MEETING REVIEW

“Cre”-ating mouse mutants—a meeting review on conditional mouse genetics

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So you want to make a mouse mutation to study the function of your favorite gene? Making a simple knockout is just the first step. The future is in complex genome engineering strategies that will allow you to knockout or misexpress your gene when and where you want, make a series of allelic alterations, and rearrange the chromosomal context in which the gene resides. However, undertaking this kind of analysis in mice is time-consuming and expensive. Many such strategies involve multiple components, each of which has to be tried and tested in a living animal. Everyone wants to know which systems work, but everyone hopes someone else will do the necessary groundwork to test them out. No wonder, then, that there was a large and attentive audience at a recent National Cancer Institute-sponsored workshop at Cold Spring Harbor Laboratory on Conditional Genetic Technologies in the Mouse, [August 31–September 2, 1998], all hoping to learn the latest successes in this area. They were treated to a series of talks by expert practitioners in the field, who not only presented their success stories but also some of the problems and failures that do not make it into the published literature. Most of the talks and their accompanying slides can be accessed online at http://www.leadingstrand.org/.

Although there is enormous potential in this area, the workshop clearly revealed that there is not fully developed, guaranteed successful kit of genetic reagents for creating conditional alterations. One reagent, however, can be considered out of the development phase and into the catalog of standard genetic tools. That reagent is, of course, the site-specific recombinase Cre. The Cre recombinase can excise DNA sequences between two loxP sites, allowing fluorescent-activated cell sorting (FACS) of Cre-transfected cells. Over 80% of fluorescent cells were found to have excised a floxed selectable marker. Several such lines were reported at the meeting (Paul Krimpenfort, The Netherlands Cancer Institute; Sally Camper, University of Michigan Medical School; Andras Nagy and Corinne Lobe, Mount Sinai Hospital, Toronto), none of which alone provides a perfect reporter for all tissues. However, collectively, along with other lines available from other labs, the mouse seems to be covered and investigators should choose their reporter mice depending on their tissue of interest.

The Cre recombinase is also the workhorse of all attempts to remodel the mammalian genome in ES cells. Many kinds of alteration can be introduced into a gene by gene targeting, but a selectable marker must always be cointroduced, which can and often does interfere with gene expression. This effect can be turned to advantage in the generation of hypomorphic alleles (Mark Lewandoski, University of California, San Francisco; A. Nagy, Toronto). However, surrounding the selectable marker with loxP sites allows its removal by Cre and generation of the required mutation, unsullied by exogenous DNA sequences. Brian Sauer (NIH), the ‘father’ of the Cre system, reported that a green fluorescent protein (GFP)–Cre fusion protein could simplify the in vitro excision process, allowing fluorescent-activated cell sorting (FACS) of Cre-transfected cells. Over 80% of fluorescent cells were found to have excised a floxed selectable marker.

Cre can also be used to make chromosomal alterations, such as large deletions and inversions (Binhai Zheng, Baylor College of Medicine), duplications and deletions by transallelic recombination during meiotic recombination (Yann Herault, University of Geneva), and inter- and intrachromosomal rearrangements to alter receptor specificity in lymphocytes (Klaus Rajewski, University of Cologne).

Cre recombinase activity also has the potential to al-
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low insertion of DNA into defined sites in the genome. When Cre catalyzes the recombination between a single loxP site in the genome and another site in an introduced circular plasmid, it promotes insertion of the plasmid into the chromosome. However, this event is inefficient, because the newly inserted sequence now is surrounded by loxP sites and subject to excision again. Three groups [B. Sauer, NIH; E. Bouhasirra, Albert Einstein College of Medicine and S. Fiering, Dartmouth Medical School; T. Imaizumi, Kumamoto University School of Medicine] presented a novel strategy to insert sequences into the genome at defined sites, making use of mutant loxP sites that can only recombine with themselves and not wild-type sites. A vector containing mutant and wild-type loxP sites flanking the sequence to be inserted can recombine efficiently into a host chromosome where the same sites lie, replacing the endogenous sequence between the loxP sites. This insertion event is not subject to further excision and is thus stable. This ‘cassette-mediated exchange’ promises to be very useful in promoting consistent insertion of different exogenous sequences into the same site in the genome, but it still requires some upfront work in establishing the target site in the genome. The most exciting and tantalizing results presented at the meeting were from Minoo Rassoulzadegan (University of Nice), who described two transgenic mouse lines that expressed Cre in the male germ line at different stages of meiosis. In eggs fertilized by male mice carrying one transgene, but not the other, transient Cre activity remaining in the male pronucleus appeared to promote efficiently the stable site-specific integration of plasmids carrying one loxP site into an endogenous loxP site after pronuclear injection. Efficiencies of up to 80% insertion in injected eggs were claimed, sparking long discussion after this talk.

Although Cre has a long head start in mammalian site-specific recombination, there is both room and need for other recombinases with different site-specificity, to broaden the strategies available. The yeast FLP recombinase has been used widely in other systems, most notably in Drosophila, but generally has been reported to be less efficient than Cre in mammalian cells [Ema Anastasiadou, Harvard Medical School; Francis Stewart, EMBL]. FLP-expressing ‘deletor’ mice do exist, however, [Susan Dynecki, Harvard Medical School; M. Lewandoski] and FLP can be a useful second tool in any complex strategy. Some of the limitations of the current FLP mice may lie in the lack of optimization of the FLP protein for function in mammalian cells. A mutant, thermostable form of FLP, FLP-c, promises to greatly enhance the utility of the FLP system [F. Stewart].

Controlled or inducible misexpression of genes is another powerful tool for analyzing gene function and several different strategies at varying stages of technological development were described. Binary systems, in which one mouse line carries the gene to be expressed in a silent form, while the other carries an activator of expression, allow for reproducible analysis of the effects of misexpression of what might otherwise be lethal in normal transgenics. Cre can be used in such strategies, its expression relieving suppression of gene expression by removing an inactivating sequence [M. Lewandoski; A. McMahon, Harvard University], but more standard transactivator strategies can also be used. Successful use of both the VP16 and Gal4/UAS activation systems was reported in transgenic mice, using either the Hoxc8 promoter driving VP16 [Claudia Kappen, Mayo Clinic, Scottsdale] or the Wnt1 promoter driving GAL4 [David Rowitch, Dana Farber Cancer Institute]. Surprisingly, there has been little reported use of either of these systems in mammals, despite the widespread use of the GAL4/UAS system in Drosophila. In the absence of extensive data, it is not yet clear whether these systems will be applicable to all tissues, or whether toxicity of the transactivator will be a major problem in certain cells. Expression of VP16 in preimplantation embryos, for example, seems to be toxic [C. Kappen]. New systems for transactivation or regulated subcellular localization of proteins using rapamycin or coumermycin-induced dimerization were also described [Gerald Crabtree, Stanford University; Michael Farrar, Merck Research Laboratories]. These systems seem to hold considerable promise but have not been tested at all extensively in intact mice.

Even more powerful than these controlled gene expression systems would be systems that induce gene expression in a temporally as well as spatially controlled manner. The best developed system of this sort is the tetracycline-regulated system developed by Hermann Bujard [University of Heidelberg]. In the presence of the drug, doxycycline, the transactivator, TTA, is bound to the tetO promoter, and gene activity is rendered silent until the drug is removed. In the so-called reverse [rTTA] system, transcriptional activation is actually induced by addition of doxycycline. Successful use of both the repressible and activatable systems has been reported in mice, but the success stories were balanced by the stories of failures to get the system to work consistently. Varying problems were reported including mosaic induction, background leakiness [particularly with the rTTA system], or no detectable expression of the transactivator. Many of the issues, as usual, may revolve around the choice of good promoters to drive expression of the tTA and characterization of suitable responder lines. However, some of the difficulties may relate to inefficient processing of the bacterial tetR gene in mammalian cells. Guerard Byrne [Nextran, Inc.] reported improved detection of protein expression and function when a genetic splice acceptor was removed from the tetR gene and the codon usage was improved to generate an optimized syntetR sequence. An additional problem is the lengthy lag phase in the in vivo response of the system following drug administration or removal. It remains to be seen whether modifications under development [H. Bujard] will make the system robustly applicable to manipulating gene expression in all tissues and in embryos in utero.

Even with its limitations, the tetracycline-based system is still the best bet for inducible misexpression. Other systems, like the interferon-inducible system,
only work in certain tissues. Another system, the ecdy-
sone-inducible system shows considerable promise [En-
rique Saez, The Salk Institute], but again has not been
tested fully in the whole animal situation. In this ap-
proach, a VP16-ecdysone receptor–glucocorticoid recep-
tor hybrid binds a synthetic response element as a het-
erodimer with the retinoid X receptor. In the absence of
ligand, the complex may actually repress transcription,
but in the presence of the ecdysteroid ligand, transcrip-
tion is activated. The precise regulation of this system
means that there is very low background expression and
very high induced expression. However, the availability
of suitable cheap synthetic ligands for use in mice is still
unclear. Also, the rapid turnover of the steroid ligands in
vivo will need to be considered when designing experi-
mental strategies that require prolonged induced expres-
sion. On the other hand, rapid ligand turnover may have
distinct advantages when rapid inactivation is desired.

The general concept of utilizing the well-studied li-
gand-binding properties of the steroid hormone receptors
in the design of inducible systems has considerable ap-
peal. Several groups presented data on the activity of
fusion proteins between the ligand binding domain of a
steroid receptor and the Cre recombinase. In cell culture,
such fusion proteins are inactive except in the presence
of the ligand, most likely because the ligand-binding do-
main is complexed with Hsp90 proteins in the cyto-
plasm, preventing the Cre recombinase from reaching its
nuclear targets. On paper, these constructs present a very
attractive means of combining tissue-specific and induc-
cible Cre-mediated excision. The Cre fusion transcript
can be driven by a tissue-specific promoter, but will only
be activated in that tissue when ligand is present. To
work in mice, the ligand-binding domains must bind
synthetic ligands that do not activate the normal steroid
hormone responses. Cre fusions with an estrogen recep-
tor mutant that only binds tamoxifen (ERt) and a proges-
terone receptor that only binds RU486 have been de-
veloped and are being tested in mice.

As pointed out by Francis Stewart, proximity to the
waters of the Rhine seems to be important for pursuit
of this approach. Several groups presented attempts
to achieve inducible excision with Cre–ERt fusions in
adult tissues [F. Stewart, Roberta Pelanda, Max-Planck
Institute for Immunobiology; Ralf Kuehn, Institute for
Genetics]. Only limited excision was reported in B cells
and the hippocampus, using promoters that had been
successful with Cre alone. Better success was reported
by Daniel Metzger (University of Strasbourg) in the
skin epidermis of mice expressing the Cre–ERt fusion.
Farther away on the banks of the Charles River, Andy
McMahon reported successful excision during uterine
development using the Wnt1 promoter to drive expres-
sion of a Cre–ERt. However, high levels of tamoxifen
were needed to activate the Cre fusion, because of low-
binding affinity of the mutated binding domain. The ef-
ective drug dose was close to that associated with tera-
togenesis and embryo loss. Limited data on the use of
the progesterone receptor–Cre fusions in mice were avail-
able [Guenter Schuetz, German Cancer Research Cen-
ter; R. Kuehn]. Clearly the Cre fusion approach appeals
because of its simplicity, but the components of the sys-
tem still need some refining. In vitro selection to en-
hance receptor–ligand interactions may improve these
systems.

As the tool kit for conditional genetic alterations in
mice becomes fully developed, mechanisms to promote
free and easy exchange of validated reagents within the
research community become critical to the rapid dis-
semination and application of the technology. Apart
from the usual need to ensure that improved reagents
like the mutated Flp and the synTetR, and the Cre-
receptor fusions are freely distributed, this area has
particular needs in terms of mechanisms to ensure easy
exchange of mouse strains. For example, once one lab
has developed a mouse strain that expresses Cre effi-
ciently in a given tissue, the same strain can be used
by many different labs to knock out their gene of interest
in that tissue, obviating unnecessary duplication of
effort. Enormous savings in time and money will be
achieved if a ‘library’ of Cre-expressing strains can be
built up and distributed. The same applies to well-
validated TetR or Flp-expressing strains, as well as to
suitable reporter strains. The audience at the meeting
was relieved by the recent agreement between Dupont,
the National Institutes of Health, and the Jackson
Laboratory, which removes constraints on the distribu-
tion of Cre-expressing mice between academic labora-
tories. This agreement, the details of which were pre-
sented to the meeting by Dupont representatives, has
come just in time to prevent a logjam of useful Cre-
related mouse strains. However, the community ex-
pressed its frustration that the previous actions of Du-
port threatened to hold back the rapid progress in the
field.

Everyone was in agreement that centralized reposito-
ries and distribution centers for widely useful strains
of mice are essential. This removes the strain on indi-
vidual investigators and [hopefully] facilitates the distri-
bution of these mice. The Jackson Laboratory in the
U.S. and EMMA, the European Mutant Mouse Archive,
are taking in and distributing Cre-expressing lines and
other useful strains and continued support of these
facilities will be essential. However, the rapid prolifera-
tion of all sorts of genetically altered mice is already
putting strain on such resource centers, a situation
that will get worse as the new technologies described
here really start to be widely used. Currently, not every
strain can be taken in and many strains will only be
maintained as frozen eggs or sperm, unless widely re-
quested. The demand for any given strain will depend
a lot on how well it is validated in terms of its tissue-
specificity, inducibility, etc. The meeting supported the
concept of maintaining a database to include all mice
used in conditional technologies and attempting to im-
pose standard criteria for assessing whether a given
strain is characterized sufficiently to warrant inclusion
in the database and distribution to the community. Cur-
rently there is one private database of Cre-expressing
lines, maintained by Andras Nagy [http://www.mshri.
on.ca/develop/nagy/Cre.html, but this database is dependent on voluntary submissions and is not complete. Expanding this database and integrating it with the activities of the mouse distribution centers should help facilitate international cooperative efforts to bring these emerging technologies to fruition. With all of the tools under development and the power of the existing technologies, there is no question that the mouse genome will be thoroughly explored and exploited in the coming years.
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