Developmental biology using purified genes

Donald D Brown

Some history

After three years of college I entered the University of Chicago Medical School in the fall of 1952 and discovered biochemistry and research. Lloyd Kozloff, a member of the bacteriophage group in the Department of Biochemistry, guided my research. While in medical school I began searching for a future research subject, thinking it should be an important medically related problem but unexplored by what were then the modern methods of biochemistry.

The field of embryology, newly named 'developmental biology', caught my attention. Reproductive biology was barely discussed, and descriptive embryology was taught in two lectures as a part of gross anatomy. In 1953, I attended a biochemistry journal club discussion of the Watson-Crick *Nature* paper describing the structure of DNA. I knew immediately that my future would involve some merger of DNA research, biochemistry and the field of embryology.

After an internship at Charity Hospital in New Orleans and my wedding, my wife and I moved to Bethesda, Maryland. My two years of service were fulfilled as a member of the first class of 'research associates' at the US National Institutes of Health (NIH). I spent two wonderful years in the Institute of Mental Health as part of a project organized by Seymour Kety to investigate systematically the metabolism of molecules that had been implicated in schizophrenia. I was assigned to study the urinary metabolites of the amino acid histidine because it was the precursor of one such compound—histamine. I ended my two years by feeding 10 microCuries of ^{14}C-histidine to ten healthy controls and ten people with schizophrenia, collecting their urine and analyzing the radioactive products. Then I published one of the few negative studies on the subject of the biochemical basis of schizophrenia.

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From the NIH I went to the Pasteur Institute in Paris to study bacterial gene regulation in 1959, the year after the Lac repressor had been discovered. Before leaving Bethesda, by the greatest luck I learned about a small research institution in Baltimore that was associated at that time with the Johns Hopkins Medical School called the Department of Embryology of the Carnegie Institution of Washington. I contacted Jim Ebert, the director, and arranged an advanced postdoctoral fellowship after my year in Paris. It is hard to imagine two more diverse research institutions.

The Pasteur Institute was at the forefront of biology, involved in the founding of molecular biology. Every day at lunch Jacques Monod, François Jacob and André Lwoff presided over an exciting discussion usually augmented by a prominent visitor. The Department of Embryology was just phasing out 50 years of describing the anatomical development of the human embryo. The faculty was principally engaged in experimental embryology. I was the first biochemist. This is how I came to spend 50 years pursuing the topic of gene regulation in development at the Carnegie Institution's Department of Embryology in Baltimore. I cannot imagine a more ideal place.

The isolation of genes before the recombinant DNA era

I choose embryos of the frog *Rana pipiens* to study because the eggs could be fertilized *in vitro* and the embryos developed synchronously and provided unlimited amounts of material for biochemistry. While I was at the Pasteur Institute, two separate groups discovered messenger RNA (mRNA), and it had become clear from studies with bacteria that ribosomal RNA (rRNA) and transfer RNAs were direct gene products. Previously, 'differential gene action' had been studied in embryos as fluctuations in enzymatic activity that was assumed to represent changes in the amount of a protein. I realized that, as RNA was closer to the gene than protein was, its analysis would be a more reliable reflection of gene expression changes.

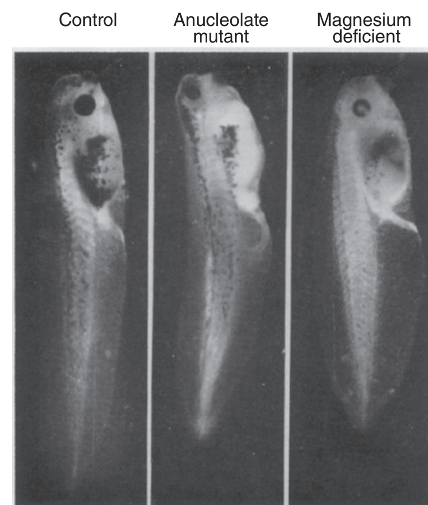

Figure 1 Comparison of control (left), anucleolate mutant (center) and magnesium-deficient (right) embryos of *X. laevis* after four days of development. Magnesium deficiency arrests embryos at the same stage of development as the anucleolate mutant. From ref. 2.

By the early 1960s, sucrose-gradient centrifugation had been devised to separate by size three general classes of RNA, the 18S and 28S rRNA, low molecular weight RNAs and a very heterogeneous DNA-like RNA that was presumed to include the newly discovered mRNA. I found that the three size classes of RNA were synthesized at vastly different rates during development. Even though the unfertilized frog egg is a single cell, it has as many ribosomes as the same weight of a multicellular somatic tissue such as the liver. New rRNA was not synthesized until the embryo had developed to 10,000 cells, and new ribosomes did not contribute substantially to the total content of ribosomes until several days later.

My first discovery at Carnegie was that frog embryos do not develop in distilled water. They need four cations—calcium, sodium, potassium and magnesium, in that order during development. Magnesium deficiency was especially interesting because, without it, embryos develop for several days and arrest as early swimming tadpoles at the same stage that a frog embryo

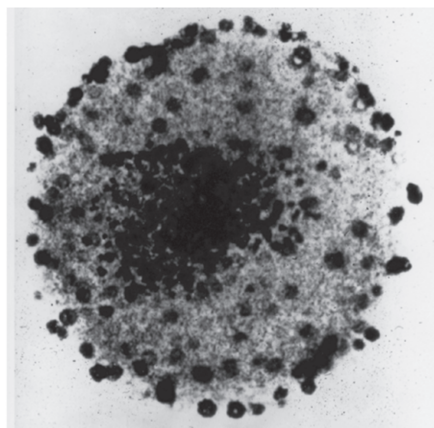

Figure 2 An isolated nucleus from a mature oocyte of *X. laevis*. The diameter of the nucleus is about 400 μm. Cresyl violet stain accentuates the thousands of nucleoli. From ref. 5.

begins to increase its total amount of rRNA. It was well known that magnesium holds ribosomes together. In 1962, I read an article about a mutant of the South African clawed toad *Xenopus laevis* that had an altered number of nucleoli[1]. The wild type had two nucleoli in each nucleus; the heterozygote had one nucleolus and was perfectly normal. Crossing two heterozygotes yielded a typical Mendelian ratio of offspring in which one-quarter of the progeny were anucleolate. These homozygous mutants arrested development exactly at the stage of a normal embryo deprived of magnesium (**Fig. 1**), suggesting to me that the mutation affected the synthesis of new ribosomes.

The only source of this mutant was the Oxford lab where it had been discovered. Meanwhile, I learned that *X. laevis* was imported from South Africa for use as a pregnancy test by an entrepreneur who lived just ten miles away in a suburb of Baltimore. I quickly switched from *R. pipiens* to *X. laevis*. John Gurdon at Oxford and I teamed up to show that the anucleolate mutant synthesized no 18S or 28S rRNA[2]. These mutant embryos synthesized the other two general classes of RNA normally. Max Birnstiel and his colleagues in Edinburgh took the next step[3]. It was well known that the base composition of rRNA has a much higher guanine and cytosine content than the bulk of the DNA. Using the method of equilibrium CsCl centrifugation developed by Jerome Vinograd, Birnstiel and his colleagues showed that the genes encoding rRNA separated from the bulk of the DNA and were absent in the anuceolate mutant. By molecular hybridization they showed that there are several hundred copies of rRNA genes per haploid complement of *X. laevis* DNA and that they must be clustered, as a single mutation deletes all of them.

Equilibrium CsCl centrifugation became the most important method for separating DNAs of different base composition for the next decade. It led to the purification of *X. laevis* ribosomal DNA (rDNA) by Birnstiel. This was the first isolation of a gene from any organism. In 1966, at a symposium on the nucleolus in Montevideo, Uruguay, Oscar Miller showed electron micrographs of genes in the nuclei of amphibian oocytes transcribing RNA[4]. These genes were the size predicted for the rRNA genes, and they came from the thousands of nucleoli in each oocyte nucleus (**Fig. 2**). As the oocyte is a tetraploid cell and should have just four nucleoli, we predicted that these extra nucleoli must contain many extra genes encoding rRNA. Igor Dawid and I[5] and, independently, Joe Gall[6] showed the 'specific amplification' of the genes encoding the 18S and 28S rRNAs in amphibian oocyte nuclei.

We learned that the genes encoding the 5S rRNAs are not linked to the rRNA genes but comprise an even greater fraction of the genome of *X. laevis* DNA than does the rDNA. We purified and studied the 5S rRNA genes (5S DNA) from the *X. laevis* genomic DNA in 1971 (ref 7), taking advantage of the unusual base composition of the many tandemly repeated 5S rRNA genes. These small genes were especially useful for detailed characterization and functional studies in the days before DNA sequencing and recombinant DNA methods were available. We learned that spacer DNA separated tandem genes (**Fig. 3**). In different species of *Xenopus* the spacers were very different, whereas the genes were highly conserved. Yet the spacers within a species were remarkably similar, a phenomenon that we called horizontal evolution. Within each 5S rRNA gene repeat there is a pseudogene.

The first eukaryotic gene to be completely sequenced was one repeat of genomic 5S DNA by Nina Fedoroff and George Brownlee[8]. We learned that there are multiple families of 5S rRNA genes in the *X. laevis* genome. The largest one is expressed only in oocytes. We called this the 'dual 5S rRNA gene system', a developmental problem that we pursued for a decade[9]. 5S DNA is a template for accurate transcription in crude extracts of oocyte nuclei[10], providing a simple assay for the *cis*-acting DNA sequences required for accurate initiation and termination of transcription. Once genes could be cloned, we made successive deletions around and into the 5S rRNA gene and then tested the ability of each mutant to transcribe 5S rRNA in the crude nuclear extract. To our amazement, the gene transcribed properly even when we had deleted one-third of either the 5′ or the 3′ ends of the gene[11,12] (**Fig. 4**). We called this the 'internal control region'. Bob Roeder and his colleagues purified the first eukaryotic transcription factor from *X. laevis* oocytes[13]. This protein, called TFIIIA, binds the internal control region of the 5S rRNA gene[14]. TFIIIA was the first example of a class of transcription factors called zinc finger proteins[15].

Silk fibroin mRNA and its gene

The discovery of gene amplification in 1968 encouraged us to ask whether this mechanism is used more broadly when a cell synthesizes a large amount of a gene product. I had read about the unusual amino acid sequence and the huge size of the protein silk fibroin from the silkworm *Bombyx mori*. Most of the protein consists of repeats of the peptide glycine-alanine-glycine-alanine-glycine-serine, which predicts an mRNA and gene with a very high guanine and cytosine content. At the end of larval development, most of the protein synthesized by the posterior end of the silk gland is this single protein. At that time, only hemoglobin mRNA in reticulocytes had been identified on a sucrose gradient as a discrete mRNA. Yoshiaki Suzuki came to my lab as a postdoctoral fellow, and we began to raise silk worms. He isolated a large ^{32}P-labeled mRNA from the posterior silk gland by sucrose-gradient centrifugation. Partial sequencing identified the predicted unusual sequence of the silk fibroin mRNA[16]. The radioactive mRNA was pure enough to hybridize with DNA from the posterior silk gland and from control silkworm tissues to show that there is a single fibroin gene in the DNA of all cell types. This ruled out DNA amplification as an explanation for the large and specific synthesis of silk fibroin mRNA by the posterior gland. Silk protein synthesis is an example of extreme translational amplification. In a period of only two to three days, each fibroin gene guides the synthesis of 10^4 copies of mRNA, each of which, in turn, synthesizes about 10^5 copies of the protein. One gene makes 10^9 protein molecules.

Amphibian metamorphosis

With the discovery of recombinant DNA technology in the late 1970s, the study of any gene became possible. In 1990, after a decade of ana-

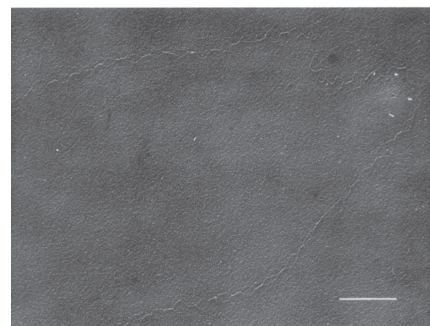

Figure 3 Electron micrograph of a partially denatured molecule with repeating units of 5S DNA. The gene for 5S rRNA comprises one-third of the native region. The average repeat length is about 700 base pairs. From ref. 7.

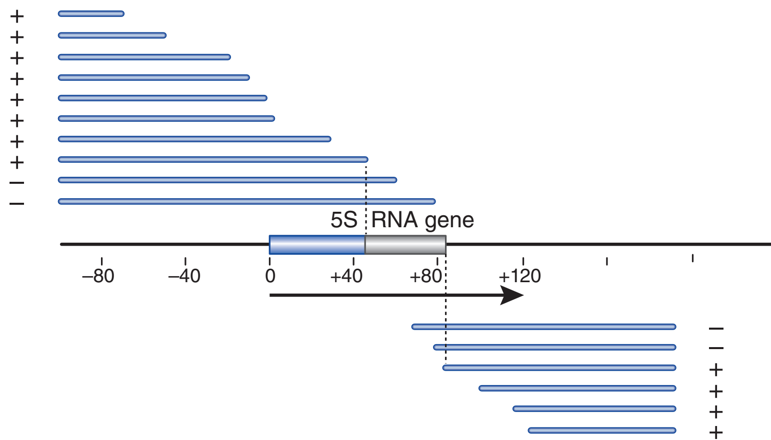

Figure 4 A transcriptional-control region within the 5S rRNA gene. The horizontal bars indicate the extent of each deletion. Mutants that still support accurate initiation of transcription are indicated as +. The region delineated by vertical dashed lines is the internal control region of the gene. From refs. 11 and 12.

lyzing the structure and regulation of the simple 5S rRNA genes, I decided to change projects. I chose amphibian metamorphosis—a wonderful biological problem that had fascinated every succeeding biological discipline but had been ignored by molecular biologists.

The question was simple. How can one molecule—thyroid hormone—change the fate of so many different cell types? The discovery of the thyroid receptors as transcription factors by Ron Evans and Björn Vennström in 1986 convinced me that the control of metamorphosis by thyroid hormone must occur by changes in the expression of many genes.

The technical advantages of the system had been well explored. Tadpoles are exquisitely sensitive to exogenous thyroid hormone. The chemicals that inhibit synthesis of the hormone by the thyroid gland in mammals work well in amphibians. The pituitary-thyroid axis is similar in all vertebrates. The disadvantages of the system were the absence of traditional genetics and the long life cycle of frogs. When transgenesis was discovered in *X. laevis* in 1996, we could overexpress transgenes driven by cell-specific promoters. Among other discoveries, this enabled us to prove that the thyroid hormone receptors are responsible for all of the many changes induced by the hormone in metamorphosis. In a recent review[17] I summarized the current state of the field.

The Department of Embryology of the Carnegie Institution for Science

Our Department has eight permanent 'staff member' slots. These positions do not confer tenure, but are renewed competitively every five years. One of the changes that I made when I became director of our department in 1976 was to establish an independent junior faculty program. A 'staff associate' position is available right after graduate school or as a second post-doc. The key to these positions is that the investigator is completely independent. A staff associate works full time in the lab with the aid of a technician. These are term positions so that staff associates know that they must leave after five years. These independent young investigators have added great diversity and energy to our department over the years. Most of them have moved to excellent jobs with great distinction. Several other research universities and institutions have initiated similar programs.

The Life Sciences Research Foundation

The simple lesson that research is best done by young people is partly responsible for my founding the Life Sciences Research Foundation (LSRF; http://www.lsrf.org/). By 1980, it had become clear that biology was beginning to replace chemistry in the pharmaceutical industry. Molecular biology, inspired by the new recombinant DNA technology, was poised to revolutionize how drugs are made. Furthermore, these discoveries were not made at companies, but at nonprofit institutions paid for by the government through the extramural grant programs of the NIH and the National Science Foundation. University scientists had begun to start companies.

I sought a way to further this interaction, expecting these companies to have a new interest in the biological research carried out in universities and research institutions. After some thought and consultation, I selected a simple postdoctoral program that I hoped would be sponsored by the major pharmaceutical companies. In addition to these companies, the Howard Hughes Medical Institute, a number of foundations, one government agency (Department of Energy–Division of Biosciences) and several philanthropic scientists have sponsored LSRF fellowships. Alas, we have never had endowment and must therefore solicit sponsors every year. We are exceedingly proud of the distinction of our 456 alumni and current fellows. In 2012, our distinguished peer-review team selected 50 finalists from 900 applications and awarded 24 fellowships. This fall we are having a celebration of alumni marking our thirtieth year.

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COMPETING FINANCIAL INTERESTS

The author declares no competing financial interests.

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