molecule; the initial transcript was mature.

Brown was impressed by the seeming simplicity of the 5S gene. A great believer in simplicity, he assumed that if he was going to learn anything about gene control, it was to come from the study of the simplest genes he could find. "I wanted to take the tiniest, simplest gene I could," he said years later, "and beat it into the ground." It is an approach, he says, of extreme reductionism. It assumes that heredity



Chromosomal sites of ribosomal genes are shown in autoradiographs, above. The 18S and 28S rRNA genes, shown at left, are present at a single site, the nucleolus, two copies of which are present in each normal *Xenopus* body cell. The 5S rRNA genes, in contrast, are found at the chromosomes' ends.

can be reduced to molecules and their biochemical interactions, and that the results can be applied to other, more complicated genes.

Although the 5S gene seemed to be just what he was looking for, he didn't completely abandon the 18S and 28S genes until late 1971. At that time, he learned that two groups of researchers in Europe discovered that *Xenopus* made not one, but two different kinds of 5S rRNA. All cells made a type called somatic-type 5S rRNA, but oocytes made, in addition, a type called oocyte-type 5S rRNA. The physical difference between the two RNAs was slight, a matter of only six out of 120 nucleotides. Functionally, they appeared to be identical.

Why did *Xenopus* have two kinds of 5S rRNA? To Brown, the answer had to have something to do with control. The toad, "in all its wisdom," must have evolved a dual system of expression to address the same problem solved by the other two mechanisms of gene control Brown had discovered: to produce an exaggerated amount of a particular gene product at a particular time in development. How the mechanism worked he didn't know. Before he could begin exploring function, he had to learn more about the physical nature of the 5S gene. In particular, he needed to learn the exact sequence of nucleotides, not just in the gene, but in the gene's spacers, for he suspected that the spacers might play a crucial regulatory role.

In 1972, the only way to learn a gene's structure was to sequence the gene's RNA product. And so Brown and his colleagues began to make test tube copies of RNA, from purified 5S rRNA genes. The procedures were as crude and arduous as they had been for the silk messenger RNA, and at times seemed endless.

Fortunately, help arrived in mid-decade with the dawn of the recombinant DNA era. Suddenly available were new and faster ways of doing experiments. No longer was it necessary to sequence RNA; DNA could be sequenced directly. Neither did Brown and his colleagues

have to isolate genes from the frog's genomic DNA; individual genes could be "cloned" quickly in order to produce the billions of copies needed for biochemical analysis. This had the added benefit, says Brown, of saving the lives of a lot of toads.

Critical to recombinant DNA technology was the discovery in bacterial cells of a group of enzymes that could cut DNA—any DNA—at specific nucleotide junctions. When added to a suspension of DNA, such an enzyme (called a "restriction enzyme") chops the DNA into isolated fragments having staggered, or "sticky," ends. The fragments can then be spliced, again in suspension, into circular molecules of DNA called plasmids, whose DNA has also been cut at complementary nucleotide positions. Under controlled conditions in bacterial cells, the plasmids replicate quickly, producing billions of identical copies, including their "passenger" DNA, in a matter of days.

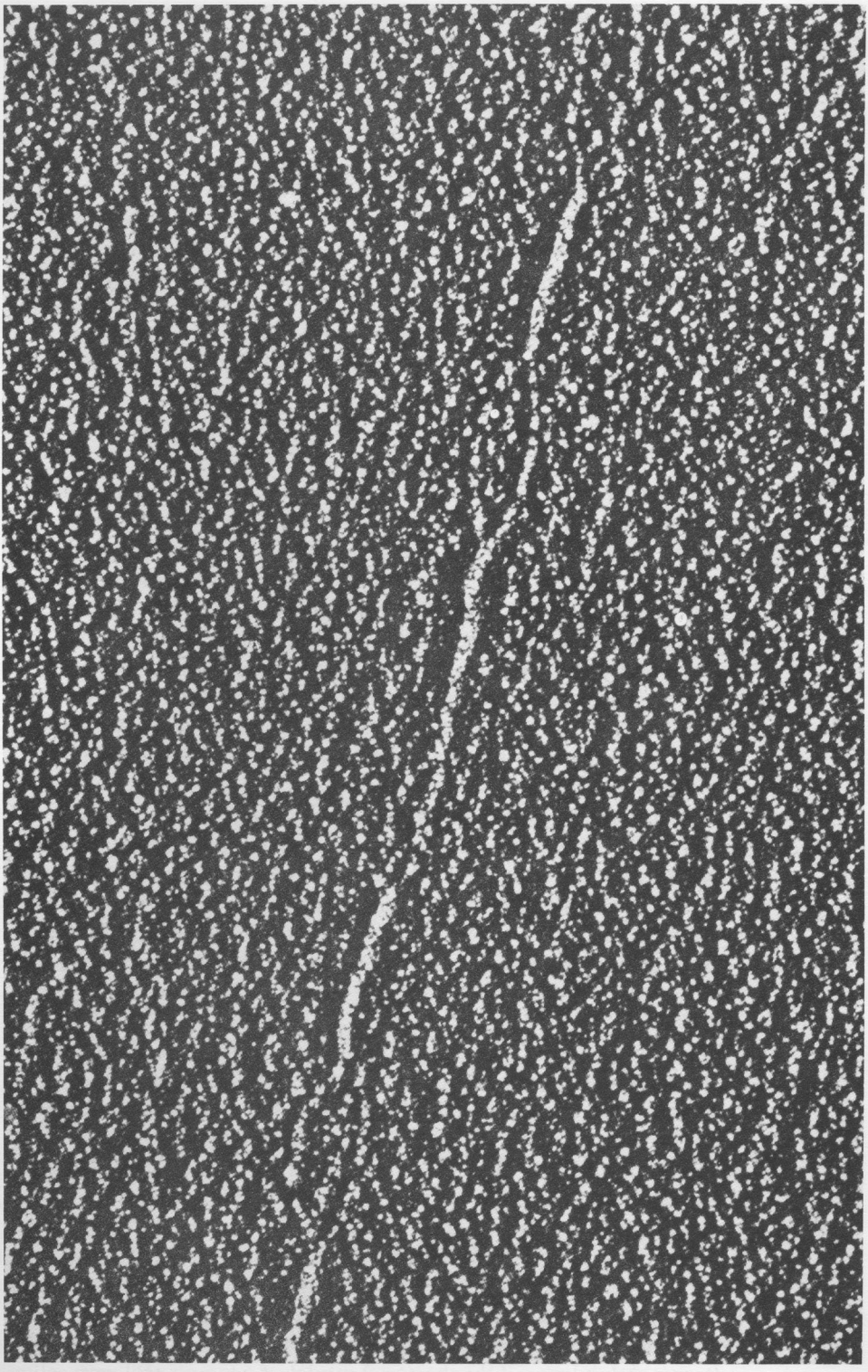
Brown adopted the new recombinant DNA techniques as they became available. He was the first, in fact, to clone a *Xenopus* 5S rRNA gene. (Brown's cloning work, unlike that of some scientists, was not held back by debates about safety, which arose shortly after the recombinant DNA techniques were introduced. He was, however, an active participant in the discussions.) Brown and his colleagues were also the first to sequence the DNA of the 5S rRNA gene. The gene, of the oocyte-type variety, was the very first animal gene to be sequenced entirely.

This was accomplished in 1977 by Brown, postdoctoral fellow Nina Fedoroff, and George Brownlee, a British collaborator. The group also sequenced the DNA of the oocyte-type gene's long spacer regions. In the process, they discovered what appeared to be an incomplete 5S rRNA gene positioned in the middle of each region. They named this fragment, identical in each spacer, a "pseudogene." A curiosity in 1977, pseudogenes are today known to be associated with many different kinds of genes. They are most likely illustrative, says Brown, of extra genes duplicated but not needed by an organism and so gone awry in its evolution.

Two years after the oocyte-type gene was sequenced, others in Brown's lab succeeded in sequencing a somatic-type 5S rRNA gene. It had taken so long because the somatic-type genes proved harder to find. It soon became clear why this was so: for every 50 oocyte-type genes in a *Xenopus* cell, there exists only a single somatic-type gene.

It was immediately apparent to Brown that the uneven distribution of the 5S genes was part of the mechanism for meeting the organism's uneven demand for ribosomes. Oocytes need an enormous supply of rRNA to make an enormous number of ribosomes. (The ribosomes are used by the embryo for up to a week after fertilization.) Thus, both the large, oocyte-type 5S rRNA gene family (containing 20,000 genes per chromosome set) and the small, somatic-type 5S rRNA gene family (containing 400 genes per chromosome set) are turned on in the oocyte. In all other cells of the organism, however, the demand for ribosomes is considerably less. In these cells, the oocyte-type genes are not required; only the somatic-type genes remain on.

Here, in these two gene families, was a gene control mechanism



Electron micrograph of oocyte-type 5S DNA from *Xenopus*. The two strands of the DNA are partly separated, or denatured. Each repeating unit consists of a spacer region (denatured) and a gene region (undenatured).

unlike any Brown had previously studied. But how did it work? How did *Xenopus* use these two families—one small, everyday family and one huge, auxiliary family—to control the production of 5S rRNA? What was different about the two types of genes, and, further, what was different about the two types of cell environments in which each worked?

By the time Brown began seriously thinking about these questions, the structural studies on the 5S rRNA gene system were done. Says Brown: "We had our genes cloned, characterized, and sequenced. We could make as many copies of each as we wanted. Now it was time to start studying their differential expression." He set a new goal for his laboratory: to reconstruct the molecular controls regulating the differential expression of 5S rRNA in living cells.

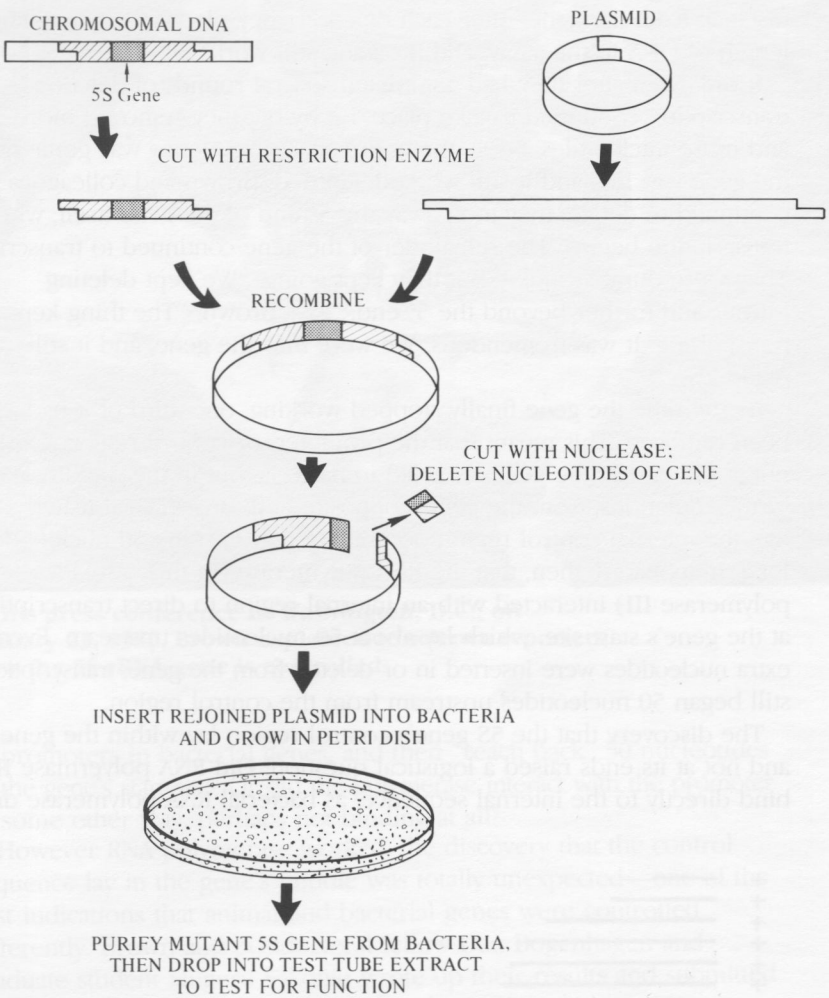
On and Off: Exploring Function

In order to study 5S rRNA gene function in a systematic way, it was necessary to have an *in vitro* (test tube) assay system in which the genes worked just as they did within *Xenopus* itself. Anticipating this need, Brown and his colleagues had begun working to develop an assay system as early as 1973. They finally perfected one in 1978. Consisting of an extract made from hand-isolated oocyte nuclei in which both kinds of genes worked, the assay provided experimental access to the 5S genes. "Combined with the new methods of recombinant DNA," said Brown later, "we could do all sorts of tricks with the genes. We could mutate them, change a nucleotide here, another one there, and then drop them into the assay and watch what happened."

As a first step in exploring function, they needed to know what signals in the DNA caused the genes to start and then stop transcribing at exactly the right spots. The DNA in eukaryotic cells consists of very long, unbroken chains of nucleotides. Which sequences, they wondered, specified the end of one 5S rRNA gene and the beginning of the next? They also needed to know what molecules in the cell "read" those control sequences to activate the genes at the proper time during development.

They had little precedent to follow. The only genes for which any understanding of control then existed were the genes of bacteria. Most bacterial genes, according to the simplest model of control, turn on when an enzyme called RNA polymerase recognizes and binds to a small region of DNA—the "promoter"—that sits immediately adjacent to the gene's start site, the so-called 5′ end. Once it binds to the promoter, RNA polymerase then trundles along the length of the gene, prompting the gene's double-stranded DNA to unzip so that RNA transcription can proceed. The gene continues to be transcribed until RNA polymerase reaches a specific nucleotide arrangement at the end of the gene (at the 3′ end) that says "stop."

Brown didn't know if this bacterial model of gene control was relevant to gene control in eukaryotic cells. Besides containing DNA more abundant and complicated than bacterial cells, eukaryotic cells were known to contain three types of RNA polymerase (designated I, II,



Cloning and deletion of 5S DNA. **DNA bearing the gene and its spacer sequences is cut with a restriction enzyme that makes the ends "sticky." This segment is recombined with the DNA of a bacterial plasmid having complementary sticky ends. Sequences are removed from the spacer sequence by another enzyme, called a nuclease, which is able to digest one or two nucleotides at a time. The plasmid is reannealed and inserted in a bacterium. In a matter of days, billions of copies of the plasmid and the inserted gene segment have been reproduced. The gene segment is purified from the bacteria and then assayed to see if it still makes 5S rRNA.**

and III). This suggested that at least three different kinds of promoters existed among eukaryotic genes.

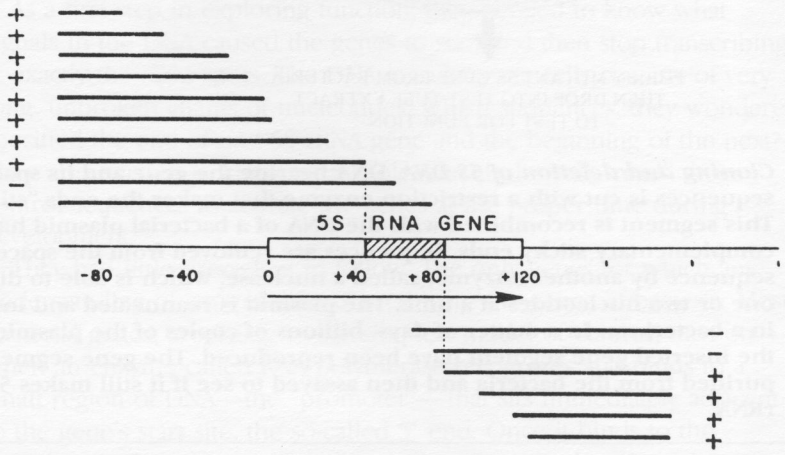
Nevertheless, DNA is DNA wherever it is, and Brown saw no reason why bacterial and eukaryotic genes should not behave in fundamentally similar ways. He thought it certain, for instance, that the 5S rRNA gene would have a promoter, and that the promoter would be adjacent to the gene's 5′ end, as in bacterial DNA. He and his colleagues thus began a systematic effort to delete (by chemical means) the nucleotides in the

spacer region near the 5S rRNA gene, a few at a time, starting at some distance from the gene. After each deletion, they placed the remaining length of DNA in the assay. Did the gene still work?

It did. Even after they had completed several rounds of deletions, transcription continued to take place. Bit by bit, they removed more and more nucleotides. Soon, the entire spacer sequence was gone; only the gene was left, and it still worked. Puzzled, Brown and colleagues continued to delete; they took away the 5′ end of the gene itself, where transcription begins. The remainder of the gene continued to transcribe! They were dumbfounded. But they kept going. "We kept deleting further and further beyond the 5′ end," says Brown. "The thing kept transcribing. It was tremendous. We were *into* the gene, and it still made RNA."

By the time the gene finally stopped working, one-third of it had been removed. This meant that the promoter, or control region, existed not at the end of the gene, as it did in bacteria, but in the middle. With further deletions, from the gene's opposite end, Brown established that the internal control region occupied a region some 50 nucleotides long. It appeared, then, that the RNA polymerase (in this case, RNA polymerase III) interacted with an internal region to direct transcription at the gene's start site, which lay about 50 nucleotides upstream. Even if extra nucleotides were inserted in or deleted from the gene, transcription still began 50 nucleotides upstream from the control region.

The discovery that the 5S gene's control region lay within the gene and not at its ends raised a logistical question. Did RNA polyermase III bind directly to the internal sequence, as bacterial RNA polymerase did



Delimiting control. **The 5S rRNA gene is represented by the rectangular box. Arrow denotes the direction of transcription. Nucleotide deletion experiments are shown as lines above and below the gene. Each line is scored with a plus or minus sign. A plus sign means that after deletion, the mutant still worked when placed in the assay system; the minus sign indicates that it didn't. When deletions fell within the cross-hatched region, the gene failed to function. This region, concluded Brown, is the internal control region.**

At his press conference in Washington, D.C., on January 23, 1980, Brown explained to science reporters how the 5S rRNA gene is controlled.

to promoters in bacterial genes, and then "reach back" 50 nucleotides to the gene's start site? Or did the polymerase interact with the promoter in some other way, perhaps not binding at all?

However RNA polymerase worked, the discovery that the control sequence lay in the gene's middle was totally unexpected—one of the first indications that animal and bacterial genes were controlled differently. Brown and postdoctoral fellow Dan Bogenhagen and graduate student Shigeru Sakonju wrote up their results and submitted the paper to the journal *Cell*. It was published in January 1980. Simultaneously, Brown held a press conference at the Carnegie Institution headquarters in Washington, D.C. The next day, stories appeared in newspapers around the world. A picture of *Xenopus* even graced the pages of *Newsweek*. The molecular genetics of eukaryotes, it was clear, was coming of age, and Donald Brown was leading the way.

Rewards and Obligations

Don Brown had just turned 49 years old. He had published dozens of scientific journal articles and given numerous talks at meetings and conferences. His work was widely known and respected. He had received his first major award—the U.S. Steel Foundation Award in Molecular Biology, from the National Academy of Sciences, in 1973. He had been elected to the Academy, itself a considerable honor, in that same year. Later had come awards from the Roche Institute for Molecular Biology (1975), the University of Chicago (1976), and the New York Academy of Sciences (1977). The citation from the Roche Institute read,

Brown received the Ross G. Harrison Prize from the International Society of Developmental Biologists in 1981. At the ceremony, which took place in Switzerland, on the banks of the Rhine River, Brown's wife, Linda, received a bouquet of flowers from Alberto Monroy, left, Doyen of the Society.

in part, "Throughout his work, Dr. Brown has demonstrated a remarkable ability to clarify problems that at first sight looked inextricable. . . . His scientific publications are models of experimental thoroughness. Dr. Brown may truly be regarded as one of the founders of experimental embryology."

That was only the beginning. In 1981, he received the first Ross G. Harrison Prize from the International Society of Developmental Biologists. Soon thereafter, he received the Rosensteil Award in Basic Biomedical Science from Brandeis University, and the Louisa Gross Horwitz Award from Columbia University.

As Brown's stature in the scientific community increased, so too did his professional obligations. He was, and still is, active in many professional societies. He has served on the editorial boards of scientific journals as well as on advisory boards of the National Institutes of Health and the National Science Foundation. He has been on visiting committees of numerous institutions and university biology departments. In 1981, he founded and became president of the Life Sciences Research Foundation, an international postdoctoral fellowship program designed to find support for young scientists working in nonprofit institutions.

In addition, he has been, since 1976, the director of the Department of Embryology. As director, he oversees seven staff members, dozens of postdoctoral and predoctoral fellows, and a small support staff. He holds the reins as loosely as possible, trying to keep paperwork and administrative duties to an absolute minimum—all so he can spend as

much time as he can in his lab, on his first and foremost commitment, research.

The Puzzle of Differential Control

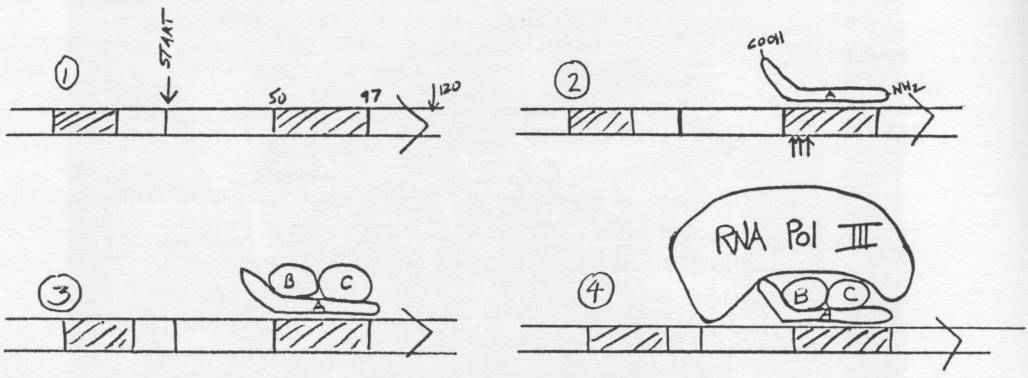
Brown and members of his lab were not the only scientists working on the 5S rRNA genes. Other investigators, many of them former postdoctoral fellows at the Department of Embryology, were also studying the system. Shortly after Brown made his major discovery, one of these former fellows, Robert Roeder (then at Washington University in St. Louis), isolated a very abundant protein from *Xenopus* ovary tissue which bound to the DNA at the center of each 5S rRNA gene—to the very region Brown had found was responsible for controlling transcription.

Teaming up immediately (collaborating by phone and express mail), Brown and Roeder soon found that the binding of the protein to the 5S rRNA gene's internal control region was essential to the gene's proper functioning; the correlation, Brown said later, was perfect. It thus appeared that RNA polymerase III did not recognize the naked DNA of the internal control region, but recognized instead the protein *bound* to that DNA. Shortly thereafter, Roeder discovered that the presence of the protein factor was not alone sufficient to turn on the gene. At least two other factors were required. Only when all factors together were present could RNA polymerase III begin its transcribing dance down the gene.

To distinguish the first transcription protein from the others, Roeder named it TFIIIA: "TF" for transcription factor, "III" because it was recognized by RNA polymerase III, and "A" because there were B and C and perhaps even more factors involved. (These other factors to this day have not been identified; indeed, no one knows exactly how many there are or how they work).

By 1982, Brown and postdoctoral fellow Dan Bogenhagen had determined that the protein factors TFIIIA, B, and C appeared to bind not only to the 5S DNA but also to each other, in some unknown way that conferred remarkable stability. When all the factors were bound together—protein to protein, and protein to DNA—the complex (on both genes, somatic-type and oocyte-type alike) stayed intact even through numerous rounds of RNA transcription.

The discovery of the stability of the 5S rRNA gene's so-called "transcription complex" intrigued Brown. In fact, he explained later, it was this discovery—more than any other—that changed forever the way he thought about eukaryotic gene control. For it was suddenly clear to him that a transcription complex might provide a molecular mechanism for memory. If so, it could explain one of the oldest mysteries of embryology—how it is that committed cells are able to maintain their differentiated states for long periods of time. It could explain why, for instance, a blood cell can make almost nothing but hemoglobin during its lifetime: its globin genes are programmed into stable transcription complexes, while its other genes are not.



When Brown gives talks about his work, he uses this series of hand-drawn sketches to illustrate the possible configuration of the 5S rRNA transcription complex. The rough appearance tells his listeners that the intimate details of the interactions among the components are still unknown. Sketch (1) shows the 120-nucleotide-long gene, with the internal control region occupying the cross-hatched area in the center (nucleotides 50–97). Sketch (2) shows possible placement of TFIIIA over the control region. The three arrows show the location of three of the six nucleotides that differ within the somatic-type and oocyte-type genes. The COOH terminal end of TFIIIA is not involved in binding but is essential for transcription. Sketch (3) shows factors B and C stabilizing TFIIIA to form a transcriptionally competent complex. In sketch (4), RNA polymerase III binds to the complex, reaching out to the start site at arrow, turning the gene on.

General speculation aside, Brown realized at this point that he could rephrase his question of differential 5S rRNA gene control in terms of the transcription complex. Why, if the somatic-type and oocyte-type 5S rRNA genes share the same transcription factors, are only the somatic-type genes bound into transcription complexes in somatic cells, while the much more abundant oocyte-type genes are not?

A definitive answer to this question required what Brown did not then have: an assay system that reproduced, in the test tube, the somatic cell environment, where only the somatic-type genes are expressed. (The assay system they were then using mimicked only the oocyte, where both genes are expressed.) Though a high-priority goal in his lab, no one had yet been able to devise such a system. That particular success would not come for several more years.

The Timetable of Control. Meanwhile, Brown and his colleagues began to study the problem indirectly. One of the first orders of business was to determine the developmental timetable of 5S rRNA gene control. In other words, they wanted to know when exactly the oocyte-type 5S rRNA genes turn off during *Xenopus* development.

With the help of postdoctoral fellow Michael Wormington, Brown determined that control is exerted within a very narrow time frame,

within only a few cell divisions. He suggested a scenario in which both oocyte-type and somatic-type genes work "flat out" in the developing oocyte. Once the mature egg is fertilized, both types of genes shut off; they remain off until the embryo reaches the 4000-cell, or mid-blastula, stage—about twelve cell divisions after fertilization. (During the first eleven cell divisions, cleavage is so rapid that there is no time for RNA synthesis; thus, not only are the 5S genes turned off, so are all the other genes.*) Once cell division slows down, all of the somatic-type genes turn back on. But only some of the oocyte-type genes resume function; Brown says these genes are "leaky." Only two cell divisions later (by the gastrula stage), all of the oocyte-type genes have turned off. Only the somatic-type genes are left on, and they remain on throughout the life of the organism.

Examining this sequence of events more thoroughly, Brown noted a curious correlation. The rapid loss of oocyte-type gene activity seemed to be closely related to a correspondingly rapid drop in cellular TFIIIA concentration. Though present in oocytes in enormous quantities (some 10 million molecules per gene), TFIIIA in mature, somatic cells was present in such low quantities as to be barely detectable. This decrease in TFIIIA concentration occurred in direct proportion to the rate of cell division: each time a cell divided, the level of TFIIIA was cut in half. By the time an embryo reached the gastrula stage, TFIIIA concentration had fallen to about one hundred molecules per gene. Since the 5S rRNA in these cells is made only by the somatic-type genes, a thousandfold differential in transcription rates existed between the somatic-type and oocyte-type genes.

What did the correlation between the rapid drop in TFIIIA concentration and the repression of the oocyte-type genes say about this large difference in transcription rates? Brown suspected that the answer had something to do with another curious phenomenon, one that Wormington and Dan Bogenhagen had discovered a year earlier, in 1982, while studying 5S rRNA gene expression in the oocyte assay.

In their studies, Wormington and Bogenhagen had found that the transcription complexes on both types of genes, once formed, were equally stable in the oocyte assay. However, the somatic-type genes appeared to be about four times more efficient in *forming* transcription complexes. This could only be due to a slight difference in the two genes' internal control regions (where TFIIIA bound), a difference, they had found, of only three nucleotides. Otherwise, the internal binding regions were identical.

The small, fourfold difference in TFIIIA binding efficiency was not nearly enough to account for the huge difference in transcription rates found in somatic cells. And so, when first considered in this context, the results were set aside. But when Brown discovered the correlation between TFIIIA concentration and loss of oocyte-type gene expression, he could not help but wonder if Wormington and Bogenhagen's results

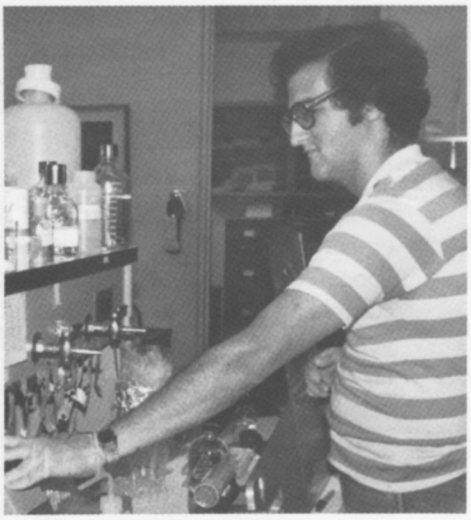
*The 4000 cells at this stage may look the same, but they have already begun the complex and poorly understood process of specialization.

were but the "tip of the iceberg." If the two genes bound TFIIIA with unequal efficiency, might it be possible that a low concentration of TFIIIA in somatic cells creates an environment so competitive for TFIIIA that only the genes most efficient at binding it (the somatic-type genes) remain on?

Brown thought so. He worked out a simple, hypothetical model explaining how such a scheme might work not only for the 5S rRNA genes but for all closely related genes that share the same transcription factors. According to the model, if two or more genes bind to a transcription factor with unequal affinity, they could be turned on and off at different times according to the concentration of that transcription factor in each cell. When the transcription factor concentration is high, all of the genes that bind to it are on. As the concentration drops, the genes turn off according to how tightly they bind. The gene that binds the least tightly is the first to be turned off. The tightest-binding gene turns off last.

Brown's model was clean and elegant, but still there was no direct evidence that it actually worked to control the 5S rRNA genes. As it would turn out, the model—or, rather, its major premise—would be proved wrong. But that was to come later. For two years, the model provided a valuable frame for further exploration.

The State of Repression. In 1984, Brown and Mark Schlissel, a Johns Hopkins University graduate student, began an effort to learn more about the repressed state of the oocyte-type 5S rRNA gene. They knew already that an active 5S gene, be it somatic-type or oocyte-type, is programmed into a stable transcription complex composed of multiple proteins. But what about a turned-off oocyte-type gene in a somatic cell? What did it look like?



Mark Schlissel

Brown and Schlissel found they could isolate a nucleus from a living adult *Xenopus* cell, and place it, very gently, in the *in vitro* transcribing extract. The genes within that nucleus were in their native chromatin state, that is, their DNA was bound up with a set of proteins called histones that are normally present in mature eukaryotic cells. The use of genes in their native state represented a significant departure for Brown. In all previous experiments, he and his colleagues had used purified DNA.

As Brown and Schlissel hoped would happen, the chromatin-bound oocyte-type genes were repressed in the *in vitro* extract, just as would occur in a mature, chromatin-containing somatic cell. The reason, they

Members of Brown's lab, 1986: Left to right: Alan Wolffe, Kent Vrana, Riccardo Losa, Eddie Jordan, and Matt Andrews.

soon found, was because of the presence in the chromatin of a common histone protein called histone 1, or H1. H1 somehow made the gene "invisible" to the transcription factors. Even when Brown and Schlissel added additional factors to the extract, the gene failed to turn back on.

A New Model

According to the model Brown had earlier devised, the fourfold difference in TFIIIA binding affinity was critical in controlling gene expression. It gave the somatic-type genes a thousandfold advantage over the oocyte-type gene when TFIIIA's concentration was low, as it is in somatic cells. The results of Schlissel's experiments explained the ultimate fate of the oocyte-type genes in somatic cells: they become bound up with H1. But the results offered no explanation why only the oocyte-type genes, and not the somatic-type genes, fall prey.

Then, in early 1986, a new postdoctoral fellow in Brown's lab, Allan Wolffe, started doing experiments on 5S rRNA gene expression using a new *in vitro* extract he had developed from unfertilized *Xenopus* eggs. In this extract, the somatic-type genes worked not four times more efficiently than the oocyte-type genes (as they had in experiments using the oocyte assay), but up to 100 times more efficiently. This was the closest anyone had come to reproducing the thousandfold differential in transcription rates present in the somatic-cell environment. It was welcome and exciting news.

As Wolffe continued to study the differential transcription of the two types of 5S genes in the new assay, however, he found an unexpected twist: unequal binding affinity for TFIIIA seemed to have little to do with transcription rates. Rather, the critical difference in gene expression

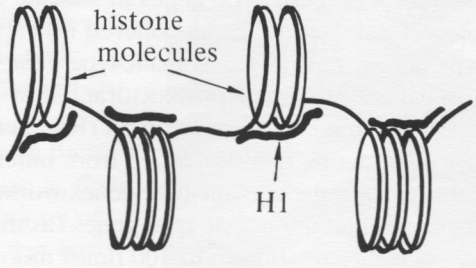
appeared to involve a difference in *stability* of the genes' transcription complexes. In the new assay, the transcription factors surrounding the somatic-type genes formed inherently stable complexes (that is, they remained physically associated with the genes), while those on the oocyte-type genes did not; even if an oocyte-type gene already in a transcription complex was placed in Wolffe's extract, the complex fell apart.

The results were confounding. In no experiments using the original *in vitro* oocyte assay had there been any evidence suggesting a difference in stability of the transcription complexes around the two types of genes: both complexes, once formed, had appeared to be equally stable. Why, then, did the oocyte-type transcription complexes disintegrate in the somatic-cell extract? And why did the same complexes remain intact on the somatic-type genes?

The answer, as this essay goes to press, is not definitively known. But Brown strongly suspects, if Wolffe's extract does indeed simulate a somatic cell environment, that the difference in stability has something to do with the formation of nucleosomes.

Nucleosomes are bundles of chromatin strung along the chromosomes of eukaryotic cells. Brown suggests that the process of nucleosome formation is a particularly turbulent one, creating a "stormy" environment that the transcription complexes on the oocyte-type genes, being inherently unstable, can't tolerate. Unable to bind to transcription factors, the oocyte-type genes instead become assembled into chromatin. Wrapped around nucleosomes, anchored by histone H1, the genes are packaged into oblivion.

Not all oocyte-type genes, however, turn off at the mid-blastula stage. The reason, suggests Brown, is twofold: (1) enough factors are still present at this stage to keep a handful of these genes on, and (2) the chromatin is not completely mature. By the time the nucleosomes are fully complexed with histone H1 (by the gastrula stage), all of the



In eukaryotic chromosomes, DNA is wound around histone proteins to form a string of nucleosomes. In Brown's model, histone H1 (solid bars) compact the chromatin and prevent the formation of transcription complexes.

oocyte-type genes have turned off. Meanwhile, the stable transcription complexes around the somatic-type genes allow these genes, as Brown says, "to weather the storm and hang on" as the cell's chromatin matures.

If this scenario is correct, it leads to another question. Why, if oocyte-type transcription complexes are inherently unstable, are oocyte-type genes able to work in the oocyte at all? Brown says the answer to this question may provide the key to the 5S rRNA gene control mechanism. It may be, he suggests, that an oocyte-type gene works in the oocyte because (1) it is surrounded by massive amounts of transcription factors, and (2) it has not yet condensed into chromatin.

Accordingly, Brown has revised his model. Instead of emphasizing differential *binding* of genes for transcription factors as factor concentration declines, Brown's new model emphasizes differential *stability* of transcription complexes as chromatin matures: those genes having the least stable transcription complexes turn off first, those with the most stable complex turn off last (if they turn off at all).

Brown is fully aware that his new model cannot explain why the transcription complexes around the somatic-type genes are more stable than those surrounding the oocyte-type genes. He is now looking for the answer to that question in the interactions of gene, TFIIIA, and the still-unidentified transcription factors (B and C) that form the transcription complex. "It's an evolving problem," he says. "It's almost at the stage where it's more complicated than it was before. It isn't simply TFIIIA and binding affinity we're looking at now, but the whole complex. The whole complex is the fundamental unit of control."

Right now, the interactions within the transcription complex and between transcription complex and gene remain mysterious. As Brown says: "We know more about the differential control of these two genes on the molecular level than we know about the differential control of any gene. But we don't know the full story yet."

Some Generalizations

Even so, Brown strongly believes that the 5S rRNA gene system offers some valuable generalizations about eukaryotic genes and their control. First, he believes that most, if not all, eukaryotic genes will be found to require transcription complexes in order to be visible to (and hence turned on by) the various RNA polymerases in eukaryotic cells. "The location of the gene control sequences may differ," he says. "They may be behind, in front of, in the middle of, or even away from the gene, but in some way all eukaryotic genes must share the same general mechanism, whereby multiple and different proteins interact with each other and with the DNA sequences of a gene to form a transcription complex."

Brown believes further that the stability of transcription complexes, besides being the crucial part of the differential control mechanism, might play a critical role in the maintenance of already-turned-on genes.

The somatic-type 5S rRNA transcription complex can exist in the same conformation for days and weeks, and conceivably for years, providing a constant DNA template for 5S rRNA production. If such complexes are stably associated with other type genes, they may explain the continued differentiated state of committed cells in, say, liver or muscle tissue.

Brown contends that eukaryotic genes might also share some general mechanisms of repression. It is likely, he says, that histone 1 interacting with nucleosomes is responsible for the stable repression of many different genes during nucleosome formation, not just the 5S rRNA oocyte-type gene. It does so presumably by keeping the unused genes wrapped up and out of the way, where they are invisible both to the polymerase and to small amounts of their transcription factors. This kind of repression, which doesn't occur in bacterial cells, may be necessary in eukaryotes, Brown suggests, because eukaryotic cells contain up to a thousand times more DNA than bacteria. By making so many genes invisible at once (by assembling them into chromatin), the cell makes it easier for the RNA polymerase to locate genes programmed into transcription complexes.

There is one further area that Brown feels might be explained by transcription complexes, and that is the puzzle of cell commitment. This has been, and remains, a central problem in embryology. For years scientists have sought the trigger that sends one cell down one developmental pathway and another cell down another. Many have thought this trigger lies in geographically distinct "determinant factors" that direct cell fate simply by virtue of their locations, or microenvironments, in the early embryo.

Brown suggests that such determinant factors, if they in fact exist, may be nothing more than transcription factors that are asymmetrically localized in the egg cell and thus unequally distributed to the daughter cells. Instead of being present in a time gradient (as occurs with TFIIIA, B, and C during 5S rRNA gene expression), the transcription factors may be present in a gradient of space. Once a cell comes in contact with a specific transcription factor, its destiny may be forever determined.

Brown cautions, however, that the puzzle of cell commitment, indeed of development in general, is complex, and no easy answers will solve it. He realizes that a simple, two-dimensional, reductionist model of gene control—such as his model of the 5S rRNA gene system—may not be able to explain the behavior or control of more-complex genes. Most eukaryotic genes, for example, contain introns, which are segments of "nonsense" DNA that interrupt a gene's continuity. The role of introns is not known. Neither is it known how whole batteries of genes are coordinated, how tissues interact with each other, how patterns form.

Donald Brown has helped set the stage on which to explore these and other questions. He has helped define the problems, and he has established some general rules. There is no doubt that future work on gene control will rest on foundations he has built. Meanwhile, he himself continues to push the limits of knowledge.

GLOSSARY

Amino acid. A molecular building block of protein. Twenty different kinds exist.

Assay. To test experimentally.

Blastula. The end result of the first stage of embryonic growth characterized by the production of a large number of cells by rapid cell division. Usually results in a hollow sphere.

Cellular commitment. The process by which noncommitted (immature) cells become committed, or dedicated, to a particular path of development leading to a highly specialized cell.

Centrifugation. The spinning of a mixture at very high speeds to separate substances of different densities.

Chromatin. The total complex of DNA and proteins in the nucleus of eukaryotic cells.

Chromosome. One piece of chromatin containing one long molecule of DNA bearing many genes.

Clone. To make many identical copies.

Codon. A group of three adjoining nucleotides that codes for a particular amino acid.

Complementarity. Characteristic of two strands of DNA and RNA products made from DNA. Nucleotides on one strand correspond to the appropriate nucleotides on the other, with adenine matching thymine (or uracil in RNA), cytosine matching guanine.

DNA. Deoxyribonucleic acid. The genetic material; contains instructions for the ordering of amino acids in proteins.

Egg. A mature female reproductive (germ, or sex) cell that has undergone meiosis and is ready to be fertilized.

Embryology. The study of the formation and development of the individual from the fertilized egg to the adult.

Eukaryotic cell. A cell containing a nucleus; found in all organisms except bacteria.

Fertilization. Fusion of the nuclei of egg and sperm.

Gastrulation. The stage of embryonic development where the cells of the blastula rearrange themselves into distinct layers; characterized by rapid RNA synthesis.

Gene. A portion of a DNA molecule that codes for a particular product; the unit of inheritance.

Gene amplification. An increase in the amount of a particular gene.

Gene purification. The separation of a stretch of DNA (corresponding to a gene) away from the rest of the cell's DNA; also called gene isolation.

Gene sequencing. Determining the order of nucleotides along a particular DNA molecule.

Genome. The total of an organism's genetic material.

Histone proteins. Basic proteins that bind to DNA and serve as the structural elements of eukaryotic chromosomes.

in vitro. Not in the living organism; in the test tube.

in vivo. In the living organism.

Meiosis. A process of nuclear division in which the number of chromosomes in a cell is cut in half; occurs during the production of sex cells.

Messenger RNA, or mRNA. The molecule that bears genetic information from the DNA to the ribosomes, where the information is used to determine the order of amino acids along a protein chain; mRNA comprises only 2–5% of a cell's total RNA.

Molecular biology. The study of life at the level of the molecules (proteins and genes) of the cell.

Mutation. A heritable change in DNA.

Nucleolus. A dense body within the nucleus, usually attached to one of the chromosomes; contains multiple copies of genes for ribosomal RNA.

Nucleosome. A bead-like unit of chromatin containing a length of DNA wrapped around histone proteins.

Nucleotide. A chemical entity consisting of a 5-carbon sugar with a phosphate group and either a purine or pyrimidine nitrogenous base attached; building-block unit of DNA and RNA.

Nucleus. A membrane-bounded organelle containing the chromosomes.

Oocyte. An immature egg cell, formed in the ovary.

Promoter. A specific nucleotide segment that directs RNA polymerase to initiate transcription at the start of a gene.

Prokaryotic cell. A cell that lacks a nucleus; found only in bacteria.

Protein. A large polymer consisting of many amino acid building blocks linked together.

Recombinant DNA. DNA spliced together from more than one source.

Restriction enzymes. Enzymes which work by cutting DNA at particular nucleotide sequences, thus breaking the chain into small pieces. Molecular biologists now use some 150 known restriction enzymes to produce recombinant DNA.

Ribosomal RNA, or rRNA. A major component of ribosomes; comprises approximately 80% of the total cellular RNA.

Ribosome. A small cytoplasmic organelle, composed of numerous proteins and RNA, which functions in protein synthesis; a cell may contain half a million, the number varying with the amount of protein the cell manufactures.

RNA. Ribonucleic acid. The direct product of DNA when a gene is expressed.

RNA polymerases. The enzymes that assemble the nucleotide building blocks into RNA using a DNA template.

Somatic cell. Any cell of a eukaryotic organism except the germ (or sex) cells.

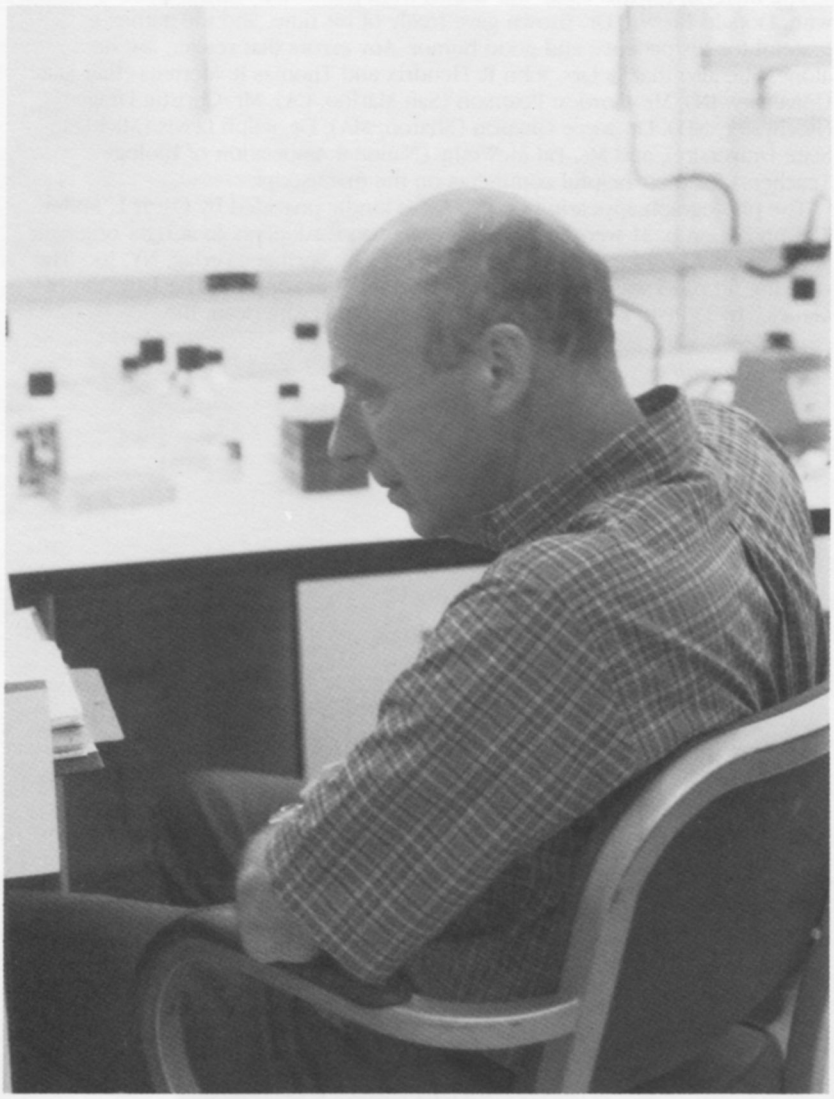
TFIIIA. The first-discovered protein transcription factor in eukaryotes; required for accurate transcription of the 5S rRNA gene.

Transcription. The synthesis of RNA along a DNA template.

Transfer RNA, or tRNA. An RNA molecule that transports amino acids to the ribosomes during protein synthesis.

Translation. The conversion of the genetic information carried by an mRNA molecule into the precise arrangement of amino acids in proteins.

***Xenopus laevis*.** The species of African clawed toad used by Donald Brown as his principal experimental organism.



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The photograph appearing on p. 17 was kindly provided by Oscar L. Miller. The photos on p. 21 were provided by Mary Lou Pardue; photo at right originally appeared in *Chromosoma 42*, p. 191, © 1973 by Springer-Verlag, NY, Inc. The drawing on p. 25 was adapted from a diagram appearing in "The Isolation of Genes," by Donald D. Brown, © 1973 by Scientific American, Inc.

Patricia Parratt
October 1988