Knockout Mice: Round Two

Clever new techniques allow researchers to achieve the long-sought goal of inactivating mouse genes specifically in selected cell types

Immunologist Klaus Rajewsky of the University of Cologne had a problem. He wanted to know how important the enzyme DNA polymerase β is for the development of the immune system's T and B cells. But when he tried to answer that question in the now-standard way—by making knockout mice that lack a functional DNA polymerase β gene—it turned out that the enzyme is vital to mouse development. As a result, the mice died as embryos, even before T and B cell development began, making it impossible for Rajewsky to get his answer. And so he found himself wishing for a way to knock out the gene in just the cells he wanted to study. Rajewsky is hardly alone; his is a common longing these days among developmental biologists, immunologists, and others who want to study gene functions in mice. But until recently, the idea of such conditional knockouts was merely a pipe dream.

No longer. On page 103, Hua Gu, Rajewsky, and other colleagues report that they've inactivated the DNA polymerase β gene in just the T cells of mice. The achievement has provided the answer to Rajewsky's problem: T cells with the dysfunctional gene seem to be at a disadvantage during development, although some of them survive. But what is much bigger news than the biological finding is the technical advance: This is the first report of a normal mouse gene being selectively knocked out in just one cell type, and the method should be generally applicable to other types as well. "I think it will be very important," says developmental biologist Janet Rossant of Mount Sinai Hospital in Toronto. With such conditional knockout methods in hand, she says, "one's imagination can run riot with ways to manipulate gene expression."

Although Gu and Rajewsky are the first into print with a tissue-specific knockout of a normal mouse gene, their team is just one of many moving toward the same goal of conditional gene knockouts. "I would guess there are minimally about 100 labs working on this," says Mario Capecchi of the University of Utah, one of the developers of knockout mice. "It is a large enterprise."

Driving this enterprise is the sense of excitement about what it can achieve. As Rajewsky found with his efforts to use traditional knockouts to study the function of DNA polymerase β, eliminating a gene in all the cells of the mouse's body won't work if the gene is needed early in development. "You can't study other aspects of that gene's function that would occur at later stages," says Capecchi. With tissue-specific knockouts, he adds, that problem can be avoided by allowing the researcher to design a mouse in which the gene is knocked out only in certain tissues, after the early critical period when the gene is needed has passed.

Variations of the conditional knockout method are allowing researchers to do other genetic tricks that have been difficult or impossible in the past, such as swapping a gene containing a point mutation of the researcher's choosing for its normal counterpart or indelibly marking all the cells that descend from a particular cell type, a process called fate mapping. "It is going to make an incredible impact on mammalian cell-fate determination," says Jamey Marth of the University of British Columbia in Vancouver, a collaborator on the Gu and Rajewsky paper.

Standard knockout mice are made by specifically inactivating the gene of choice in cultured cells known as embryonic stem (ES) cells. The ES cells are then injected into mouse embryos, where they have the potential to develop into all the different mouse cell types (*Science*, 5 June 1992, p. 1392). The resulting animals are then bred, and those whose germ cells—the eggs and sperm—are derived from the ES cells will pass the inactivated gene to their progeny. To create mice that lack the gene only in certain tissues, the gene must be knocked out not in the ES cells but later, in cells of the developing embryo or even adult mouse. To accomplish that, researchers have resorted to clever techniques using enzymes borrowed from bacterial viruses and yeast.

For their work, Gu and Rajewsky used an enzyme called Cre recombinase from bacteriophage P1, a virus that infects the bacterium *Escherichia coli*. During that infection, Cre's job is to separate any phage genomes that become joined to one another. To do that, Cre lines up short sequences of phage DNA called *loxP* sites and removes the DNA between them, leaving one *loxP* site behind.

Molecular biologist Brian Sauer, then at du Pont, first drew the attention of genetic engineers to this seemingly esoteric enzyme in the mid-1980s, when he showed it could be made to work in cells of higher organisms such as plants and mice. Sauer introduced a gene flanked by *loxP* sites into the genomes of cultured cells that were engineered to express the Cre protein as well. The result: The *loxP*-flanked DNA was removed.

The next step was to see whether the Cre system would work in the cells of living mice. Two groups, one led by Marth in Vancouver and the other by Heiner Westphal at the

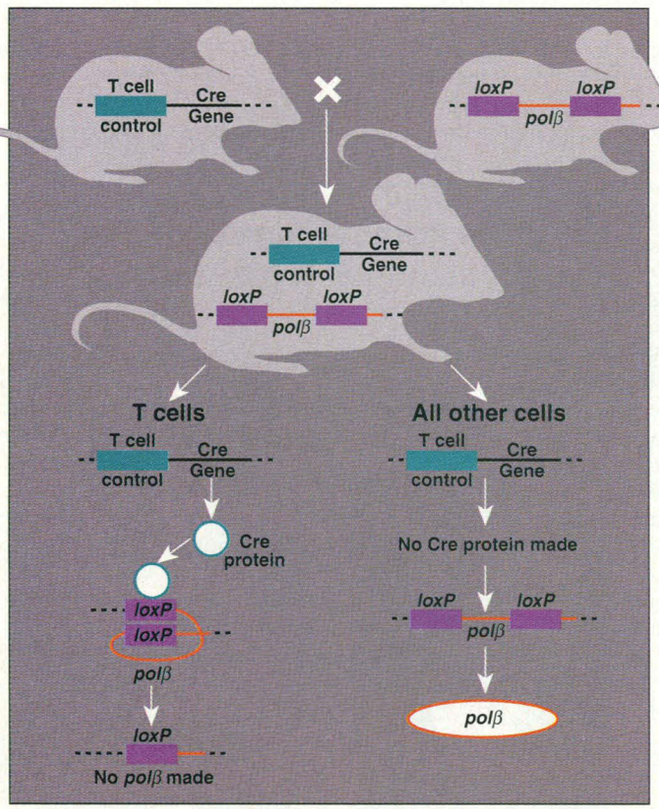
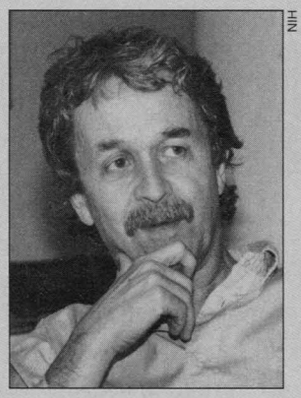


ILLUSTRATION: C. FABER SMITH

Cutting out. The Cre enzyme, which clips out the polymerase β gene segment between the *loxP* sites, is made only in T cells, and thus inactivates the gene only in those cells.

Researchers Devise a Master Gene Control Switch

It's been a busy decade in mouse genetics; researchers have flooded into the field to take advantage of new techniques for making transgenic mice that contain foreign genes and knockout mice in which normal mouse genes are eliminated. But rare is the researcher who has been satisfied with the tools at hand. Those working with knockout mice wanted to be able to deactivate genes only in specific tissues; that dream has now come true (see main text). And even though researchers working with transgenic mice have long been able to turn foreign genes on selectively in certain cell types, they want yet more control—the equivalent of a manual master switch with which they can turn genes on and off at will. And now they, too, are beginning to get their wish.



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Developed a "hot item." Hermann Bujard.

Ideally, this master switch would be a chemical or hormone that can control a foreign gene in mice without affecting normal mouse genes, thus avoiding stray biological effects that might cloud the experimental results. One promising system was reported in 1992 by Hermann Bujard and his student Manfred Gossen at the Center for Molecular Biology in Heidelberg, Germany. It borrows its working parts from bacterial genes that are turned on by the antibiotic tetracycline.

In bacteria, these genes are normally kept in the off position by a repressor protein that sits on control sequences at the start of the genes. But when tetracycline is around, the drug binds to the repressor, pulling it off the control sequences and switching the genes on. Bujard and Gossen modified this system so that it could be used to control gene activity in animal cells—but with a twist. In the modified system, tetracycline turns genes off instead of on.

Bujard and Gossen transformed the tetracycline-repressor protein into a mammalian gene activator by splicing its gene onto the gene for a viral protein that turns on mammalian genes. This new hybrid gene makes a hybrid activator protein that, in mammalian cells, will bind to and activate any gene linked to the bacterial control sequences recognized by the tetracycline repressor—but only when tetracycline is absent. Add the drug and it will bind to the hybrid protein, pulling it off the genes and shutting them down. Gossen and Bujard soon found they had a hot item on their hands: After publication of their 1992 report in the *Proceedings of the National Academy of Sciences* showing that the scheme works in animal cells, "we were swamped with more than 800 requests [for the system's components] in one-and-a-half years," says Bujard.

Many of those requests came from research groups eager to try to turn genes on and off in transgenic mice. One such research team includes Lothar Hennighausen of the National Institute of Diabetes and Digestive and Kidney Diseases, and Priscilla Furth of the University of Maryland. While on sabbatical in Peter Gruss's lab at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, they teamed up with Bujard to test the tetracycline system in mice.

They made transgenic mice that contain both the gene for Bujard's hybrid activator protein and a bacterial gene for the enzyme β-galactosidase, which is linked to the control sequences from bacterial tetracycline-controlled genes. They found that the genes work just as expected in the animals: "With this system, you can actually control the expression of the [β-galactosidase] transgene," says Hennighausen. "You can keep it silent [by treating the mice with tetracycline] until the time when you want to turn it on." Now that they have shown that the technique works in mice, Furth and Hennighausen plan to use it to study what effects cancer-causing oncogenes have when they are turned on for the first time in the mammary tissue of adult mice.

One drawback of the tetracycline system is that turning the genes on can be a slow and potentially imprecise process. "In this system, we use tetracycline to keep the gene off," says Furth. "To make a gene turn on, you have to wait for the tetracycline to leave the tissue, so you have a biological half-life problem." For the oncogene experiments she plans, that shouldn't be an issue, since precise timing is not crucial. But in some developmental applications, when a researcher wants to time the gene's activation very precisely, the slow draining of tetracycline from the tissue could present difficulties. Bujard says some groups have already gotten around this problem by using tetracycline to control the production of an "antisense" message that then acts to turn off the gene of interest. That way, he says, tetracycline can be used to indirectly turn a gene on by blocking the production of the antisense message. Soon, he says, there may be a more direct solution: His lab is working toward developing a second-generation system in which tetracycline turns the genes directly under its control on instead of off, working in mammals as it does in bacteria.

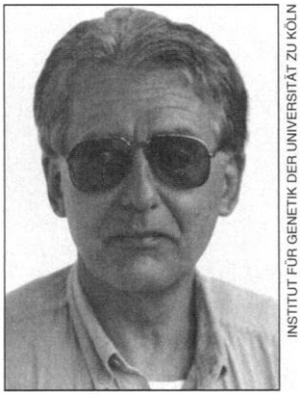
Soon there will be other choices for controlling transgenes in addition to tetracycline. Ronald Evans of the Salk Institute is working on a system to turn genes on in mice with the insect hormone ecdysone. Ecdysone is a steroid hormone that binds to a receptor, which then goes to the nucleus and binds to DNA sequences to turn on its target genes. Evans chose ecdysone for the same reason that Bujard chose tetracycline: Normal mouse genes don't respond to the insect hormone, so there is no risk of unwanted biological effects. But because mice don't normally make the ecdysone receptor either, Evans' group needed to double-engineer mice as Furth and Hennighausen did with the tetracycline system, putting in two genes: one that codes for the gene-activating hormone receptor and another coding for a second foreign gene, under control of an ecdysone-responsive regulatory sequence from fruit flies.

Evans' group has already put the system through its paces in cultured cells and is now waiting for the first transgenic mice to be born. "I have high hopes," for the system, he says. He plans to use it, for example, to study the effects of various steroid hormone receptors in mice. Attempts to do this by knocking out the receptors have failed because the animals die as embryos. But by adding back the knocked-out gene under ecdysone control, the Evans team will be able to turn it on during the critical early stages of development and then turn it off to see how lack of the gene's function affects the animal later in life.

And that is just one of a seemingly infinite range of possibilities, says Evans. These on/off switches can be used to selectively knock out genes by putting enzymes capable of deleting genes (see main text) under tetracycline or ecdysone control. And it's possible to engineer a mouse in which tetracycline or ecdysone only turns its target genes on and off in certain tissues, by setting up the gene-activating protein to be made only in those tissues. With all these possibilities, the field of mouse molecular genetics is likely to get even busier than it has been so far.

–M.B.

National Institute of Child Health and Human Development, independently demonstrated in 1992 that the answer is yes. The Marth group's experiment was similar to those done in cultured cells. They made transgenic mice whose DNA contained both the *cre* gene and, in a separate location, a bacterial gene flanked by *loxP* sequences. They found that the bacterial gene was excised from the genome in cells where the *cre* gene was active. "We showed that Cre is effective in mammals in vivo and could be used at a high efficiency to excise chromosomal DNA," says Marth.



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Makes cell-specific knockout. Cologne's Rajewsky.

Westphal and his colleagues took a different tack. They showed that Cre's DNA-excising capabilities could be used to turn on a foreign gene that they had put into mice. For this experiment, they took the tumor-causing oncogene coding for the large-T (for tumor) antigen from simian virus 40 and hooked it up to DNA control sequences that would turn the gene on in the lens of the eye. But the mice didn't get eye tumors because Westphal's team threw a monkey wrench into the gene: They spliced onto the oncogene a *loxP*-flanked piece of DNA containing stop signals that block production of the large T antigen. Next, they mated these mice with mice carrying the *cre* gene. All the offspring that inherited both genes got eye tumors, because the Cre enzyme excised the *loxP*-flanked stop signals and turned on production of large-T. Westphal's experiment showed that "you can take any gene, [put it into mice], and essentially activate it... whenever you want," says Sauer, now at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), who was a collaborator on the Westphal paper.

Just as Westphal and his colleagues used Cre to remove a piece of DNA and activate a foreign gene, Gu and Rajewsky used the enzyme to *inactivate* a normal mouse gene. Their technique was similar to that for generating normal knockouts, but with a significant change. Instead of simply inactivating the DNA polymerase β gene in ES cells, they replaced part of it with a cloned copy of the corresponding gene segment, flanked by *loxP* sites. Mice that have this engineered gene are perfectly normal, because they are able to make an active DNA polymerase β despite the presence of the *loxP* sequences.

Now, the challenge was to delete the gene, but only in the animals' T cells. To do this, Rajewsky and Gu mated their mice with a transgenic strain produced by British Columbia's Marth, in which the *cre* gene is expressed only in developing T cells. The T cells of the offspring survived, even though a high percentage couldn't make the DNA polymerase, as Cre had excised a chunk of the gene. This result indicates that the polymerase isn't absolutely needed throughout T cell development, says Rajewsky.

A more interesting biological question, which the group plans to address next, is how loss of the polymerase will affect B cell development, where the enzyme is thought to play a key role in generating high-affinity antibodies. Rajewsky says the biological conclusion from their present experiment was less important than the evidence that the technique worked. "The paper was really meant to show that here is a strategy by which this kind of goal can be achieved, namely cell-type specific inactivation."

Already, researchers are using the Cre/*loxP* strategy not only to achieve tissue-specific inactivation of genes, but also to track the fates of cells during development—something that has been impossible to do in such a controlled way in mice. One example comes from Alexandra Joyner's group at Mount Sinai Hospital in Toronto, which is studying the effects of a pair of genes called *engrailed* on mouse brain development. Joyner's group found that knocking out the *engrailed* genes results in mice that are missing parts of their mid- and hindbrains. But they couldn't tell whether the missing cells are the descendants of the cells that expressed *engrailed*, or whether *engrailed* has an effect over a wider range of cells.

To try to resolve this issue, Joyner and her co-workers wanted to indelibly mark the cells that express *engrailed*, as well as all their progeny, and see if those are the same cells missing in *engrailed* knockout mice. To do that, they are using a variation of the Westphal group's method for tissue-specific activation of foreign genes in mice. Joyner's group set up their experiment to express Cre in the cells that make *engrailed*, where it then removes *loxP*-flanked blocking sequences from a gene that makes the bacterial enzyme β-galactosidase. As a result, the enzyme is made in those cells and all their progeny, and it can be detected by a color assay that turns the cells blue. The method seems to have worked in the first group of mice, says Joyner: "We are getting blue in the mid-hindbrain region."

Cre and *loxP* also provide a handy way to make point mutations in specific genes in mice, says Mount Sinai's Rossant. With standard knockout technology, a normal gene is replaced with a copy of itself that contains the bacterial antibiotic resistance gene, *neo*. The *neo* gene not only inactivates the target gene, but also helps the researcher to identify cells that have taken up the engineered gene. Using the Cre/*loxP* method, that approach can be modified, says Rossant, by introducing a point mutation into the replacement gene as well as flanking the *neo* sequence with *loxP* sites. Once the ES cells that contain the replacement gene have been selected, Cre can be used to slice out *neo*. "Now you have gotten rid of the selectable marker, and you just have a point mutation," says Rossant. Rossant and her colleague Andras Nagy have been using this scheme to make point mutations in the *N-myc* proto-oncogene, and she says it "works very well.'

Despite the popularity of the Cre/*loxP* system, it is not the only game in town. Yeasts have an enzyme called FLP, which works similarly to Cre, snipping out pieces of DNA flanked by sequences called "frt," FLP's version of *loxP*. Stephen O'Gorman, then a postdoc with Geoffrey Wahl at the Salk Institute, showed several years ago that FLP works efficiently in cultured animal cells. The enzyme subsequently got a bad reputation when several groups tried to use FLP to make knockout mice, because they had trouble getting it to work well in ES cells.

While others gave up on FLP, O'Gorman, now a staff scientist at Salk, has not. He has shown that the enzyme works nicely in transgenic mice, which can be made without the use of ES cells, and has now returned to ES cells to do a head-to-head test of FLP and Cre. "We know FLP works [in mice]. That is absolutely clear-cut," O'Gorman says. And he believes it can be made to work in ES cells as well. It would be useful to have both the Cre and FLP enzyme systems available, he says, for complicated engineering schemes that require two different DNA excisions.

IRIS HARTMANN

Cre/*loxP* developer. NIDDK's Brian Sauer.

"This is real genetic engineering," says Ronald Evans of the Salk Institute about both the new knockout technologies and methods he is working on to gain greater control over the foreign genes inserted into transgenic mice (see box on p. 27). "As soon as you get to a certain state of technology, you can think of nice tricks and questions you wouldn't normally think about, and that is fun." It seems that with genetically engineered mice, the fun is about to begin.

–Marcia Barinaga