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Transgenic mice – the early days

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Prologue

In 1979 our lab was shifting our research emphasis from studying regulation of egg white protein synthesis in chick oviduct to studying regulation of metallothionein genes by metals and glucocorticoids. We thought that the availability of mouse cell lines and mouse genetics would be advantageous for dissecting the mechanisms by which ligands such as steroid hormones and metals regulate gene expression in eukaryotic cells. In the summer of 1979, Ralph Brinster called to request some ovalbumin mRNA that he wanted to inject into mouse oocytes. Ralph explained that he wanted to determine if chicken ovalbumin mRNA could be translated by mouse oocytes and whether it could be secreted. We had lots of ovalbumin mRNA because of our studies that were directed towards finding the signal sequence responsible for secretion of ovalbumin (Palmiter *et al.*, 1978) and thus were willing to send him an aliquot. However, they never arrived because the package was mistakenly addressed to 35th and Locust Streets which is the location of the University President's office.

In the fall of 1979, I went to Pierre Chambon's lab in Strasbourg with a presumptive metallothionein cDNA in hand. I went there because we did not have expertise in recombinant DNA techniques and our biosafety committee made doing these experiments locally virtually impossible. My goal was to clone the *metallothionein (MT)* gene by screening a mouse lambda library. I returned two months later with two lambda phage, genomic subclones in pBR322, and a heteroduplex map indicating that the entire gene was about 1 kb and had two introns (Durnam *et al.*, 1980). When I returned, Ralph called to inquire about the ovalbumin shipment. We got the correct address and sent it again. Later in 1980, Ralph called to tell me that ovalbumin was expressed and that it was the only protein secreted by oocytes. He had run two-dimensional gels of oocytes and medium that had been incubated with radioactive amino acids. In the oocyte fraction there was one additional spot and in the medium there was only one spot - ovalbumin (Brinster *et al.*, 1981b). He inquired about the availability of other mRNAs and I offered RNA fractions enriched in conalbumin (transferrin) mRNA. I recall telling him that we were shifting our research emphasis to metallothionein and wondered whether he would be able to inject genes instead of mRNA into oocytes. He told me about experiments involving expression of *5S* ribosomal RNA genes that he had obtained from Don Brown (Brinster *et al.*, 1981a). He also said that he was collaborating with Paul Berg in an attempt to express *globin* genes in eggs but was not having any success. I mentioned that we could send him a plasmid containing a metallothionein gene, but he thought that the lack of a sensitive assay would make detection of metallothionein transcripts or protein very difficult. We were preparing to transfect the *MT* gene into tissue culture cells and planned to use the Herpes Simplex Virus thymidine kinase (*TK*) gene as a selectable marker in TK-null cells. Thus, we cloned an intact *TK* gene into a plasmid that already carried the MT gene. Ralph did an experiment in which this plasmid was injected into oocytes and then they were labeled with ^{35}S-cysteine but there was no indication of MT when the products were electrophoresed on an acrylamide gel.

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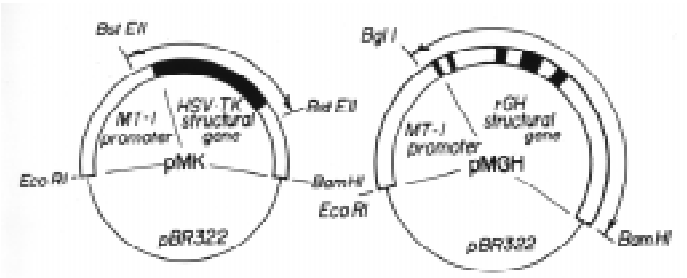

Fig. 1. **Left, plasmid pMK has the metallothionein-I promoter region (open box) fused to the coding region of HSV thymidine kinase (solid) in the classical plasmid backbone, pBR322.** *This plasmid was injected into mouse oocytes or fertilized eggs to measure thymidine kinase activity (see Brinster et al., 1982). Right, plasmid pMGH, that was constructed by Ron Evans and myself. It has the metallothionein promoter (open box) fused to the rat growth hormone (rGH) gene which has 5 exons (solid boxes).*

Success with pMK

Allen Senear, a post-doc in my lab, pointed out that it would be easy to make a fusion gene in which the *MT* gene promoter would drive the expression of *TK* because both genes had unique *Bgl*II sites just upstream of their initiation codons. Thus, by simply cutting with *Bgl*II and religating one could generate the desired plasmid. We talked about this plasmid, called *pMK* (Fig. 1), in late fall of 1980. He was very enthusiastic to try it because he had been testing a *TK* gene that he had obtained from Carlo Croce and had worked out a sensitive thymidine kinase assay based on conversion of ^{3}H-thymdine to ^{3}H-thymidine monophosphate. In late 1980, I sent an aliquot of *pMK* to Ralph. Because we knew that the mouse *MT* gene was inducible by heavy metals, Ralph tested the sensitivity of mouse oocytes and eggs to zinc or cadmium and determined that they could survive in up to 50 μM cadmium. In the first experiment with this plasmid in January 1981, he injected *pMK* and incubated some of the eggs overnight in cadmium and some were kept in the normal medium. The next day when TK activity was measured, there was 10 times more activity in the eggs that had been incubated with cadmium (Fig. 2). These experiments were conducted before transient transfection assays in tissue culture cells were considered feasible; thus, for us this result opened the way for mapping the regulatory elements involved in cadmium regulation of *MT*. In fact, in our paper describing these initial results, we also tested a series of promoter deletions and concluded that 90bp of *MT* sequence was sufficient to confer some regulation by cadmium but that there were additional elements further upstream (Brinster *et al*., 1982). Because early versions of this paper were not accepted, the revised versions ended up being published after our first transgenic mouse paper which was rushed into print. We continued to use this assay to define a 12bp sequence, the metal regulatory element, that conferred responsiveness of *metallothionein* genes to cadmium (Stuart *et al*., 1984).

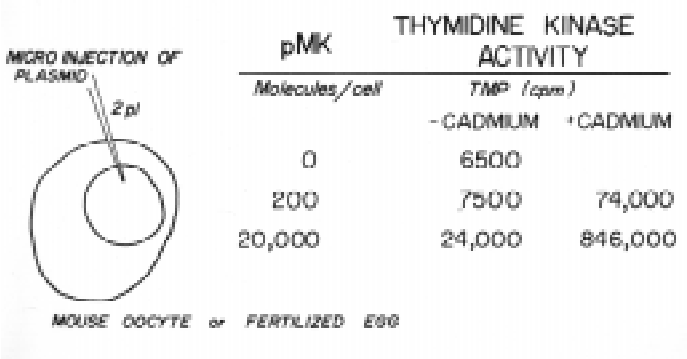

Fig. 2. An example of the thymidine kinase activity observed when plasmid pMK was injected into oocytes and enzyme activity (expressed as ^{3}H- thymidine monophosphate, TMP, produced) was measured the next day. *Oocytes were incubated plus or minus 50 μM cadmium sulfate, which is an inducer of the metallothionein gene transcription. I often showed a slide of this figure in my seminars. My earlier work on synthesis of chicken egg white proteins probably explains why the mouse egg in this picture has an oval shape!*

While we were pleased to have an efficient assay for mapping *cis*-acting regulatory elements, Ralph realized the further potential of these results and set up experiments in which some of the injected eggs were transferred to pseudopregnant foster mothers. By late spring of 1981 he had produced mice from fertilized eggs injected with *pMK* that had 100 times more thymidine kinase activity in their livers than control mice. The results of these experiments were submitted to *Cell* and on September 17, 1981 we received a note from Benjamin Lewin saying that "Of course, we shall be glad to publish your paper". That paper (Brinster *et al*., 1981c) was one of four papers published in the fall of 1981 indicating that foreign DNA could be integrated into genome of mice. A year earlier, Ruddle's lab published the first paper demonstrating the feasibility of the approach (Gordon *et al*., 1980). Our paper provided convincing evidence for expression of the foreign DNA. The plasmid used by Gordon *et al.* (1980) only contained a cDNA and hence couldn't be expressed; the mice with the *TK* gene described by E. Wagner *et al.* (1981) had barely detectable expression. The globin genes tested by E. Wagner *et al.* (1981) and Costantini and Lacy (1981) were not expressed, but T. Wagner *et al.* (1981) claimed that a β-globin gene they used was expressed, based on immunological data. However, their founder animals were killed, the samples were pooled, there was no molecular data to substantiate expression, and the experiments were never repro-

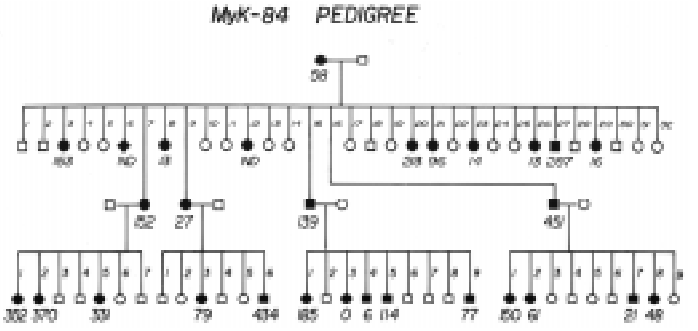

Fig. 3. The MyK-84 pedigree. *MyK-84 was a female founder mouse that carried about 100 copies of a tandem head-to-tail array of a 2.1 kb fragment derived from pMK that included about 350bp of metallothionein promoter and the intact TK gene. This founder female transmitted the transgene to half here offspring (solid symbols) and they continued to transmit the gene in a normal Mendelian manner. However, the TK activity (measured in liver samples; values under the symbols) was extremely variable. See Palmiter et al. 1982 for details.*

duced. Furthermore, lots of subsequent work revealed that regulation of globin genes expression is very complex. We went on to show that many copies of the foreign DNA were integrated in tandem, that the *pMK* DNA became methylated, that it could be transmitted thought the germline, and that expression persisted in some lines of mice but was highly variable (Fig. 3; Palmiter *et al.*, 1982b). In our second paper we began to refer to the mice carrying foreign DNA as "transgenic."

Face-to-face

Ralph and I first met in the fall of 1981, shortly before our first *Cell* paper was published. We met at his lab on the third story of an old warehouse on Locust and 25th Streets (Fig. 4). I had the opportunity to meet Barbara Paynton, who was studying protein synthesis and secretion, Howard Chen, who was doing the TK enzyme assays and Myrna Trumbauer, who did the DNA microinjections. Ralph had arranged to have our pictures taken on that occasion (Fig. 5). We discussed our goals and agreed that we wanted to use this gene transfer technique to correct a genetic disease.

Growth hormone

I had given a seminar at Roswell Park just before going to Philadelphia and learned about *little* mice from William Held. Although the genetic defect in *little* mice was unknown, it was known that they lacked growth hormone (GH) and would respond to injected GH (Beamer and Eicher, 1976). Thus, we decided that introducing a *GH* gene under the control of the *metallothionein* gene promoter might rescue the growth defect in these mice. Ron Evans had just given a seminar at the University of Washington describing the cloning and characterization of the rat GH gene, so I enlisted his help in executing this experimental plan. The only problem was that Jackson Laboratory did not have many *little* mice in stock, so it took a long time to obtain mice and then build up a colony. Meanwhile, Ralph decided to inject the *metallothionein-rat GH* gene (Fig. 1) that Ron Evans and I made into normal mouse eggs. I can still remember his excitement when he told me that some of the mice derived from injected eggs were growing larger than normal. When these experiments were published (Palmiter *et al.*, 1982a), scientists, cartoonists, comedians and animal rights activists were aroused to the potential of transgenic technology. The ability to change the phenotype of an animal was so dramatic that everyone took note even though the experiments we published a year earlier clearly demonstrated the potential of the technique. We subsequently showed that human, bovine and porcine GH worked just as well as rat GH (Hammer *et al.*, 1985a). I always wished we had used a *GH* gene from an animal smaller than a mouse, because many people mistakenly thought that the transgenic mice grew larger than normal because we used a *GH* gene from a rat. Thus, some people missed the salient point that directing expression of a gene to a more abundant cell type (such as hepatocytes) enhanced accumulation of protein in the blood and prevented normal feedback regulation (Fig. 6).

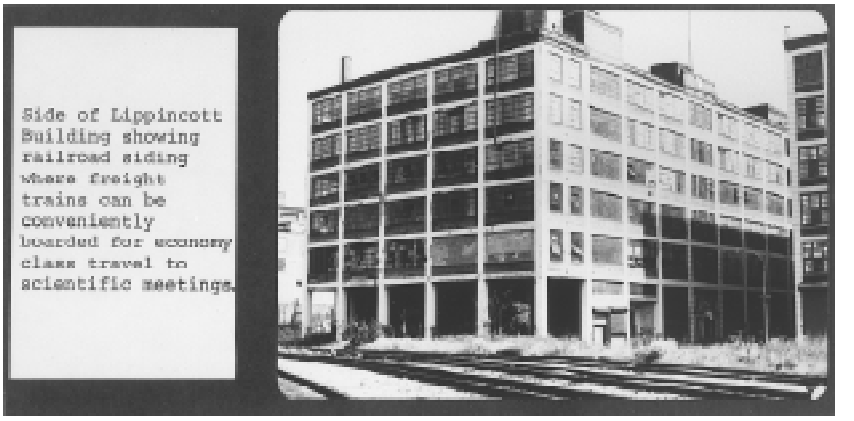

Fig. 4. The Lippincott Building where Ralph did all the pioneering transgenic experiments. *I was so amazed when I visited in 1981 that I asked Ralph for some pictures of the building which sent along with some annotations. Ralph's lab was on the 3rd floor, which one reached by ascending in a freight elevator.*

Fig. 5. When I visited in 1981, Ralph (on the right) had a professional photographer scheduled to take our picture.

Robert Hammer joined Ralph's lab when the GH experiments were beginning and he ultimately introduced the *metallothionein-GH* gene into *little* mice and showed that it corrected their growth defect. In fact they grew larger than normal (Hammer *et al.*, 1984). Only later was it discovered that the genetic defect in *little* mice lies in the receptor for growth hormone regulatory factor (GRF). Robert Hammer collaborated with Kelly Mayo, a former student of mine, to show that expression of GRF under the control of the *metallothionein* promoter also stimulated growth by enhancing the production of mouse GH by the pituitary (Hammer *et al.*, 1985b). We also pursued growth control in mice by making transgenic mice that expressed insulin-like growth factor-I (IGF-1) and showed that they too grew larger than normal (Mathews *et al.*, 1988). Meanwhile, Richard Behringer used a cell ablation technique (Palmiter *et al.*, 1987) to generate mice in which the somatotroph cells that make growth hormone were ablated by expression of diphtheria toxin. These mice were dwarfs (Fig. 7; Behringer *et al.*, 1988).

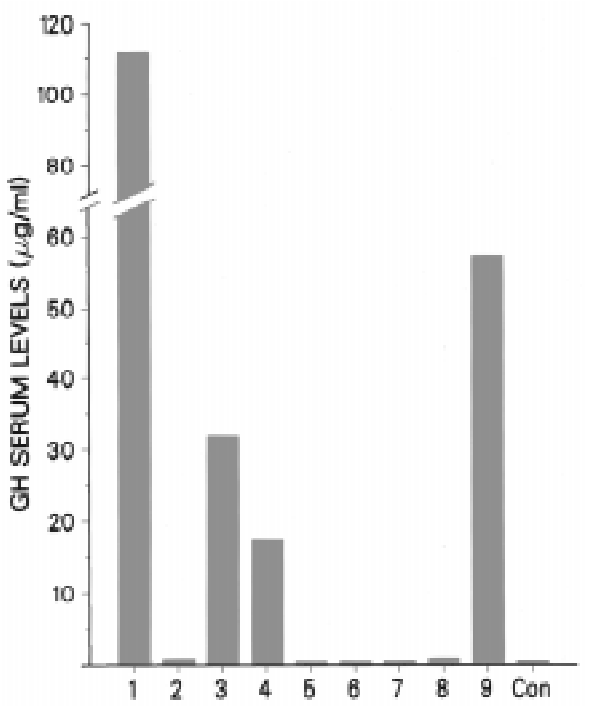

Fig. 6. Incredible amounts of growth hormone accumulated in the blood of some of the transgenic mice carrying the MT-rGH transgene. *Growth hormone is normally present at a few ng/ml. See Palmiter et al. (1982) for details.*

Domestic animals

The potential of using this technology in domestic animals was indicated in the concluding paragraph of the first GH paper (Palmiter *et al.*, 1982) where we stated that "the ability to introduce into mice, an by extrapolation into other animals, functional genes of selected construction offers wide ranging experimental as well as practical opportunities." Indeed, we went on to make transgenic rabbits, sheep and pigs (Hammer *et al.*, 1985c) and some of them expressed the foreign *GH* genes. I never suspected that I would be a co-author on a paper with pork chops prominently displayed (Pursel *et al.*, 1989).

Cell-specific expression

Gene constructs with the *MT* promoter were expressed in many different cell types of transgenic mice, which was expected because the endogenous *MT* gene is expressed in most organs (Palmiter *et al.*, 1983). The idea of trying to restrict expression of transgenes to specific cell types came naturally from experiments aimed at identifying promoter/enhancer elements responsible for cell-specific expression. The first experiments of this type were conducted by Ralph in collaboration with Ursula Storb, a colleague of mine at the University of Washington. They showed that an immunoglobulin *kappa chain* gene was expressed exclusively in B cells (Storb *et al.*, 1984). Our first experiments involving cell-specific gene expression were performed on the rat *elastase* gene, which is normally expressed in pancreatic acinar cells. These experiments were initiated by Ray MacDonald, who I had met on a trip to Dallas, and later pursued by David Ornitz a graduate student in my laboratory. We were able to identify a 200bp region that was sufficient to achieve abundant acinar cell-specific expression of GH (Fig. 8), which had become a convenient reporter molecule (Ornitz *et al.*, 1985a). The fact that the mice expressed copious amounts of GH but did not grow larger than normal was compelling biological evidence that GH was being secreted into the pancreatic juice rather than the bloodstream. Expression of diphtheria toxin from the same *elastase* promoter ablated acinar cells without affecting other cells, again indicating that the promoter is expressed selectively in acinar cells (Palmiter *et al.,* 1987). Similar experiments were subsequently conducted to identify the regulatory elements responsible for erythroid-specific expression of human β-globin (Townes *et al.*, 1985; Ryan *et al.*, 1989), liver-specific expression of albumin (Pinkert *et al.*, 1987), and spermatid-specific expression of protamine (Peschon *et al.*, 1987).

Neoplasia

Our venture into oncogenesis was initiated by accident. I made plasmid constructs in which the *SV40 T-antigen* gene was placed in the same plasmid as a *MT-growth hormone* gene. The reason for constructing this plasmid was to see if T-antigen would stimulate gene amplification in cultured primate cells. Ralph and I subsequently decided to test this construct in mice to see if the SV40 enhancer, that was included in the construct, would boost expression of GH. The unexpected result was that the mice developed brain tumors (Fig. 9; Brinster *et al.*, 1984). Albee Messing, then a fellow at the University of Pennsylvania, showed that the tumors were derived from the choroid plexus. When I told Arnold Levine, who I met at a meeting at the Salk Institute, about these unexpected results, he became intrigued and offered to help because of his long-standing interest in T-antigen. Although the *T-antigen* gene was present in all cells, the choroid plexus

Fig. 7. Dwarf mice were produced by directing diphtheria toxin A chain expression to somatotroph cells of the pituitary using the rat growth hormone gene promoter. *The mouse on the left has virtually no somatotroph cells in the pituitary and hence cannot make adequate amounts of growth hormone. The mouse on the right is a control littermate.*

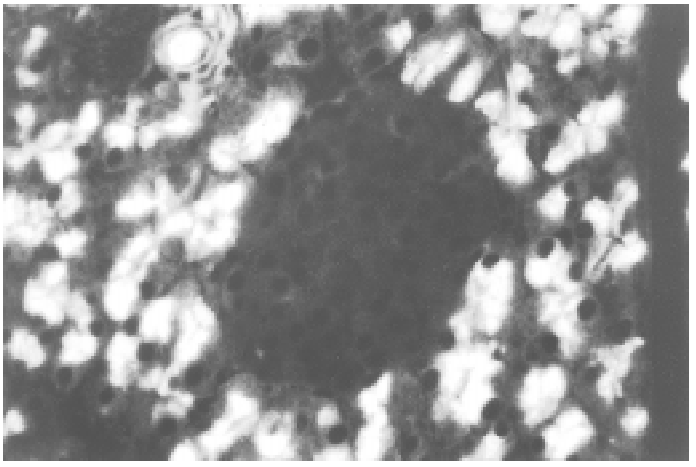

Fig. 8. Immunocytochemistry of a section through the pancreas of a mouse carrying an elastase-human growth hormone gene construct. *The FITC-labeled antibody detects growth hormone (white areas in this black and white photo) that is present in the acinar cells and pancreatic duct (upper left), but not in the islet cells (dark oval in center).*

appeared to be particularly vulnerable to transformation by T-antigen. This experiment led to many more in which SV40 T-antigen was directed to other cell types including pancreatic acinar cells (Ornitz *et al.*, 1985b,1987) and hepatocytes (Sandgren *et al.*, 1989). In these cases, the transgenic mice invariably developed pancreatic or liver tumors, respectively, when they were a few months old. Other oncogenes were also tested including the *myc* gene (Adams *et al.*, 1985) and an activated *ras* gene (Quaife *et al.*, 1987), and they too invariably produced tumors in the mice. The results with an activated *ras* gene under the control of the *elastase* promoter were particularly striking because the mice were born with pancreatic tumors, suggesting that *ras* alone was sufficient to drive uncontrolled proliferation in embryonic acinar cells (Quaife *et al.*, 1987). In most other examples, the transgenic mice bearing oncogenic transgenes manifest cellular hyperplasia. Then focal nodules of more transformed cells emerge, suggesting that additional genetic insults are necessary to give them a proliferative advantage.

Disease models

Several mouse models of human diseases were produced by transgenic techniques, including diabetes (Lo *et al.*, 1988) and sickle cell anemia (Ryan *et al.* 1990). The sickle cell model, in particular, required extensive prior transgenic experimentation. Another transgenic mouse model that has been particularly useful is one that resembles the active carrier state of chronic hepatitis B virus infection. Working with Frank Chisari at Scripps, we made transgenic mice that expressed the surface antigens of hepatitis B virus in the liver under the control of either the mouse albumin or *metallothionein* gene promoters. When the large envelope protein is produced in liver it accumulations in the endoplasmic reticulum and leads to cellular injury, death and regeneration. This process is associated with constant inflammation. These lesions are characteristic in people with chronic hepatitis (Chisari *et al.*, 1987). When the mice are over a year old, they develop hepatocellular carcinomas (Chisari *et al.*, 1989). We proposed that the hepatocellular regeneration exposes dividing cells to excessive oxygen radicals generated by the chronic inflammation and that this leads to DNA damage which culminates in neoplasia.

Germ cells

Most of the results described above emanated from projects that were initiated in the three years after our successful expression of *pMK* in fertilized mouse eggs. It was readily apparent that transgenic methodology could be used to explore many different experimental questions and we could not resist the opportunities to collaborate with specialists throughout the country on these various projects. During this time we also laid the groundwork for research areas that we wanted to pursue. Ralph was particularly interested in germ cell development. We began in that area by studying a transgenic mouse carrying a *pMK* transgene that resulted from one of our first experiments. It had the unusual property of not transmitting the transgene through the male germline even though the males were fertile (Palmiter *et al.*, 1984). By cloning the transgene insert, we learned that it was flanked by identical 5 kb DNA sequences that were only present once in the preinsertion sequence (Wilkie and Palmiter, 1987). We ultimately discovered that the *TK* gene was toxic to male germ cells and homologous recombination between the duplicated DNA sequences eliminated the transgene and restored fertility (Wilkie et al., 1991). We also pursued the germ cell interests by cloning the mouse *protamine* gene and studying its regulation (Peschon *et al.*, 1987; Braun *et al.*, 1989a,b) and then turned to the *ZFY* gene (Zambrowicz *et al.*, 1994).

Catecholamines and neurobiology

Meanwhile, Ralph convinced me that we should use transgenic techniques to study the development and function of the nervous system. In 1985, Ed Baetge, applied for a post-doctoral position and he came to the lab with several cDNAs for genes involved in catecholamine biosynthesis that he had cloned as a graduate student. Using those cDNAs as probes, we cloned the genes (Baetge *et al.*, 1988, Mercer *et al.*, 1991), identified their regulatory regions (Hoyle *et al.,* 1994) and used them to direct the expression of reporter genes (Kapur *et al.*, 1991,1992), oncogenes and growth factors (Hoyle *et al.*, 1993) to developing catecholaminergic neurons . More recently, some of these genes were inactivated by gene targeting in embryonic stem cells (Thomas *et al.*, 1995; Zhou *et al.*, 1995). Our interest in neurobiology continues.

Modus operandi

Starting in 1982, we began to have frequent phone conversations that were necessary to coordinate all the transcontinental experiments. This evolved into a regular schedule in which we would talk for about 2 hours every Saturday afternoon and continue on Sunday afternoon, if necessary. Each week, tail samples for DNA analysis and tissue samples for mRNA analysis would arrive by Federal Express - typically 200 to 500 samples. I would relay the results of the DNA and RNA analyses to Ralph and then we would discuss current research directions and overall strategy. This mode of operation continued for about 13 years. During that time about 900 DNA constructs were made and sent to Philadelphia for microinjection. In return, we received over 125,000 tubes with tissue samples in sodium dodecyl sulfate and proteinase K for analysis of DNA or mRNA!

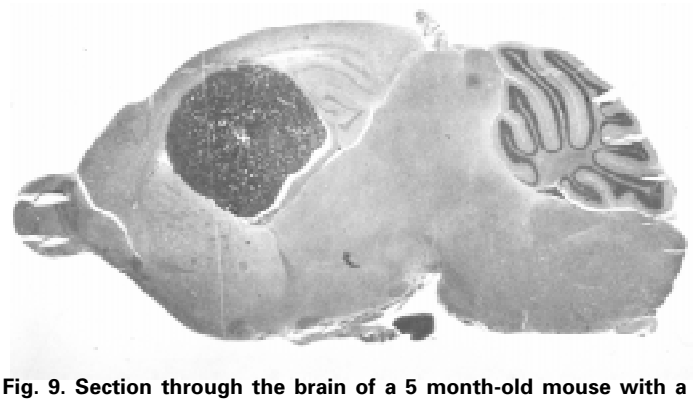

Fig. 9. Section through the brain of a 5 month-old mouse with a choroid plexus tumor (dark mass on the left) that was caused by expression of SV40 T-antigen. *See Brinster et al. (1984) for details.*

During our "strategy sessions," we often talked about methods to improve the efficiency of basic transgenic technology and many different ideas were tested. The resulting paper (Brinster *et al.*, 1985) included a wealth of data (representing hundreds of transgenic animals produced) that we accumulated related to the influence of DNA concentration, its form, buffer composition, choice of male or female pronucleus and genetic strain of the recipient eggs. Some of the things we learned are summarized in Figure 10. Perhaps the most remarkable outcome from all these studies was that the original choice of conditions were close to optimal.

Homologous recombination

Another topic that was often discussed was the desire for site-specific integration or even better, homologous recombination.

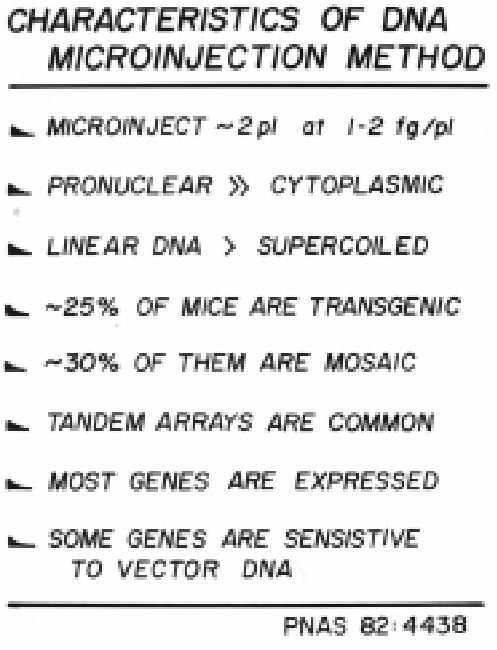

Fig. 10. Some of the characteristics of the DNA microinjection method that are important for efficient production of transgenic mice. *I often showed a slide of this figure during seminars when I was asked to talk about transgenic mice.*

One idea that we pursued was the use of FLP recombinase. We engineered a FLP recognition sequence into an intron of the *GH* gene and then made a line of transgenic mice carrying the *MT* promoter and the first half of the *GH* gene with the FLP recognition sequence. This gene was expressed but not functional because the C-terminus of GH was missing. Then, eggs from these mice were injected with an overlapping piece of DNA that included the FLP recognition sequence but was nonfunctional because it lacked the N-terminus of GH. Purified FLP recombinase, obtained from Michael Cox, was coinjected with the hope that it would recognize the FLP recognition sequences in the injected DNA and recombine it with the other half of the gene that was already integrated. Despite a valiant effort, this experiment did not succeed. However, in the process of doing controls in which both pieces were coinjected together, we learned that recombination between DNA fragments that overlapped by just a few hundred basepairs was quite efficient

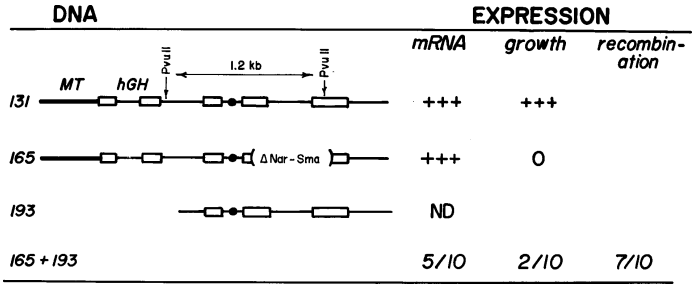

Fig. 11. Recombination of coinjected DNA molecules. *In this experiment, two DNA fragments (#165 and #193) that overlap by about 300 basepairs were coinjected. Neither fragment alone is capable of making functional growth hormone, but if they recombine then they can produce a functional gene (equivalent to construct #131). The FLP recognition sequence (solid circle) was included as part of an experiment to achieve site-directed recombination (see text).*

because 7 or 10 transgenic mice produced had recombined the two DNA fragments and 2 of the mice grew much larger than normal indicating that they produced functional GH (Fig. 11; Palmiter *et al.*, 1985). Recombination among injected fragments prior to integration was the explanation we offered for the presence of tandem, head-to-tail, arrays in our first transgenic paper (Brinster *et al.*, 1981) but this result suggested that one could actually build large transgenes by injecting overlapping fragments. We used this observation to our advantage when studying *ZFY* genes some years later (Zambrowicz *et al.*, 1994).

We often discussed how to achieve homologous recombination. Most of the transgenes we were using were composed of pieces of DNA from different genes that were derived from different species; thus, they were not good substrates for homologous recombination. However, in a few cases, we tested marked transgenes derived entirely from mouse genes; but when the DNA from the resulting transgenic mice was examined by Southern blotting there was no indication of homologous integration. In 1988, we decided to try in earnest to achieve homologous recombination and chose to target the histocompatibility class II Eα locus because the endogenous Eα gene in the strains we routinely used had a 630 bp deletion that removed its promoter and first exon. Because of

our collaboration with Rick Flavell, the DNA needed to make a targeting construct was available (Pinkert *et al.*, 1985). If the experiment worked it would also correct a genetic defect. We ultimately succeeded in obtaining one mouse in which targeting had occurred, but only after examining 500 transgenic mice derived from thousands of injected eggs (Brinster *et al.*, 1989). We also noted numerous mutations had occurred in the recombined allele. We subsequently tried to target constructs to the *MT* locus and to the *LFA-1* locus by direct injection. The latter was chosen because the targeting frequency in ES cells was very high; thus, we knew what was potentially possible. Although we ultimately obtained one fetus where Southern blotting indicated that recombination had occurred, the frequency was too low to be practical. By this time, ES cell gene-targeting had become routine and we switched to that approach. Subsequently, Susulic *et al.* (1995) reported that they had achieved correct targeting of the adrenergic β3 receptor by direct injection of DNA into fertilized eggs with a frequency of 9% (2/23); the frequency in ES cells for the same construct was 56% (43/77). Direct injection still seems like a good idea in that it would speed up the process and there would be little uncertainty about germ line transmission, but the efficiency needs to be improved. When rate-limiting factors for homologous recombination are identified they could be coinjected into fertilized eggs along with the targeting construct. Perhaps even injecting DNA along with a nuclear extract form ES cells would facilitate targeting in eggs.

Introns

In the process of testing many transgenes, we began to realize that natural genes (e.g., growth hormone) were expressed much better than constructs that incorporated cDNAs. This led to the idea that the introns in natural genes were important for efficient transgene expression. Ralph suggested that we should study this *phenomenon systematically*, which we did. We compared several natural genes, with and without their introns, and clearly demonstrated that introns increased the frequency of obtaining transgenic mice that expressed the genes and the level of expression (Palmiter *et al.*, 1988). We went on to determine whether certain introns were more important than others and whether heterologous introns would work (Palmiter *et al.*, 1991). We concluded that the role of introns was not in splicing *per se*, but that they helped phase nucleosomes appropriately relative to important cis-acting regulatory elements. We speculated that after injection the transgenes would be packaged into chromatin and would be inactive until some later time in development and that the nucleosomal phasing was important for the efficient activation of the transgenes.

Epilogue

Collaborations are often difficult. I think that we succeeded in our transcontinental collaboration for so many years because we (i) had non-overlapping talents and respected each others abilities, (ii) had similar work ethic and pushed the projects we agreed upon to publishable ends, (iii) communicated regularly, and (iv) tried to share the credit equally between ourselves and with our students.

Ultimately, our research interests began to diverge. We wanted to use mouse genetic techniques to explore the function of metallothioneins and to pursue our growing interest in neurobiology. Ralph, on the other hand, wanted to pursue his long-standing interest in male germ cells. Our collaboration changed the scientific direction of my lab and exposed me to ideas and biological problems that I would not have encountered otherwise. There is no question that my collaboration with Ralph was an exhilarating adventure that I will always cherish.

KEY WORDS: *transgenic mice, growth hormone, thymidine kinase, neoplasia, recombination*

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