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Twists and Turns: A Scientific Journey

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Abstract

In this perspective I look back on the twists and turns that influenced the direction of my scientific career over the past 40 years. From my early ambition to be a chemist to my training in Philadelphia and Bethesda as a molecular biologist, I benefited enormously from generous and valuable mentoring. In my independent career in Philadelphia and Princeton, I was motivated by a keen interest in the changes in gene expression that direct the development of the mammalian embryo and inspired by the creativity and energy of my students, fellows, and research staff. After twelve years as President of Princeton University, I have happily returned to the faculty of the Department of Molecular Biology.

Contents

METAMORPHOSIS OF A STUDENT INTO A SCIENTIST 2
CLONING THE β-GLOBIN GENE AND FINDING A SURPRISE 4
EXPLORING THE STRUCTURE AND EXPRESSION OF THE MOUSE α-FETOPROTEIN GENE 6
THE MYSTERIOUS *H19* GENE 10
GENOMIC IMPRINTING OF *H19* 11
EXPLORING THE EVOLUTION OF GENOMIC IMPRINTING 13
REFLECTING ON HAPLOINSUFFICIENCY OF *PAX6* 14
FROM PHENOTYPE TO GENOTYPE: CHROMOSOME WALKING IN THE MOUSE 15
AND NOW FOR SOMETHING COMPLETELY DIFFERENT 17
CONCLUDING THANKS AND APOLOGIES 18

METAMORPHOSIS OF A STUDENT INTO A SCIENTIST

I cannot remember a time when I did not want to be a scientist, but despite that unwavering conviction, the path has been anything but a straight one. In fact, a more apt description of my scientific journey would be one of twists and turns, where both life and science regularly intervened with midcourse corrections and, to mix metaphors, threw me curveballs.

My ambition to pursue a life in science was borne of an early fascination with numbers and puzzles. I thought math would be my calling until I encountered chemistry in high school and fell head over heels in love with the periodic table. It made so much sense to me—the orderly accumulation of electrons, neutrons, and protons as atoms grew from left to right and top to bottom in the table. Once I learned the rules underlying covalent bond formation, I discovered the fun of designing the smallest number of reactions to transform one molecule into another—a new kind of puzzle to solve.

So I went off to Kingston, Ontario, and Queen's University in the fall of 1964, fully intending to become a chemist. Queen's did a lot to encourage this ambition, by giving me both a full scholarship and plentiful opportunities to actually do research. I spent my sophomore year and the summer following it in the laboratory of Professor Saul Wolfe, a distinguished organic chemist who was working on the de novo synthesis of penicillin. The experimental challenge was a deceptively simple one—find the reaction conditions under which a biologically inert anhydropenicillin could be converted into the biologically active antibiotic. This was a perfect test of my commitment to the bench, as it required months of painstakingly changing one reaction condition after another, all to no avail. Finally, one morning I opened the incubator and saw a clearing in the *Escherichia coli* lawn caused by the latest reaction. Any doubts I had about my decision to pursue a life in science evaporated. My first paper in the *Journal of the American Chemical Society* soon followed (Wolfe et al. 1966).

Several studies have shown that one of the best predictors of persistence in science is early success, especially for women, who may need the extra encouragement that comes with making an original discovery. I had more opportunities to work in faculty labs, publish another paper, and write a senior thesis. But by the end of my third year, I knew I was not going to be a chemist.

As much as I enjoyed chemistry, it did not come naturally to me. So began the first twist and turn in the road—I went in search of another scientific calling. Fortuitously, I happened upon the

beautiful experiment by Matt Meselson & Frank Stahl in which they used isotopes of nitrogen to establish that DNA replicates semiconservatively (Meselson & Stahl 1958). This is, of course, one of the greatest experiments of the twentieth century. Not only did it address a central question in molecular biology but it was brilliantly designed so that Meselson & Stahl would know the answer no matter what the experimental result or outcome.

I was hooked, but there was one problem. I had not taken a single biology class in my life. My high school in Winnipeg, Manitoba, discouraged good students from taking biology, which was taught as an exercise in the memorization of body parts. At Queen's I managed to talk my way into a biochemistry course in my senior year and spent the first month completely lost—"What's a phage?" was a common refrain in my head.

After graduation I took another twist and turn by spending two glorious years in Sierra Leone, West Africa, teaching chemistry and English in a small secondary school for boys. I was inspired by the spirit of the 1960s to be sure, but I was also burned out after years in the classroom and the lab. I now encourage my own undergraduates to take a gap year or two before graduate or professional school, as I found the experience completely restorative, both emotionally and intellectually.

I returned to North America, and to graduate school at Temple University, determined to become a molecular biologist. Luckily for me, my thesis advisor, Richard Hanson, the Chair Emeritus of Biochemistry at Case Western Reserve (**Figure 1*a***), was willing to give me my head, and I went in search of the molecular basis for the changes in levels of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step in gluconeogenesis, in response to dietary changes. We showed that the elevation in hepatic PEPCK after starving and its rapid decline upon refeeding rats were due to changes in the level of its translatable messenger RNA (mRNA) and that the inducer was cyclic adenosine monophosphate (Kioussis et al. 1978, Tilghman et al. 1974).

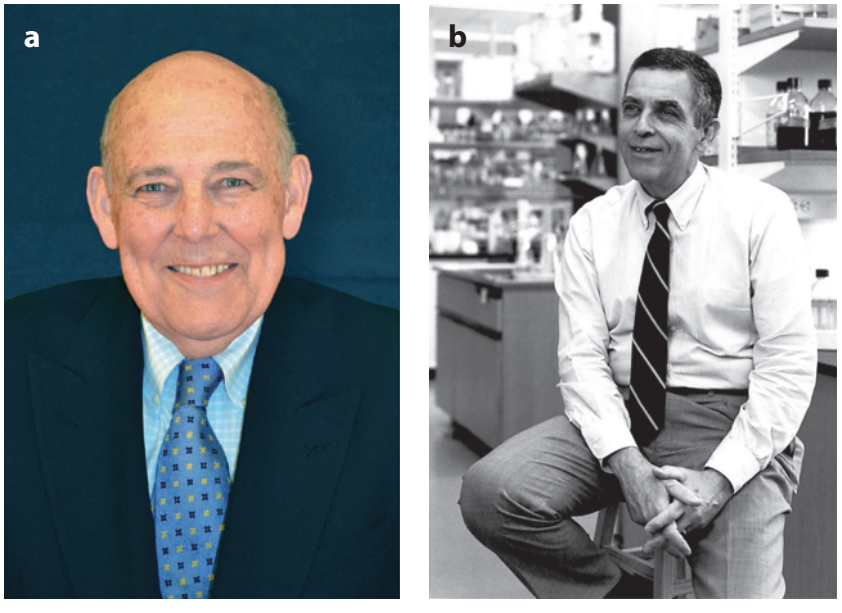


Figure 1

My mentors, (*a*) Dr. Richard Hanson and (*b*) Dr. Philip Leder.

Richard was a superb mentor, in part because he treated me like a colleague, as opposed to a student. He encouraged me to think for myself and to take the initiative in the lab. He was also a dedicated and inspiring teacher who made his field of expertise, metabolic regulation, seem like the most fascinating subject in the world. Richard and I learned the molecular biology trade together. But as I began to consider my postdoctoral options, I knew that I needed to study with a master. After hearing Philip Leder, the Chair Emeritus of the Department of Genetics at Harvard (**Figure 1*b***), give a seminar, my choices went from many to one.

CLONING THE β-GLOBIN GENE AND FINDING A SURPRISE

At the time, Phil was the chair of the Laboratory of Molecular Genetics at the National Institutes of Health (NIH), and he offered me a Fogarty fellowship, which is restricted to foreign nationals, in his lab. I was never so grateful for my Canadian citizenship! Nor have I ever stopped being grateful for Phil's immense learning, support, and mentorship. For many years, when I was asked to speak at a meeting or contribute an article to a volume, or to participate in an important committee, more often than not, Phil was pulling strings behind the scenes. He is a superb scientist and a model mentor.

I arrived in Phil's lab in the summer of 1975, which was one of those tipping points in the history of molecular biology. Recombinant DNA was in the process of being invented, and one could begin to contemplate the prospect of isolating and studying in detail one of the thousands of genes in a complex eukaryotic genome. My assignment was to collaborate with David Tiemeier, another fellow in the lab, and develop the technology to clone the mouse β-globin gene.

That, as it turns out, was a tall task, both scientifically and politically. In February of 1975 a group of influential scientists had gathered at Asilomar in Northern California to discuss the potential hazards associated with recombinant DNA. They drew up a set of guidelines under which recombinant DNA experiments were to be conducted until the safety of recombinant organisms could be more carefully assessed (Berg et al. 1975). In the case of mammalian genes, they proposed that cloning be restricted to bacterial host and vector systems that could not survive outside the laboratory. Such a system did not exist, and David set out to develop one using bacteriophage λ. (Luckily, by then I knew what a phage was!) Working closely with phage geneticists Lynn Enquist and Nat Sternberg in Bob Weisberg's lab, David introduced a series of mutations into the λ genome that met the high regulatory threshold for cloning our mouse β-globin gene target (Leder et al. 1977).

In the meantime, my project was to sufficiently purify the 7-kilobase (kb) *Eco*R1 restriction fragment that contained one of the two β-globin genes so that it could be picked out of a complex population of recombinant phage. Given the three billion bases of the mouse genome, and the 4-kb average size of an *Eco*R1 fragment, I needed to devise a purification scheme that would enrich the globin gene at least a hundredfold. The relatively homogeneous chemical composition of DNA significantly limited the possible options, other than size fractionation. A solution presented itself at a Nucleic Acids Gordon Conference in the summer of 1976. Bob Wells described a method for purifying DNA using RPC-5—a resin that used reversed-phase chromatography to separate transfer RNAs (Hardies & Wells 1976).

The column worked like a charm, and using that and size fractionation, I was able to enrich the 7-kb restriction fragment approximately 500-fold, sufficient for cloning into David's modified λ vector. Clothed in the mandatory gowns, masks, and booties in our newly constructed P-3 biosafety facility, David and I picked 5,000 phage plaques while arguing over music selection. Three contained the prize—a recombinant bacteriophage harboring the major β-globin gene of the mouse (Tilghman et al. 1977).

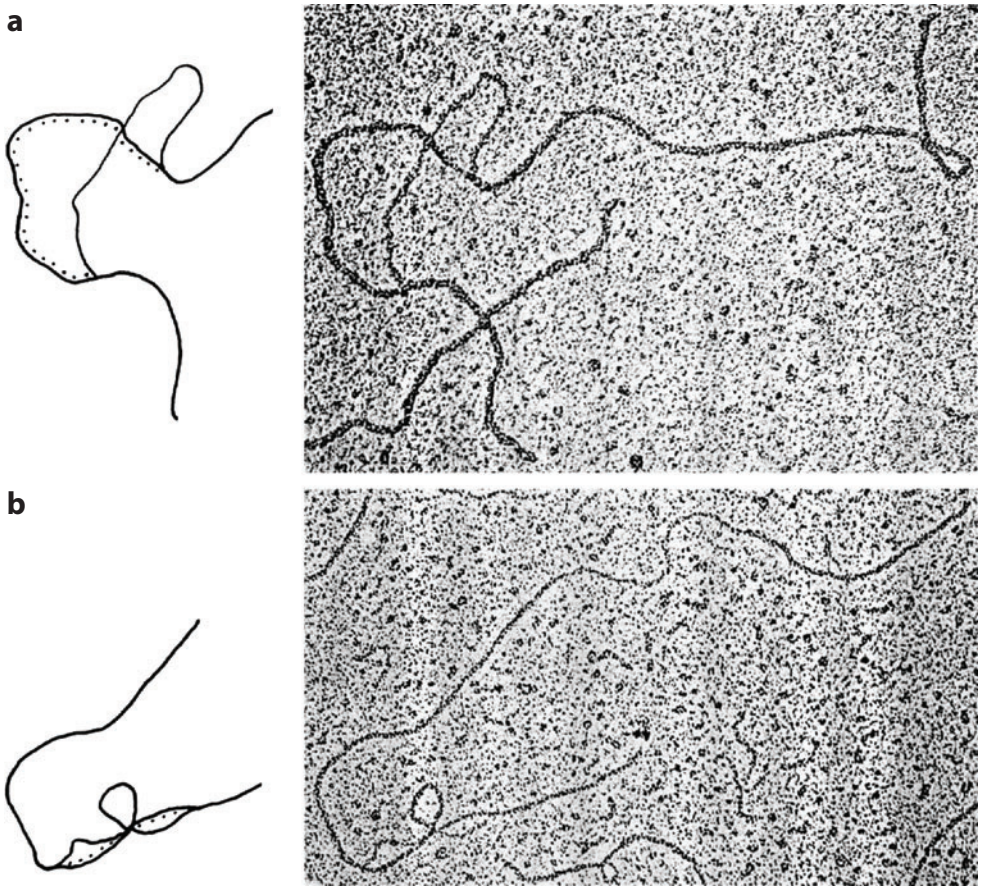


Figure 2

R-loop mapping of the β-globin gene. (*a*) Hybrid between the β-globin gene and the 15S β-globin nuclear precursor RNA. (*b*) Hybrid between the β-globin gene and the 10S mature β-globin mRNA. Adapted from Tilghman et al. (1978a).

Little did we know what surprises that gene had in store for us. DNA sequencing was in its infancy, so our first glimpse into the organization and structure of the globin gene was R-loop mapping using electron microscopy (Thomas et al. 1976). In this method, RNA and double-stranded DNA are heated together under conditions that favor RNA-DNA hybrids. When there is base complementarity, the RNA will intercalate between the two DNA strands, creating an R-loop that can be visualized by electron microscopy. Based on what was known about bacterial genes, we expected to see a single large loop where globin mRNA hybridized to its complementary strand of DNA. Instead, we observed over and over again a Kilroy structure, in which two large R-loops in the middle of the fragment were separated by a double-stranded DNA loop that appeared to interrupt the coding sequence of the mRNA, with a smaller loop at one end (**Figure 2*b***).

Phil's first reaction when I showed him the images was to send me back to repeat the experiment, but the result remained the same: Kilroys galore. The only conclusion we could reach was that the mRNA was not contiguous with the DNA, but why remained a mystery. I will never know

how long we would have puzzled over those images, had not a colleague returned from a visit to Cold Spring Harbor Laboratory with the startling news that Richard Roberts's group had just discovered that the 5′ ends of multiple late adenovirus mRNAs were noncontiguous with the genome (Broker et al. 1978). Their findings, which implied an entirely novel mechanism for mRNA biogenesis, were simultaneously reported by Philip Sharp's group at the Massachusetts Institute of Technology (Berget & Sharp 1977).

There was, of course, the plausible explanation that the noncontiguous organization of gene sequences in adenovirus was the result of intense evolutionary pressure on a complex virus with a small genome. Once we interpreted our Kilroy structures in light of the adenovirus work, however, it was immediately apparent that their finding had much broader implications, and that eukaryotic genes were organized in the genome in far more complicated ways than we could ever have imagined (Tilghman et al. 1977).

If the globin mRNA was encoded in three different segments of the genome, how could a mature mRNA be produced? Several groups had recently shown that the nuclear precursor to globin mRNA was larger than the mature form—15S versus 10S in sedimentation studies (Curtis & Weissmann 1976, Ross 1976)—but it had been assumed that the extra sequences were trimmed from either the 5′ or 3′ ends of the precursor. Two additional possibilities now presented themselves: The mRNA was transcribed discontinuously, or the precursor was internally spliced to form the mature RNA.

To resolve this critical question, we collaborated with Peter Curtis, a fellow in Charles Weissmann's lab, who had prepared minute quantities of the rare 15S precursor, sufficient for just one R-loop experiment. When I visualized the RNA-DNA hybrids in the electron microscope, I saw only perfect single R-loops (**Figure 2*a***), demonstrating that the 15S RNA nuclear precursor was contiguous with the gene. To produce the mature mRNA, the precursor must be cleaved and spliced in two places (Tilghman et al. 1978a). The splicing revolution was under way.

The discontinuous organization of most eukaryotic genes, and the intricate splicing mechanism needed to produce mature mRNAs from larger precursors, raised fascinating evolutionary questions that are still being pondered today. With the sequencing of the human genome, it is now clear that much of the genetic diversity that is responsible for the complexity of *Homo sapiens* is generated by the facility with which exons can be duplicated and rearranged by genetic recombination to generate new genes. Perhaps more surprising has been the discovery that alternative splicing has exploded the one-gene-one-protein paradigm and that some genes are capable of encoding dozens, and in some cases hundreds, of different proteins.

EXPLORING THE STRUCTURE AND EXPRESSION OF THE MOUSE α-FETOPROTEIN GENE

As I began to contemplate the prospect of striking out on my own, it was clear to me that I would be wise to leave the very crowded globin field. So began the next scientific twist and turn in the road. At a Gordon Conference in the summer of 1977, Gail Martin gave a remarkable talk about the ability of mouse teratocarcinoma cells to differentiate into multiple cell types under defined conditions in culture (Martin & Evans 1975). I was intrigued by the possibility of using this model system to study mouse development and went in search of a gene that was activated and abundantly expressed during the first stages of embryogenesis.

After a little digging in the library, I settled on α-fetoprotein (AFP), which was known to be a major component of fetal serum and amniotic fluid in the mouse. AFP is synthesized at high levels in both fetal liver and the yolk sac that surrounds the developing fetus, and as its name suggests, its synthesis declines dramatically in liver after birth. It is replaced by albumin, a protein closely

related in size and function. Most importantly, AFP is activated in the visceral endoderm of the mouse embryo prior to implantation.

I left the NIH in 1978 for the Fels Research Institute at Temple University School of Medicine, but shortly thereafter I moved to the Institute for Cancer Research at the Fox Chase Cancer Center. Thanks to the protein's abundance in amniotic fluid and the mRNA's abundance in visceral endoderm, we soon accumulated the antibodies, mRNA, and genomic clones that we needed to study its transcription in the mouse (Gorin & Tilghman 1980, Tilghman et al. 1978b). By then it was no longer big news that a gene was encoded discontinuously—within 15 exons in the case of AFP—but there was a surprise awaiting us in the analysis of the structure of the gene and its sequence.

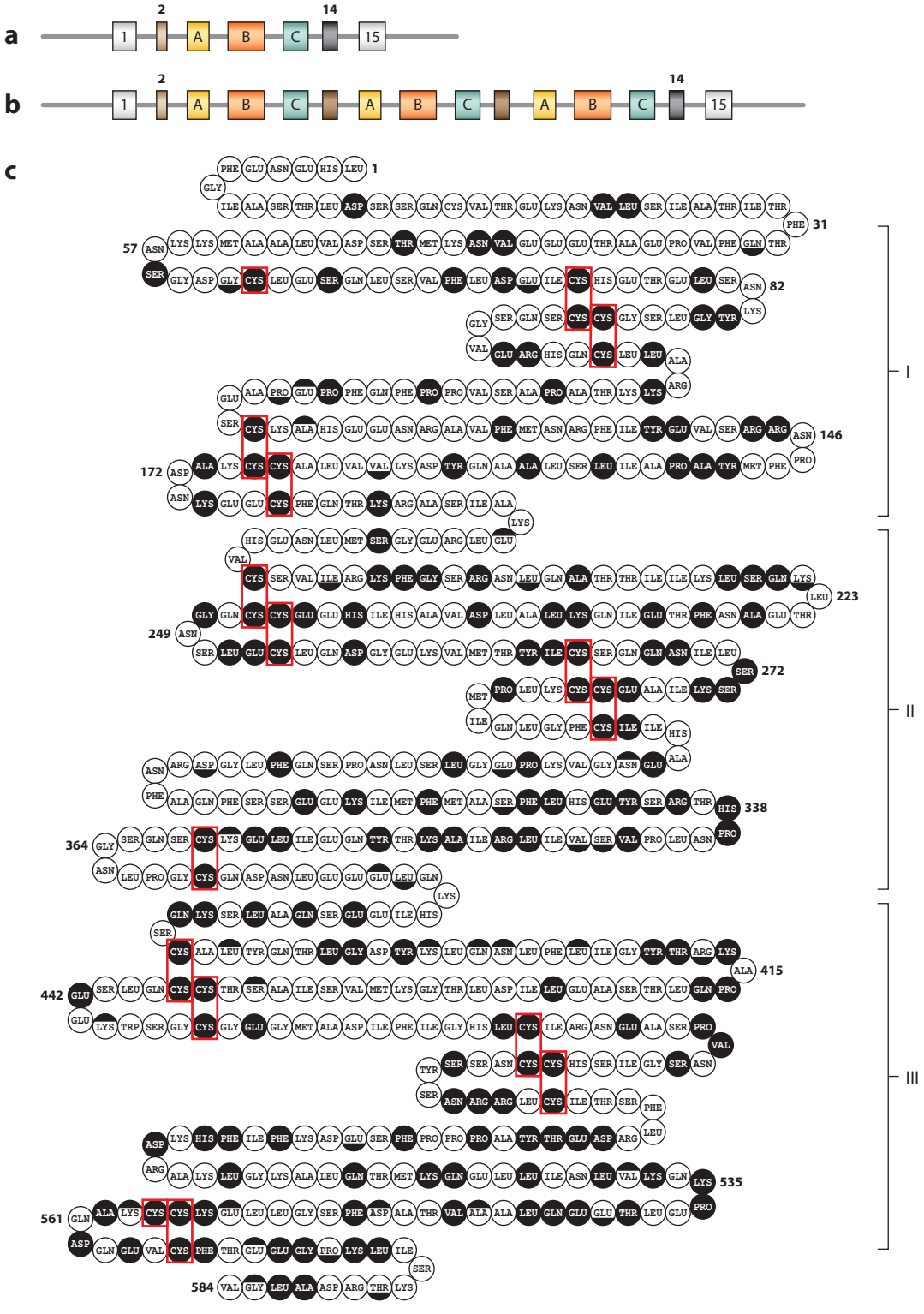
We first confirmed the evolutionary relatedness of AFP and albumin by cloning the latter and showing that its exonic structure was identical to that of AFP (Gorin et al. 1981, Kioussis et al. 1981). Furthermore, the two genes had remained closely linked in tandem on mouse chromosome 5 over a period of approximately 400 million years (Ingram et al. 1981), suggesting the possibility that they shared regulatory sequences—an issue that postdoctoral fellow Sally Camper was later able to confirm (Camper & Tilghman 1989).

However, the duplication of an ancestral albumin/AFP progenitor was not the end of the evolutionary story. In 1976, Brown (1976) had proposed, based on the sequence and putative three-dimensional structure of bovine serum albumin, that the protein was composed of three repeating segments corresponding to different binding domains of the protein. Albumin binds a variety of metabolites, and its function is to sequester molecules that might be harmful to the organism and to transport metabolites, such as steroid hormones and fatty acids. Once we had the amino acid and nucleic acid sequences of both genes, it was clear that Brown had been correct and that the ancestral progenitor had itself evolved from the triplication of a single 3-exon protein-coding domain (**Figure 3*a–c***) (Alexander et al. 1984, Eiferman et al. 1981). With each duplication and divergence, the protein acquired the capacity to bind new molecules. The AFP/albumin genes provided a vivid example of the evolutionary nimbleness that came with the development of exons and introns.

With the necessary molecular reagents in hand, we set out to describe the *cis*-acting regulatory sequences and the *trans*-acting factors responsible for the developmental regulation of the AFP and albumin genes. Postdoctoral fellow Rick Scott stably introduced a modified AFP transgene containing the entire 14-kb intergenic region between the 3′ end of the albumin gene and the 5′ end of the AFP gene into F9 teratocarcinoma cells and showed that the minigene could be reliably induced upon differentiation, although not to levels comparable to that of the endogenous gene (Scott et al. 1984). That led us to conclude that we had the majority of the regulatory domain in hand, but we needed a better expression system to make further progress.

The technological breakthrough that moved the field forward came with the arrival of transient transfection of DNA, in which the expression of a nonintegrated transgene is assessed in a population of transfected cells shortly after DNA transformation. Using this faster and more reliable method, a postdoctoral fellow, Roseline Godbout, showed that the intergenic region between AFP and albumin contained three distinct enhancers spread over a region of 6.5 kb. Each enhancer was capable of enhancing tissue-specific expression of a reporter gene in liver-derived Hep3B cells when combined with a region proximal to the AFP promoter (Godbout et al. 1986, 1988). This was one of the first studies to demonstrate that transcriptional regulatory domains of eukaryotic genes contained redundant elements that were capable of acting over large distances.

The cell culture analyses of regulatory elements were critically dependent upon the degree to which the phenotype of the cell lines, which were largely derived from tumors, reflected that of the cells of origin. The development of transgenic mice (Brinster et al. 1981a,b) overcame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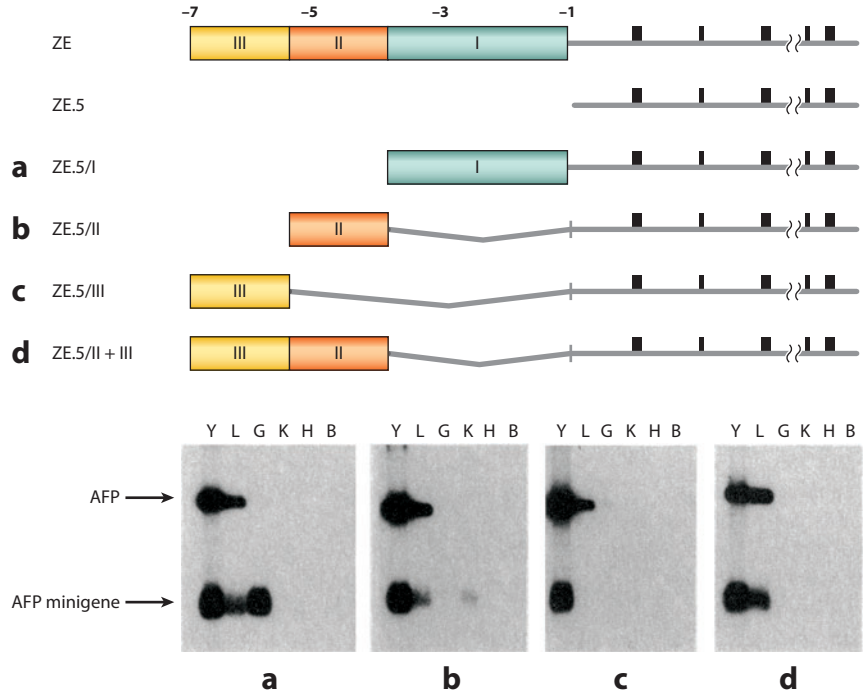

Figure 4

Tissue-specific expression of an α-fetoprotein (AFP) transgene in transgenic mice. An AFP minigene composed of the first three and last two exons of the AFP gene (*black rectangles*) was introduced into transgenic mice along with different segments of the 7 kb of DNA flanking the 5′ end of the gene. Enhancers I (at −2.5 kb), II (at −5 kb), and III (at −6.5 kb) were tested together (ZE) or separately for their abilities to direct expression of the minigene in yolk sac (Y), liver (L), gut (G), kidney (K), heart (H), and brain (B). The expression of both endogenous AFP mRNA and the minigene was monitored by Northern blot analysis. Adapted with permission from Hammer et al. (1987).

this limitation and allowed us to examine the activity and specificity of transcriptional elements in vivo. In collaboration with Ralph Brinster at the University of Pennsylvania and his fellow Bob Hammer (Hammer et al. 1987, Krumlauf et al. 1985), Robb Krumlauf showed that the three intergenic enhancers displayed exquisite tissue specificity by directing expression of an AFP minigene reporter exclusively in the liver, yolk sac, and gut. However, each enhancer had a different spectrum of activity, with the most proximal enhancer I directing high-level expression in all three tissues and the most distal enhancer III primarily in yolk sac (**Figure 4**). Transcription of all transgenes was repressed after birth and reactivated upon liver damage, demonstrating that they were responsive to both developmental and environmental cues in a manner identical to the endogenous gene.

Figure 3

The structure and evolution of the α-fetoprotein (AFP) gene. (*a*) The proposed exon structure of the primordial AFP/albumin gene before triplication. (*b*) The exon structure of the AFP/albumin gene after triplication. (*c*) The amino acid sequence of mouse AFP, drawn to emphasize the repeated nature of the protein held together by cysteine disulfide bridges (*closed circles bracketed in red*). Redrawn with permission from Alexander et al. (1984) and Gorin et al. (1981).

THE MYSTERIOUS *H19* GENE

While one part of the lab was identifying the *cis*-acting sequences that control the expression of AFP, another group of students and fellows was beginning to think about approaches to the factors that mediate their effects. Prior to having the *cis*-acting sequences in hand, we turned for clues to classical mouse genetics. In a screen of inbred strains of mice, Olsson et al. (1977) reported a 20-fold elevated basal level of AFP in the adult liver of BALB/cJ mice compared with 26 other strains they examined. They showed that the BALB/cJ trait was recessive and named the polymorphic gene *raf*, for regulation of AFP (later renamed *Afr1*). Alexandra Belayew, a fellow in the lab, determined that the *raf* gene is unlinked to AFP and affects the adult basal levels of AFP mRNA, suggesting that *raf* could encode a repressor of AFP transcription after birth (Belayew & Tilghman 1982). However, later we showed with nuclear run-on experiments that *Afr1* acts post-transcriptionally on AFP and its other targets (Vacher et al. 1992).

And here we come to a major twist and turn in the road. With the discovery that *Afr1* affects the postnatal repression of AFP mRNA, the next obvious question was whether *Afr1* has other gene targets. That question led Alexandra Belayew and a graduate student, Vassilis Pachnis, to screen a mouse fetal liver complementary DNA (cDNA) library for clones that displayed higher levels of expression in BALB/cJ liver compared with other strains of mice. That is how *H19*, a fascinating gene that dominated the rest of my career, was identified—in the H column and nineteenth row of a cDNA library (Pachnis et al. 1984).

What made *H19* so mysterious? First and foremost, it does not code for a protein, despite the fact that it has all the hallmarks of an mRNA, including a 5′ cap, a polyA tail, and a canonical exon/intron structure, and is transcribed by RNA polymerase II. Convincing ourselves of that simple fact was not easy; there was no precedent at the time for an mRNA-like RNA without an open reading frame. The most compelling evidence came from a sequence comparison of the mouse and human genes. Despite the conservation of the sequence, expression, and organization of the genes, the homologs do not share even a short open reading frame (Brannan et al. 1990). The other striking thing about *H19* RNA was its abundance in the fetus. It may well be the most abundant mRNA-like transcript in the mouse embryo.

The *H19* gene posed an interesting dilemma. When do you pursue a conundrum like *H19*, and when do you cut your losses and move on to projects that look more promising? As one of my colleagues advised me at the time, "There are too many other interesting things to study to take a chance on a gene that is likely to be a completely uninteresting pseudogene." In the end I relied on intuition that this gene must be doing something interesting to be conserved in mammalian evolution, transcribed at such a high level, and developmentally regulated. But truthfully, I could easily have been wrong.

In 1986, I moved the lab to the brand-new Department of Molecular Biology at Princeton University. The Institute for Cancer Research had been an ideal place to begin a scientific career. Like many research institutes, it was organized so that its investigators could spend the majority of their time doing experiments. Because of the administrative support at the Institute, I spent seven years focused entirely on my own experiments, as well as overseeing those of my students and fellows, without teaching or administrative duties. Furthermore, the Institute was highly unusual in having a group of strong women lab heads, including Helen Berman, Susan Astrin, Beatrice Mintz, and Jenny Glusker. Helen, in particular, was immensely supportive as I managed the high-wire juggling act of starting my lab and raising two children. However, by 1986 I yearned for a larger intellectual and scientific community and opportunities to teach. Princeton offered both, along with a small community with good public schools for my children, who were approaching school age. It was a twist and turn that changed my life in ways I could not have imagined at the time.

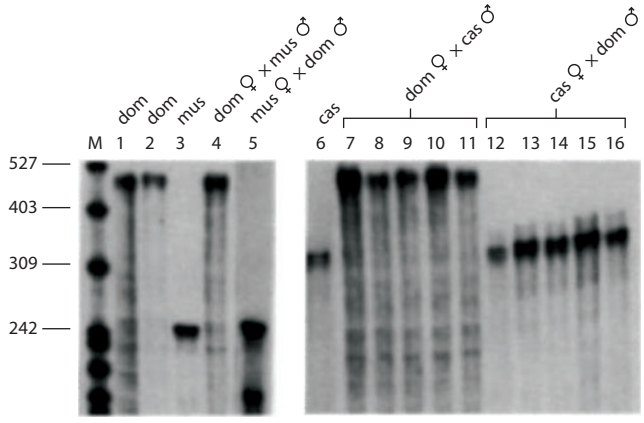

Figure 5

The imprinting of the mouse *H19* gene. The expression of *H19* RNA was detected in inbred and F1 hybrid neonatal liver using an allele-specific RNA protection assay that distinguished the alleles of *Mus domesticus* (dom), *Mus musculus* (mus), and *Mus castaneus* (cas) mice. In each hybrid, expression of only the maternal allele is detected. Adapted with permission from Bartolomei et al. (1991).

GENOMIC IMPRINTING OF *H19*

At Princeton we continued to puzzle over *H19*'s function. Ironically, the breakthrough came about through a mistaken observation. Mary Brunkow, a graduate student, had been tasked with generating gain-of-function mutations in *H19* by overexpressing a marked version of the gene in transgenic mice. After many failed attempts, we concluded that overexpression of the gene was lethal in mice (Brunkow & Tilghman 1991). Around this time, I attended another Gordon Conference and heard Argiris Efstratiadis speak about his remarkable and serendipitous discovery of the first imprinted gene, *Igf2*, encoding insulin-like growth factor II. Together with Elizabeth Robertson at Columbia, Arg had just shown that a loss-of-function mutation in *Igf2* caused a dwarf phenotype, but only when the mutation was inherited from fathers (DeChiara et al. 1991), a hallmark of an imprinted gene that is paternally expressed. Furthermore, he showed in situ hybridization patterns of *Igf2* mRNA that were virtually superimposable onto ones we had generated for *H19*. Lastly, I knew that both genes mapped to the distal end of mouse chromosome 7.

Putting all these observations together with the apparent dosage sensitivity of *H19* RNA, I leaped to the idea that *H19* might be imprinted as well. I raced back to the lab and persuaded a very skeptical Marisa Bartolomei to do the experiment to test this idea. She quickly capitalized on the evolutionary distance between inbred strains of mice to distinguish alleles of *H19* and demonstrated conclusively that *H19* was indeed imprinted, but in the opposite direction from *Igf2*; that is, it is expressed exclusively from the maternal chromosome (**Figure 5**) (Bartolomei et al. 1991). Ironically, a former postdoctoral fellow, Jennifer Schmidt, was later able to produce mice overexpressing *H19*, so the original prompt to think that *H19* was imprinted was incorrect (Carr et al. 2006).

The discovery began an enormously exciting and productive time in the lab, as we raced to unravel the mechanism underlying the imprinting of *H19*. That question became inextricably linked to the mechanism of *Igf2* imprinting when Sharon Zemel showed that the genes are next-door neighbors, separated by just 75 kb (Zemel et al. 1992). The first functional evidence for the linkage of their imprinting came when Philip Leighton generated a germline deletion of the *H19* gene, along with its 5′ flank. To our surprise, mice that inherited the mutation from their mothers

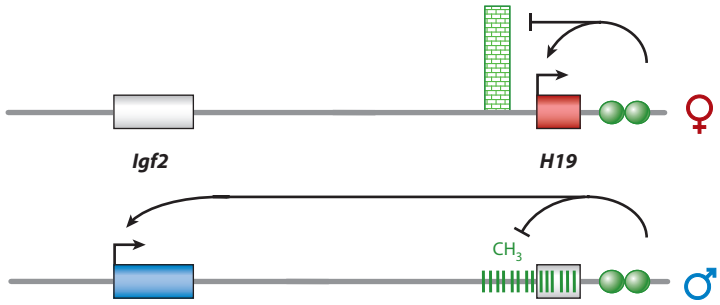

Figure 6

The mechanism of *H19* and *Igf2* imprinting. On the maternal chromosome (*top line*), the *H19* gene is activated by two endodermal enhancers (*green spheres*). The enhancers are prevented from activating the *Igf2* gene by the binding of CTCF to the imprinting control region (ICR), creating a chromatin boundary (*brick wall*). On the paternal chromosome (*bottom line*), the ICR is heavily methylated, which prevents CTCF binding and allows the endodermal enhancers to activate the *Igf2* gene. The spread of methylation inhibits *H19* transcription.

were 30% overgrown, a consequence of the loss of silencing of the maternal *Igf2* gene (Leighton et al. 1995a). The second functional link was established when Phil deleted two endoderm-specific enhancers that had been mapped to the 3′ end of *H19* (Yoo-Warren et al. 1988). Mice that inherit the enhancer mutation from mothers lose expression of *H19* in endodermal tissues; mice that inherit it from fathers lose expression of *Igf2* (Leighton et al. 1995b).

After an enormous amount of work by several groups and a few models abandoned along the way (Schmidt et al. 1999), the picture of how *H19* and *Igf2* are imprinted gradually came into focus (**Figure 6**). A region just upstream of the *H19* promoter is a CpG-rich bifunctional imprinting control region (ICR, green brick wall in **Figure 6**) that is heavily methylated during spermatogenesis and unmethylated during oogenesis (Bartolomei et al. 1993, Tremblay et al. 1995). When unmethylated, the ICR binds CTCF, a zinc-finger protein that has been implicated in chromatin boundary function at *H19* as well as many other loci in mammals (Bell & Felsenfeld 2000, Bell et al. 1999, Hark et al. 2000, Schoenherr et al. 2003). The boundary forms on the maternal chromosome, blocking the ability of the enhancers downstream of *H19* to activate the *Igf2* gene. With DNA methylation blocking CTCF binding on the paternal chromosome, the boundary does not form, and the enhancers can access the paternal *Igf2* promoter. The silencing of the paternal *H19* gene is due to the spreading of the ICR methylation downstream through the promoter.

Thus, we had found one function for the mysterious *H19* gene: Its transcription provides a foil to prevent *Igf2* transcription on the maternal chromosome, thereby controlling the level of expression of a critical fetal growth factor. But what role, if any, does the product of the *H19* gene play in all this? The fact that Beverly Jones was able to generate an apparently wild-type mouse that expressed the luciferase gene in place of *H19* RNA implied either that it had no function or that the function was redundant (Jones et al. 1998).

Since that time, there have been a plethora of studies implicating *H19* RNA in everything from tumor growth (Matouk et al. 2007) and suppression (Hao et al. 1993, Yoshimizu et al. 2008) to development and differentiation (Gabory et al. 2010, Keniry et al. 2012). At least some of these effects likely are mediated by MiR-675, a microRNA that is encoded within the first exon of *H19* (Cai & Cullen 2007). In keeping with its status as the first long noncoding RNA to be described, *H19* continues to provide interesting and novel scientific challenges.

EXPLORING THE EVOLUTION OF GENOMIC IMPRINTING

Genomic imprinting has become one of the most intensely studied epigenetic mechanisms in mammals, but its function and the rationale for its evolution exclusively in placental mammals have been hotly debated (Wilkins & Haig 2003). One reason for the debate is that imprinting presents geneticists with a paradox: An imprinted gene foregoes the obvious advantage of diploidy. On first principles, one would have predicted that imprinting would be selected against in evolution, especially for genes like *Igf2* that have profound effects on fetal growth.

The most persuasive (at least to me) explanation was proposed by David Haig in 1991 (Moore & Haig 1991), a remarkable accomplishment given that his ideas were developed before a single imprinted gene had been identified. Haig proposed that imprinting arose as a parental tug-of-war over the allocation of maternal resources to their progeny. The conflict requires two preconditions. The first is an asymmetry in parental allocation of resources for the embryo and the ability of both genomes to affect that allocation after fertilization. That explains why imprinting arose only in placental mammals, where the allocation of maternal resources continues throughout development and in the presence of the paternal genome. The second is polyandry, in which females mate with multiple males—a condition that holds for all but a handful of placental mammals. The best strategy for mothers to optimize their own reproductive fitness and the number of their future progeny is to allocate their resources evenly among all offspring, including future offspring. Fathers optimize their reproductive success by ensuring that their progeny receive more than their fair share of maternal resources by, for example, silencing the *Igf2r* gene that encodes a receptor that sequesters IGFII (Barlow et al. 1991). This conflict over maternal resource allocation is especially acute in species with multiple paternity within a litter, which is common among wild rodents and presumably their mammalian ancestors.

It is challenging to devise definitive tests of evolutionary theories. Paul Vrana, a fellow who came to the lab from the Museum of Natural History in New York, was intrigued by Haig's theory and proposed that we test it by studying a species of deer mouse, *Peromyscus polionotus* (P), that comes very close to being that rarest of beasts, a monogamous mammal. Most other *Peromyscus* species are polyandrous. It had been observed that if a *P. polionotus* female is crossed to a distantly related polyandrous male, *Peromyscus maniculatus* (M), the resulting offspring (P × M) are oversized and rarely survive. The reciprocal cross, in which *P. polionotus* males are crossed to *P. maniculatus* females (M × P), produces dwarfs (Dawson 1965). These parent-of-origin effects could be explained if the monogamous *P. polionotus* had lost imprinting, a finding that would lend strong support to Haig's theory.

What Paul Vrana found, however, was something quite different from our prediction. Both *Peromyscus* species exhibited all the hallmarks of imprinting, but there was widespread disruption of imprinting in the hybrid animals (Vrana et al. 1998, 2000). In a survey of eight normally imprinted genes, seven were biallelically expressed in one or both of the hybrids.

Paul's work implied that the mechanisms responsible for genomic imprinting, which appeared with the evolution of placental mammals approximately 60 Mya, are rapidly evolving. The two *Peromyscus* species diverged from one another only 100,000 years ago, but they have a limited ability to recognize and maintain each other's imprinting signals. These imprinting incompatibilities in closely related species are probably not unique and may explain the over- and undergrowth phenotypes of other mammalian interspecific hybrids, such as those between horses and donkeys or tigers and lions. Thus, imprinting may be accelerating the rate of mammalian speciation (Bush et al. 1977) by increasing the likelihood of hybrid dysgenesis between two populations that recently became reproductively separated.

REFLECTING ON HAPLOINSUFFICIENCY OF *PAX6*

Not all my intuitive leaps landed on gold mines like *H19*. After hearing Richard Axel describe his work with Andrew Chess on the monoallelic expression of olfactory receptor genes (Chess et al. 1994), I became obsessed with the idea that the haploinsufficiency of several developmentally important genes could be explained if the genes exhibited random-choice allelic exclusion. I imagined that in heterozygous animals, half the cells would be missing expression of the gene, a condition that could explain their heterozygous loss-of-function phenotypes. Testing this idea was not simple—single-cell PCR was just being developed. Eventually a graduate student, Catherine van Raamsdonk, unambiguously disproved my idea by using RNA-DNA fluorescence in situ hybridization of *Pax6* expression in the developing eye placode (**Figure 7**) (van Raamsdonk & Tilghman 2000). But she did far more than disprove her advisor's crazy idea. She went on to show that the haploinsufficient phenotype was caused by a failure to reach a critical threshold concentration of PAX6 protein at a critical time in the development of the lens placode. Timing, she showed, can be everything in development.

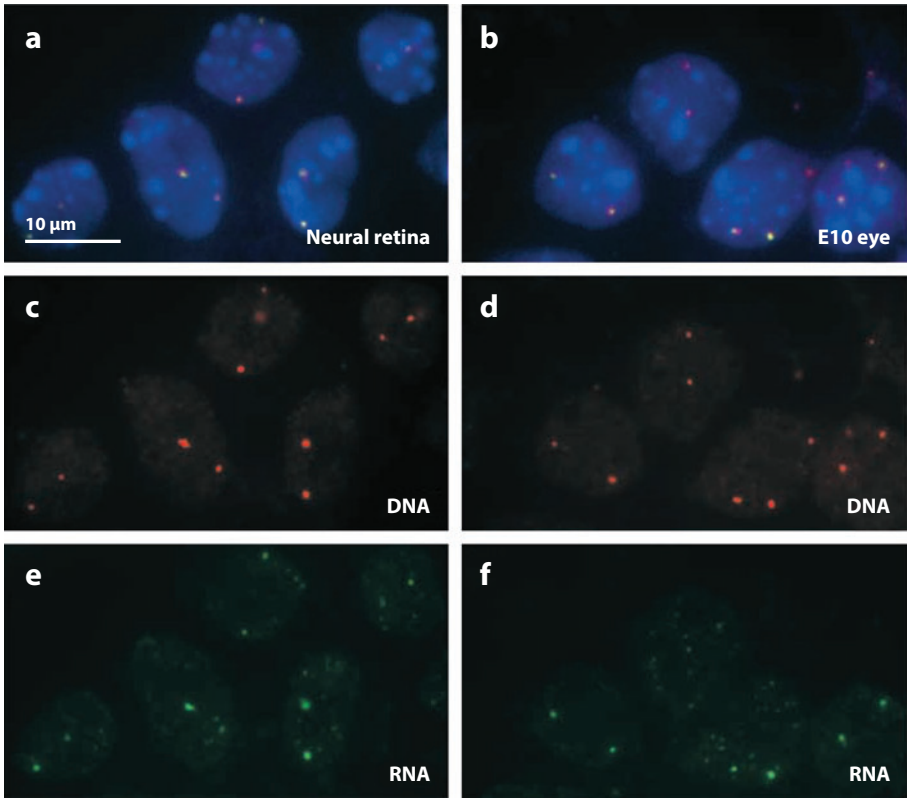

Figure 7

Biallelic expression of *Pax6*. RNA-DNA fluorescence in situ hybridization analysis of *Pax6* expression in (*a*,*c*,*e*) E12.5 neural retina cells and (*b*,*d*,*f*) E10 eye cells. RNA (*green*), DNA (*red*), and DAPI (*blue*) shown. (*c*,*d*) DNA signal. (*e*,*f*) RNA signal. Adapted from van Raamsdonk & Tilghman (2000).

FROM PHENOTYPE TO GENOTYPE: CHROMOSOME WALKING IN THE MOUSE

Bruce Alberts is responsible for another very significant twist and turn in the road. Not long after I arrived in Princeton in 1986, Bruce asked me to serve on the National Research Council (NRC) committee that he was assembling to study the feasibility of launching a major effort to sequence the human genome. Although I was following the debate in the scientific community, I was not an active discussant, and I was not certain why I was being asked to serve. When I arrived in Washington for the first meeting, it became clear that not only was I the only female on the committee, I was its youngest member. As it turns out, I was also the only member who had actually sequenced DNA with my own hands!

Serving on that committee, and watching Bruce ably craft a consensus report among very strong-minded individuals, was a tremendous learning experience. My major contribution to the outcome was advocating, alongside David Botstein, that the project cut its teeth and develop the necessary improvements in technology by sequencing model organisms. Not only did this strategy win for the project the support of skeptical biologists, it accelerated research on those organisms by decades and provided moments to celebrate along the way, thus avoiding the political fate of the superconducting supercollider.

The experience on the genome committee opened my eyes to the possibility of using genomics to identify genes involved in mouse development. Through the committee I met Eric Lander and recruited David Burke, a graduate student from Maynard Olson's lab who developed yeast artificial chromosomes (YACs), to build the first mouse YAC library (Burke et al. 1991, Rossi et al. 1992). With that library in hand, it was now thinkable to clone interesting genes by positional cloning.

Thinkable, yes, but madness without the support of the Howard Hughes Medical Institute (HHMI). I had just received one of those career-changing phone calls from HHMI Vice President Purnell Choppin, offering an investigatorship that came with very significant financial support for seven years. Suddenly, I had the freedom from grant worry to pursue new projects without a single piece of preliminary data—a scientific dream come true.

The mother lode for mouse mutants is housed at the Jackson Laboratory in Bar Harbor, Maine. For many years I had been attending its scientific meetings and serving on its Board of Scientific Overseers, becoming well versed in the collection of naturally occurring and induced mutant strains that had been assembled by generations of scientists at the lab. With positional cloning in its infancy and the only mouse YAC library in our freezer, it was only a matter of choosing which mutants to tackle.

We chose two: *Fused* (*Fu*), a dominant mutation that affects tail development in heterozygotes and results in embryonic lethality with axis duplications in homozygotes, and *piebald*, a recessive spotting mutant that affects neural crest development. I chose them in part because multiple alleles of both genes were available, which I thought might help in the endgame of conclusively identifying the genes. Although both had been mapped in the mouse genome, the resolution was in tens of megabases, so fellow Janice Rossi and graduate student Danika Metallinos generated 1,000-animal backcrosses of *Fu* and *piebald*, respectively, to narrow the intervals to regions that were tractable (Metallinos et al. 1994, Rossi et al. 1994). Then began the arduous job of walking from YAC to YAC to close the interval between the closest genetic markers. No one who has embarked upon such a marathon project could ever doubt the value of genome sequences.

The good news is that both genes turned out to be of great interest to developmental biology. The bad news is that others got to the genes before we did—and by other means. In the case of *Fu*, I got a phone call from Frank Costantini at Columbia, who had just generated a transgenic mouse line to study the expression of the embryonic globin gene. The insertion caused a homozygous

phenotype that was very similar to that of *Fu* and mapped near *Fu* on chromosome 17. We joined forces and showed by complementation mapping that Frank's transgene had indeed landed in the *Fu* gene (Perry et al. 1995). The product of the *Fu* gene is Axin, an inhibitor of Wnt signaling that regulates an early step in embryonic axis formation (Zeng et al. 1997). I am very grateful to Frank for generously asking us to collaborate in working out the function of the *Fu* gene.

In the case of *piebald*, Masashi Yanagisawa and his colleagues at the University of Texas Southwestern generated a targeted disruption of the endothelin-B receptor gene (*Ednrb*) in mice that turned out to display all the hallmarks of a *piebald* null allele, including aganglionic megacolon and loss of melanocytes (Hosoda et al. 1994). They showed that the induced allele failed to complement a naturally occurring *piebald* allele, establishing that the two were identical. EDNRB is a member of the G protein–coupled, seven–transmembrane domain protein family and is required for the development of both melanocytes and enteroneurons.

As we put the disappointment of not discovering the gene ourselves behind us, Myung Shin, a fellow in the lab, set out to identify when in development the expression of the *Ednrb* gene is required, as its expression profile is not particularly informative. He took advantage of the newly developed tetracycline-inducible system to engineer mice in which he could control the expression of *Ednrb* by administering the drug to pregnant mice. In an absolutely beautiful series of experiments, Myung showed definitively that the receptor is exclusively required for a brief

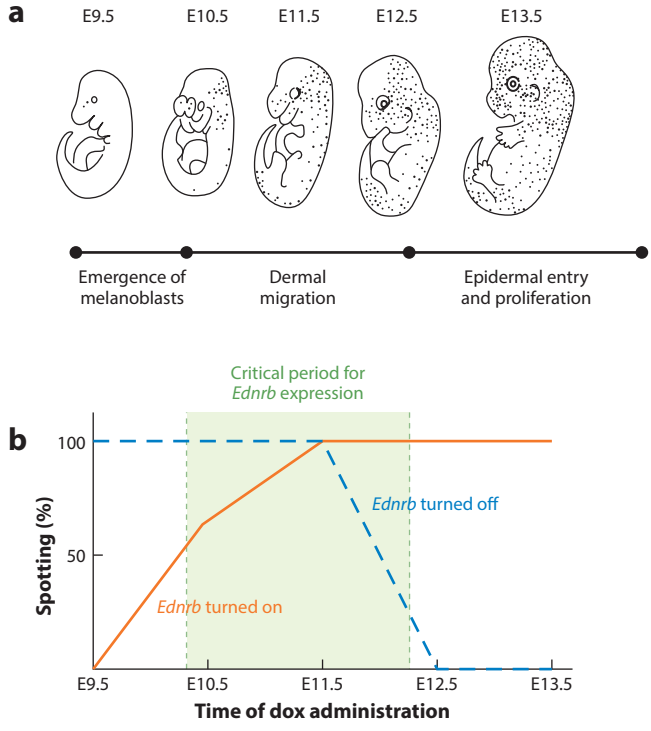

Figure 8

EDNRB signaling is required during melanoblast migration. (*a*) The appearance of melanoblasts in mouse embryos at different stages of embryogenesis. (*b*) The degree of white spotting in offspring in which tetracycline was administered at the times indicated (*orange*) or the time when tetracycline was removed (*blue*). White spotting indicates a loss of melanocytes. The critical period when EDNRB signaling is required is indicated by the green shaded region. Redrawn with permission from Shin et al. (1999).

period of neural crest development between embryonic days 10 and 12 (**Figure 8**). Furthermore, he could deduce from the timing that signaling through the receptor activates the migration of both neural crest derivatives away from the spinal cord (Shin et al. 1999). It was a tour de force of mouse engineering, and it gave us a glimpse into the possibilities for manipulating gene expression to understand mouse development.

AND NOW FOR SOMETHING COMPLETELY DIFFERENT

In October of 2000, I was elected by the faculty to the committee charged with selecting the next president of Princeton. In March, the committee asked me to step aside and become a candidate, and in early May, I was offered a most abrupt and unexpected twist and turn in the road.

I am often asked what prepared me to be the nineteenth President of Princeton University. As I look back on the 23 years of my scientific career prior to June 15, 2001, I can now see a growing interest in participating in the world outside the confines of my lab, chairing the Molecular Biology Study Section at the NIH; chairing NRC committees that studied the career prospects of young investigators (a topic in which I continue to have a deep interest); joining scientific advisory boards and committees of the Jackson Lab, the Whitehead Institute, and Genentech; serving as a trustee of the Cold Spring Harbor Laboratory and Rockefeller University; and becoming the founding chair of Princeton's Lewis Sigler Institute. I would not have agreed to these extra assignments if I did not enjoy them, and it is also probably true that I would not have been asked if someone did not have confidence that I could do the job.

Figure 9

Tilghman Laboratory reunion 2002.

When it came to the moment when I had to decide whether to give up the only professional world I knew—and one that I loved—I did not spend much time agonizing. I had loved Princeton from the moment I saw her beautiful verdant campus and began interacting with her impressive students and faculty. The presidency was an extraordinary opportunity to give back to an institution that made it possible for me to have a successful and rewarding career while raising two children as a single mother. I also suspected that I had already done the best science I was ever going to do, and moving on to something new where I might have a significant impact on an institution that had become home felt much more rewarding than treading water or falling behind in science. It is a decision that I have never regretted for one moment—even when the university's endowment fell 23.5% in 2009!

CONCLUDING THANKS AND APOLOGIES

I wish to thank the many students, fellows, and staff who were critical players in the story I have told in this essay (**Figure 9**). It was their energy, creativity, and hard work that produced the scientific advances that I have described. To those I did not mention by name, or whose projects I did not have space to highlight, I apologize. This essay is not meant to be an exhaustive review of the fields I have worked in, and I have left out many references to the work of scientific colleagues who contributed significantly to our own thinking. For that I also apologize.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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