Chapter 8

Strategies for Designing Transgenic DNA Constructs

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Abstract

Generation and characterization of transgenic mice are important elements of biomedical research. In recent years, transgenic technology has become more versatile and sophisticated, mainly because of the incorporation of recombinase-mediated conditional expression and targeted insertion, site-specific endonuclease-mediated genome editing, siRNA-mediated gene knockdown, various inducible gene expression systems, and fluorescent protein marking and tracking techniques. Site-specific recombinases (such as PhiC31) and engineered endonucleases (such as ZFN and Talen) have significantly enhanced our ability to target transgenes into specific genomic loci, but currently a great majority of transgenic mouse lines are continuingly being created using the conventional random insertion method. A major challenge for using this conventional method is that the genomic environment at the integration site has a substantial influence on the expression of the transgene. Although our understanding of such chromosomal position effects and our means to combat them are still primitive, adhering to some general guidelines can significantly increase the odds of successful transgene expression. This chapter first discusses the major problems associated with transgene expression, and then describes some of the principles for using plasmid and bacterial artificial chromosomes (BACs) for generating transgenic constructs. Finally, the strategies for conducting each of the major types of transgenic research are discussed, including gene overexpression, promoter characterization, cell-lineage tracing, mutant complementation, expression of double or multiple transgenes, siRNA knockdown, and conditional and inducible systems.

Key words Transgene, DNA construct, Position effects, BAC, Plasmid, Overexpression, Insulator, siRNA, Recombinase, Cre–loxP, FLP-FRT, Conditional, Inducible, Animal model

1 Introduction

Since its development over 30 years ago [1], the pronuclear microinjection method for making transgenic mice has been widely used to address a variety of biological questions, including lipoprotein and atherosclerosis research (see reviews 2–5). Transgenics enables the in vivo modulation of gene activity in a spatial- and temporal-specific manner, greatly enhancing our ability to analyze the functions of genes involved in lipoprotein biology as well as to build animal models for atherosclerosis and other disorders of lipoprotein metabolism. Transgenic models also complement well with

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the increasing number of available knockout/knockin and other mutant mouse lines generated by both ethyl nitrosourea (ENU) and gene-trapping mutagenesis. These loss-of-function and gain-of-function approaches synergistically increase our ability to understand complex physiological processes and mechanisms of disease.

Pronuclear microinjection is an efficient and facile method for delivering foreign genes into the mouse genome, although extensive training is required to become proficient at it. Recent advancements in site-specific recombinase systems, such as Cre-loxP, FLP-FRT and Dre-rox systems [6], and site-specific endonucleases, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (Talens) [7], have begun to launch the era of site-specific or targeted transgenics [8]. However, as of today, the great majority of transgenic projects are still carried out using the conventional random integration method. In spite of three decades of experimentation and optimization, our ability to predict and control the expression of randomly inserted transgenes is still far from satisfactory. Nevertheless, some important knowledge and general guidelines for controlling transgene expression have accumulated over the past three decades. Following these guidelines can significantly improve, although not guarantee, the odds of proper transgene expression. This chapter is mainly aimed at providing beginners with a broad perspective on the applications of the conventional transgenic technology. It is impossible to discuss each topic in much detail, and therefore many important innovations and contributions are not mentioned here.

2 Major Challenges for Controlling Transgene Expression

Microinjected transgenes integrate into the genome at random positions. One important lesson learned from the past three decades is that the genomic environment surrounding the site of integration can exert a profound influence on expression of the transgene. In the cell nucleus, chromatin interacts extensively with the nuclear matrix and other proteins to form looped and topologically constrained domains. Distinct boundary elements or insulators embedded in the genome separate it into regions where gene expression is often facilitated or repressed. Consequently, it is not surprising that the promoter activity of the inserted transgene is subject to the influence of the local genomic environment as well as distant transcriptional enhancers or repressors.

These chromosomal position effects are typically represented in the following forms. First, it is quite common that some of the transgenic lines do not express the transgene at all or express it at a very low level. These silenced transgenic lines are often eliminated during the initial period of transgenic mouse characterization. Second, altered transgene expression refers to transgenic lines with

spatial or temporal expression patterns that do not match those expected based on the promoters used to drive the transgenes. This is often a result of the influence of a strong promoter/enhancer/repressor near the integration site. Third, mosaic transgene expression is another often-encountered problem, i.e., the transgene is expressed only in a portion of cells of the same tissue. Fourth, progressive extinction also happens to some transgenes. In this case, the transgene gives correct and robust expression when the transgenic line is first established, but its expression decreases or even completely stops after multiple generations of breeding. Chromosomal epigenetic modifications such as DNA methylation and histone acetylation probably play important roles in diminishing the expression of these transgenes. Most researchers carefully characterize their transgenic lines when they are first created, but then do not reexamine transgene expression thereafter. Many scientists who receive mice from another laboratory tend to trust the published initial characterization results. Therefore, failure to periodically check transgene expression can potentially lead to erroneous scientific conclusions.

Some of the patterns of uncontrolled transgene expression cannot be explained by chromosomal position effect alone. For example, genes of mammalian origin usually express better than genes of nonmammalian origin [9]. Prokaryote-derived cloning vector sequences often strongly suppress transgene expression if they are not removed from the transgene. Genomic DNA usually expresses better than cDNA [10, 11]. When many copies of transgenes are inserted as tandem repeats at the same location, they are more likely to be silenced [12, 13]. While introns may facilitate the export of mRNA out of the nucleus, which at least partially explains why genomic DNA is better than cDNA for achieving gene expression, the other observed phenomena still remain to be explained, although chromosomal epigenetic modifications are believed to be the main suspect.

The importance of ensuring correct DNA construct design for transgenic projects can be puzzling to beginners, because their experience is often grounded in transfecting and expressing foreign genes in cultured mammalian cells. After all, shouldn't cultured cells and cells in live animals have similar, if not exactly the same, genomic organization and transcriptional machinery? To help clear the confusion, it should be pointed out that the majority of in vitro transfection experiments involve transient transfection. The DNA vectors do not even integrate into the genome, and therefore they are not subjected to complicated genomic environmental influences. In fact, for generation of stable cell lines, the same problems of chromosomal position effects and epigenetic modifications do exist, as reviewed by Kwaks and Otte [14]. However, because hundreds of thousands or millions of cells are normally used in each cell transfection experiment, it is usually not very difficult to find some cells with the

expected gene expression. In the case of transgenic animals, creating and maintaining each transgenic line is labor-intensive and costly. Most scientists do not have the luxury of selecting several perfect lines from many available transgenic lines. Furthermore, each transgenic animal contains hundreds of different cell types, whereas each cell transfection experiment normally uses only one type of cell. Therefore, unexpected patterns of tissue-specific gene expression are far more likely to show up in transgenic mice than in a particular cell line. Lastly, useful transgenic models need to be maintained for a very long time, for multiple generations. Moreover, authentic transgene expression must also be properly maintained through the changes in gene activities and overall genomic modifications associated with the different stages of embryonic and postnatal development. In contrast, the changes in gene expression and epigenetic modification in stably transfected cell lines are rather limited.

3 Commonly Used Transgenic Cloning Vectors

One of the key advantages of the pronuclear microinjection method over the newer lentiviral method is that there is essentially no limit on the size of DNA constructs that can be microinjected, whereas the lentiviral vector can only accept inserts of 10 Kb or smaller [15]. Currently, the most commonly used vectors for cloning transgenes are plasmids and bacterial artificial chromosomes (BACs).

3.1 Plasmid Vectors

Plasmids are convenient and efficient cloning vectors for carrying out a variety of recombinant DNA procedures. Generating a typical transgenic construct involves assembling three basic DNA elements: (1) a promoter and/or enhancer which confers the desired spatial and temporal pattern of transgene expression; (2) the gene to be transcribed, which may or may not encode a protein; and (3) a transcription termination or polyadenylation signal sequence to stop transcription and enable 3′ end processing. An optional fourth DNA element is a genomic boundary element for reducing position effects. The promoter and transcribed region will be discussed in more detail in Subheading 4.1. Commonly used polyadenylation sequences are derived from SV40, the growth hormone (GH) gene, and the β-globin gene, and are quite effective at terminating transgene expression.

One controversial area is the inclusion of genomic boundary elements for combating chromosomal position effects. Plasmid-based transgenes are relatively small, and hence they are prone to influences of the neighboring chromatin environment. Over the years, several genomic DNA elements have been employed to reduce chromosomal position effects. The locus control region (LCR) initially derived from the β-globin gene can improve

transgene expression by positively stimulating transcription, presumably by imposing a chromatin configuration that favors gene expression [16]. Another class of elements is the so-called matrix- or scaffold-attachment regions (MARs/SARs). They appear to mediate their effects by acting as a barrier to both positive and negative signals from the surrounding environment [17]. Enhancer-blocking (EB) insulators and barrier insulators [18] have also been tested for improving transgene expression. Many positive results have been reported with these elements, but negative results are also frequently found (see reviews 19, 20). Therefore, use of boundary elements in transgenic constructs is not yet widespread. Further characterization of these genomic boundary elements should increase their use not only for controlling transgene expression but also in gene therapy.

Prokaryote-derived cloning vector sequences often interfere with adjacent promoter activity when inserted into a mammalian genome, so it is necessary to remove the cloning vector from the transgene before microinjection. Therefore, when designing the DNA construct, it is important to leave suitable restriction enzyme digestion sites at both ends of the transgene so that the transgenic DNA fragment (promoter + transgene + polyadenylation signal) can be separated from the plasmid backbone. At the time of transgene integration, it is possible that a few base pairs at the ends of the DNA fragment may be deleted [21]. This usually will not affect the expression of the transgene. However, if the ends of the released transgenic fragment are critical to the function of the transgene, then restriction enzymes that leave at least several base pairs of polylinker sequence at the ends should be used.

Although plasmid vectors do not have an absolute limit on the size of DNA that can be cloned, both the transformation efficiency and plasmid stability decrease dramatically when the DNA fragments become too big (>25 kb). Cosmids are modified plasmids which contain the cos sequences from bacteriophage lambda [22]. Cosmids can be handled and propagated in *Escherichia coli* similar to plasmid vectors, but they can accept DNA fragments up to 47 kb.

After finishing the transgenic DNA construct, it is recommended that it be tested by transfecting it into appropriate mammalian cell lines. Correct expression in cell lines cannot guarantee the same in transgenic mice, but negative results are usually indicative of problems with the constructs.

3.2 Bacterial Artificial Chromosomes (BACs) and Other Large Pieces of DNA

One of the by-products of the genome sequencing projects was the creation of a series of overlapping BAC clones that essentially covers the entire human and mouse genomes. For the great majority of mouse and human genes, BAC clones are readily available from repositories such as the BACPAC Resource Center at Oakland Children's Hospital (http://bacpac.chori.org/). These fully

sequenced BAC clones are usually 100–300 kb long, and can be used directly for generating transgenic mouse lines. The main advantage of BACs is that they are more likely to contain all the genomic regulatory elements required for mimicking the endogenous gene expression pattern. Also, large pieces of DNA can better shield the transgene from unwanted position effects. When overlapping BAC clones containing the same gene are available, it is best to choose the clone that contains not only the entire transcribed region of the gene but also as much 5′ and 3′ flanking regions as possible. When more than 50 kb of genomic region flanks both the 5′ and 3′ ends of the gene, the probability of faithful and reproducible transgene expression is as high as 85 % [23]. However, one potential problem with using large BACs is that larger clones are more likely to include neighboring genes. Whether these extra full-length or partial genes pose any complications to the study will depend on the purpose of the project.

The recombineering method [24–26] has enabled the precise modification of BAC DNA molecules. Therefore, BAC clones are not only good for overexpressing native genes, but they also can be used to drive reporter gene or modified gene expression. The open reading frame of a reporter gene can be inserted into the 5′ UTR or the coding region to replace the native gene, or be inserted into the 3′ UTR for expression in addition to the native gene. BACs can be used for driving expression of Cre and FLP recombinase genes in desired tissues, but as will be discussed in Subheading 4.6, the commonly used BAC vectors already contain loxP sites, which can be problematic when crossbred to floxed mouse lines.

Unmodified mouse BAC clones are exactly the same as the endogenous genomic DNA except for the linked cloning vector. Some knowledge of vector DNA sequence is needed for designing probes or primers with which to genotype the transgenic mice. The mRNA and protein expressed from the transgene are also indistinguishable from their endogenous counterparts, which makes it difficult to assess the level of transgene expression although quantitative real-time RT-PCR can be used to determine relative expression of a transcript in a transgenic mouse compared to a control, nontransgenic mouse, as described in Chapter 2 of this volume. BAC clones from a different species such as human or rat can be used, which are often different enough to allow differentiation of the transgene from the endogenous gene. Alternatively, molecular tags such as FLAG tag or GFP can be added to the transgene.

Removal of the BAC vector sequence from the transgene is generally not required for microinjection, because (1) the influence of the prokaryote-derived vector sequence is often diminished by the large eukaryotic DNA fragment in the BAC and (2) releasing and separating the large BAC insert from its cloning vector can be technically challenging. It has been reported that linear and circular BAC DNAs were equally efficient in resulting in transgenic mice

[27], but in our hands linear BACs appear to be more efficient for producing positive founders (unpublished observation). Shearing is a potential problem during preparation, storage, and microinjection of BAC as well as other large DNA molecules. BAC DNA should be stored in TE buffer or microinjection buffer supplied with salt and polyamines to make the long DNA strands more compact and hence less prone to shearing. Wide-bore pipette tips should be used for transferring BAC DNA solutions. When microinjecting linearized BACs, slightly larger needle openings and lower injection pressures are preferable for minimizing shearing.

Besides BACs, other large DNA fragments have also been successfully used to generate transgenic mice. For example, the bacteriophage P1 cloning system can accept DNA inserts of 100 kb, and its modified form, PAC (P1 artificial chromosome) vector, can typically carry 100–250 kb of DNA. For a small portion of mammalian genes, even these large vectors are not large enough to contain the entire gene or the flanking regions that are important for the desired expression pattern. Yeast artificial chromosomes (YACs) can accommodate a couple of megabases of DNA [28], and human artificial chromosomes (HACs) can carry more than 10 Mb [29]. Both vector types have been successfully used to generate transgenic mice. Gene-targeting methods are also increasingly being used to ensure that a transgene assumes the expression pattern of the endogenous gene, which is the ultimate method for recapitulating in vivo gene expression.

4 Main Applications of Transgenic Technology

Transgenic methods have been used to address a wide range of biomedical questions. A good transgenic construct must suit the purpose of the study. Below we discuss the strategies for each of the major applications of transgenic technology.

4.1 Gene Overexpression

An obvious application of transgenic methods is to increase the level of gene expression and then observe what effect this has on phenotype. If the goal is to modestly increase the expression of an endogenous protein in its native environment, then BAC DNA is a good choice, particularly for genes whose promoters have not been extensively characterized. This approach is not ideal for producing severe phenotypes because both the level and tissue specificity of gene expression are not dramatically changed. Nevertheless, it is very useful for studying subtle physiological differences, and for building animal models for disorders resulting from overexpression of a gene, such as Down syndrome.

Thus far, most overexpression constructs are assembled in plasmid vectors using heterologous promoters. Both genomic DNA and cDNA (minigenes) can be used, but genomic DNA is

preferable because introns contained in the genomic DNA can facilitate export of the mRNA from the nucleus to the cytoplasm [10]. Unfortunately, the genomic DNAs of many mammalian genes are too large to be conveniently manipulated in plasmid vectors. Consequently, full-length cDNAs are often used to make transgenic constructs, and many of them are expressed well. However, many experienced transgenic researchers prefer to incorporate a heterologous intron, such as introns from β-globin, SV40, or adenovirus, into their constructs to increase the odds of successful expression. For instance, the pCI and pSI vectors marketed by Promega contain a chimeric intron (β-globin donor site and immunoglobulin acceptor site) at the 5′ end of the cloned gene. Alternatively, a chimeric gene can be created by creating an in-frame fusion between the cDNA and genomic DNA of the same gene [30].

Besides full-length cDNA, mouse and human full-length ORFs (open reading frames) are also commercially available. These clones contain the entire coding region, but not the 5′ and 3′ untranslated regions. It is not recommended to use ORFs to directly make transgenic animals, because it has been well documented that the 5′ and 3′ UTRs can play important roles in regulating mRNA stability, intracellular localization, and efficiency of protein translation. However, it is perfectly acceptable to insert these ORFs into other genes, such as the UTRs of BACs, for expression as transgenes.

When choosing a promoter, it is recommended to consider promoters that have been shown to be able to drive heterologous gene expression. A promoter that can drive its native gene expression may not necessarily correctly drive heterologous gene expression, because some regulatory genomic elements may lie downstream of the transcription initiation site. It is noteworthy that some of the most commonly used promoters for transfecting cultured cells are not the best choice for directing ubiquitous gene expression in transgenic animals. Such promoters are small in size and very convenient, but they are subject to heavy position effects. For example, CMV is considered a ubiquitous promoter for transfecting a wide variety of cultured mammalian cells. However, in transgenic mice its effects are not as ubiquitous [31]. For ubiquitous transgene expression, good results have been achieved using the ROSA26 and the CAGGS (chicken β-actin promoter with CMV early enhancer) promoters. For tissue-specific expression, proven promoters for most major tissues or cell types can be found in the literature. For instance, the Tie2 promoter has been shown to be able to direct transgene expression specifically in the vascular endothelium [32]. Tissue-specific promoters for liver, intestine, and macrophage, which are of particular interest to lipoprotein researchers, are discussed in Chapter 12 of this volume. If an untested promoter needs to be used, it is advisable to use as big a piece of genomic DNA as practically possible, such as an entire BAC clone.

Mutated genes can also be expressed in transgenic animals, which may result in knockdown or dominant negative phenotypes, depending on the dominance or recessiveness of the mutation.

An extreme form of gene overexpression is to use transgenic animals as bioreactors for producing recombinant proteins (see reviews 33–35). Although the main purpose of this approach is to use large farm animals to produce biopharmaceutical products, transgenic mice have also been used to test DNA constructs and to conduct biomedical research. For example, the mouse ZP3 protein has been successfully produced in mouse milk by expressing it under the control of the goat β-casein promoter [36].

4.2 Promoter Characterization and Cell Linage Markers

Transgenic animals are often generated for characterizing the temporal and spatial patterns of gene expression governed by promoter elements. Knowing the exact cell types and developmental stages in which a particular gene is normally expressed can shed light on its physiological functions. Generating and characterizing transgenic mice is the most efficient method for examining in vivo gene expression patterns. A good starting point is to place several kilobases of promoter region in front of a suitable reporter gene. Depending on whether the reporter gene expression is able to match the pattern of endogenous gene expression, the length of the promoter can be subsequently increased or decreased to identify important genomic regulatory elements. For most genes a few kilobases or even several hundred base pairs of DNA upstream of the transcription initiation sites are enough to confer tissue-specific gene expression. However, for some other genes, dozens of kilobases of promoter region or even entire BACs are not sufficient for recapitulating the in vivo expression pattern of the gene. In the latter cases, targeting a reporter gene into the native genomic locus has increasingly been used for recapitulating the expression pattern of the endogenous gene.

Various reporter genes have been successfully used for characterizing promoter activity [37]. The firefly luciferase and chloramphenicol O-acetyltransferase (CAT) reporters are excellent for quantitative measurement of gene activity in various organs at various time points of development, while beta-galactosidase, alkaline phosphatase, or fluorescent proteins can provide a direct visualization of promoter activity at the tissue and cell levels. Fluorescent proteins are particularly good for providing a live and dynamic view of gene activity, because no tissue fixation and disruption are required. Concerns over GFP toxicity have been raised [38, 39], but a large number of healthy GFP-expressing transgenic lines have been successfully generated.

It should be pointed out that the presence or absence of reporter proteins may not precisely represent the true state of the native protein, because mRNA and protein stabilities as well as the regulation of mRNA translation may be different between the native and reporter genes.

The promoter–reporter systems described above are not only useful for analyzing promoter activity but also very useful as cell-lineage markers. For example, there are many types of neurons in the brain which synthesize different neuropeptides or neurotransmitters. It is very difficult to distinguish them morphologically. The GENSAT (Gene Expression Nervous System Atlas) project is aimed at labeling various neurons with fluorescent protein using neuron-specific promoters [40]. Besides marking specific cell types, GFP can also be fused to various subcellular localization signals as well as to individual proteins to track individual cellular organelles or proteins in animal tissues. It is recommended to verify cellular localization of endogenous proteins using antibody-based methods, if possible.

4.3 Gene Knockdown by RNA Interference

RNA interference (RNAi) technology has been widely used in cultured cells to knock down gene expression, but its use in transgenic animals is still not widespread. This is partly because gene knockout technology has been firmly adopted as the gold standard for developing loss-of-function animal models. Another reason is that the uncontrolled transgene expression described in Subheading 2 seems also to affect RNA polymerase III promoters, causing large variations in shRNA expression among different tissues [41]. Nevertheless, it has been shown that the shRNA method can work well in transgenic animals [42, 43]. Both the H1 and U6 promoters can direct RNA polymerase III to transcribe enough shRNA to knock down endogenous gene expression by as much as 80–90 %. RNA polymerase II-dependent promoters can also be used to drive the expression of double-strand RNA, which opened the door for utilizing many well-characterized RNA polymerase II promoters for directing tissue-specific siRNA knockdown [44–46].

Much more work is needed before siRNA transgenesis can challenge the knockout method. However, it should be noted that the siRNA method is a much quicker (one generation vs. three generations of mouse breeding) and easier (small transgenic construct vs. large multifragmented gene-targeting construct plus tedious ES cell work) than the gene knockout approach. One of the main problems of the siRNA approach is that it can only reduce, but never completely abolish, endogenous gene expression. However, in the cases when complete knockout results in lethality, the knockdown approach can actually be advantageous. As will be discussed in Subheadings 4.6 and 4.7, the conditional and inducible systems developed for RNA polymerase II promoters also appear to work for the U6 and H1 promoters, which significantly enhance the flexibility and usefulness of the siRNA knockdown approach [47].

4.4 Complementation and Mutation Mapping

For spontaneously occurring or chemically (such as ENU) or physically (such as irradiation) induced mutant mouse lines, extensive genetic mapping is required to identify the mutated gene(s).

Transgenic animals can be used to confirm or nullify the mapping results. Large pieces of DNA, such as BAC or YAC clones, from the suspected genomic regions can be used to create transgenic mice for rescuing the mutant phenotype if the phenotype is recessive. If the mutant phenotype is dominant, the mutated form of the gene can be introduced into wild-type mice to recreate the mutant phenotype. Some users of transgenic core facilities request that their transgenic lines be made directly on the mutant background, but it is usually easier and faster to create the transgenic lines in standard wild-type mice and then cross them into the mutant background. Even if transgenic mice are created using the mutant mice as egg donors, it is likely that the mutated gene and the newly inserted transgene will segregate in subsequent generations. The BAC and YAC clones used in the complementation studies may contain multiple genes. When interpreting experimental results, the possibility that these extra genes may be contributing to the phenotype needs to be considered.

Transgenic mice can also be used to rescue knockout mouse lines, and more interestingly tissue-specific transgenic lines can be used to restore gene expression to only some of the knockout tissues. This strategy is particularly useful for studies of gene function in a specific tissue, when floxed conditional knockout mouse lines are not yet available.

4.5 Double or Multiple Transgenes

Sometimes it is necessary to co-express two or more transgenes in the same mouse line. Of course, this can be achieved by crossbreeding two or multiple transgenic lines together. The disadvantage is that extensive breeding and genotyping are required; furthermore, the obtained genotypes are not stable and hence the transgenes may segregate in subsequent generations. Several alternative methods exist for incorporating multiple transgenes into the same transgenic line. First, two or more separate transgenes can be mixed and co-microinjected. Because multiple copies of transgenes are often cointegrated at the same genomic location, there is a good chance that both transgenes are integrated in the same locus in the same transgenic line. Such transgenic lines can be stably maintained without tedious crossbreeding and genotyping. When mixing DNA constructs for microinjection, the DNA concentration for each construct should be proportionally reduced so that the total DNA concentration remains in the normal 1–2 μg/ml range. Otherwise the elevated DNA concentration may reduce the survivability of the injected embryos. Second, two transgenes, each containing its own promoter and polyadenylation sequence, can be cloned into the same transgenic vector. Third, an internal ribosomal entry site (IRES) can be used to create bi- or polycistronic mRNA, which enables the production of two or more proteins from the same mRNA. However, the efficiency of the polycistronic system still varies greatly, depending on the sources of the IRESs, the genes to be

expressed, and the distance between the two genes relative to the IRES sequence, as if the cistrons interfere with each other. The rules for governing IRES efficiency are not clear, but it is generally recommended to place the more important gene in front of, and the less important gene (such as the reporter or marker gene) after, the IRES. Fourth, 2A peptides have been increasingly used as alternatives to the IRES in expressing multiple proteins from one DNA construct. The 2A sequences were first identified in picornaviruses, which encode relative short peptides (~20 amino acids) that can cause ribosomal skipping and consequently result in multiple polypetides being made from the same mRNA. They function efficiently in a wide variety of eucaryotes, ranging from yeast to human cells, including in transgenic mice [48].

4.6 Site-Specific Recombinase and Conditional Transgenic Lines

Cre and FLP transgenic mouse lines are extremely useful tools for carrying out conditional knockout and knockin studies. Cre [49] and FLP [50] are site-specific recombinases that can specifically recognize the loxP and FRT DNA sequences, respectively. FLPe is the most used variant of FLP recombinases thus far, but the codon-optimized FLPo has been shown to be more efficient at mediating homologous recombination between FRT sites [51]. When two copies of loxP or FRT sites exist in the same mouse line, the homologous recombination events mediated by these recombinases can result in deletion, inversion, and translocation, depending on the orientation and location of the two sites [52].

Cre or FLP transgenic lines can be generated following standard transgenic methods using a wide variety of ubiquitous or tissue-specific promoters. Because BAC clones are excellent for blunting chromosomal position effects and maintaining authentic gene expression patterns, they are candidates for driving recombinase gene expression. This can be done by inserting Cre into the 5′ UTR of the BAC clones. However, it must be pointed out that the commonly used BAC vectors (RPCI-23 library from the C57BL/6 J mouse strain and RPCI-22 from the 129/SvEv mouse strain) already possess loxP sites. These sites can potentially cause serious troubles when crossed into the floxed conditional knockout mouse lines. Therefore, it is strongly recommended to eliminate the loxP sites embedded in the BAC vector before using it to drive Cre gene expression.

The expression pattern of Cre and FLP transgenic lines must be systematically characterized by crossing them to commonly used recombinase reporter mouse lines, including R26R [53], Z/AP [54], and Z/EG [55] for Cre and R26:FRAP for FLPe [56]. Generally, these reporter lines contain a floxed (for Cre) or flirted (for FLP) translation STOP cassette between a ubiquitous promoter and a reporter gene. In the absence of the recombinase, the reporter protein is not expressed. However, in tissues where recombinase is expressed, the reporter gene product is produced because the STOP cassette has been deleted by the recombinase.

It is important to note that the pattern of tissue-specific promoter activity revealed by ordinary reporter gene assay, as discussed in Subheading 4.2, can only report the status of promoter activity at the moment when the tissues were harvested. However, the patterns revealed by the recombinase reporter mice are more like fate mapping. Recombinase-mediated DNA excision is irreversible, and therefore even brief expression of the recombinase can permanently delete the DNA flanked by the loxP or FRT sites not only in that particular cell but also in all of its progeny. This characteristic makes it more difficult to conduct tissue-specific knockout experiments in certain cell types, because many seemingly tissue-specific promoters in adult mice also transiently express in some other cell types during embryonic development. That is why inducible recombinase transgenic lines are often needed to achieve tissue-specific knockout (*see* Subheading 4.7).

All the adverse consequences of chromosomal position effects and epigenetic modification discussed in Subheading 2 also apply to Cre and FLP transgenic lines. Because the recombinase lines usually need to be crossbred with other conditional knockout mouse lines to conduct complicated studies, the importance of thoroughly and rigorously examining the pattern of Cre or FLP expression cannot be overemphasized [57]. Sometimes the Cre protein can be transported from one cell to another, or even from the uterine lining of the mother to an early embryo. Sometimes, males and females from the same transgenic line behave differently in terms of Cre expression [58]. The genetic background of the mouse can also apparently influence Cre expression [59]. To avoid potential problems, some researchers even characterize each individual mouse that will be used for certain conditional mutagenesis experiments [60].

Of note, germline-specific Cre transgenic lines, ZP3-Cre for oocytes and prm-Cre for spermatids, can be used to specifically knock out genes in reproductive cells. This feature can be used to bypass the sterility or lethality of heterozygous mice to generate homozygous knockout embryos [61].

Besides being used as a tool for achieving conditional gene knockouts, the Cre and loxP-STOP cassette can also be used to generate conditional transgenic mice. The loxP-STOP cassette can be inserted between the gene of interest and its promoter. The transgene can be turned on in tissues where Cre recombinase is expressed. This approach is more complicated than making conventional tissue-specific transgenic lines, but it is a useful strategy when constitutive expression of your gene causes lethality or sterility, which makes it impossible to maintain the transgenic lines. Furthermore, the same STOP cassette-containing transgenic line can be crossed to various Cre lines for achieving different expression patterns.

A similar Cre–loxP strategy has also been shown to function in RNA polymerase III-transcribed transgenes [62, 63]. LoxP-flanked stuffer DNA sequences (similar to the STOP cassette) can be inserted between the Pol III promoter and the shRNA sequence to block gene expression. Upon excision of the stuffer sequence by Cre recombinase in specific tissues, the shRNA will be activated to exert its knockdown effects only in those tissues where Cre is expressed. Alternatively, the entire shRNA transgene can also be flanked by two loxP sites. In this case, the shRNA knockdown is absent in the tissues where Cre is expressed, and is present in other tissues. These conditional systems allow tissue-specific gene knockdown using the ubiquitous H1 and U6 promoters.

All of above strategies rely on the recombinases' capability to delete DNA sequences. As in all reversible chemical reactions, the catalysts/enzymes should also be able to facilitate the reverse reaction to an equal degree, i.e., to insert DNA sequence into a specific site. However, Cre and FLP are not efficient for inserting DNA, mainly because the inserted DNA is quickly deleted by the same enzyme due to the reversible nature of the reaction. For achieving site-specific insertion, another site-specific recombinase, ϕC31, is utilized. It catalyzes homologous recombination between two nonidentical sites, attB and attP [64]. When one DNA molecule containing one site is inserted into another molecule containing the other site, recombination results in two chimeric sites which cannot be recognized by the ϕC31 recombinase, and therefore are not subsequently deleted. This feature has made it possible for targeted insertion of transgenic constructs with the attB site into specific genomic loci, where the attP docking site had been previously inserted through gene-targeting process [8].

It should be noted that new site-specific recombinase-mediated conditional systems [65, 66] as well as new variants of existing recombinases [67] or recognitions sites are continually being developed. These variations in recombinases and recognition sites enable the insertion of nonidentical targeting sites into the genome, which can dramatically facilitate the insertion of transgenes in a site-specific and direction-specific manner, through recombination-mediated cassette exchange (RMCE). In this respect, it should also be mentioned that the ZFN, Talen, as well as the recently emerged RNA-guided CRISPR genome-editing methods [68] have substantially increased our ability to insert transgenes into specific genomic loci without using any site-specific recombinases and embryonic stem (ES) cells. These latest technological developments are beginning to launch the era of site-specific transgenics, but they are clearly out of the scope of this chapter.

Site-specific recombinases can also result in DNA inversion when the two recognition sites are in reverse orientation. Livet et al. [69] took advantage of this feature and generated transgenic lines that can stochastically recombine the expression of several

fluorescent proteins to label brain neurons with many different colors, which greatly facilitated the identification of individual neurons in a complicated neural network.

4.7 Inducible Transgene Expression

Over the years, various inducible gene expression systems have been developed and applied in transgenic research, including metallothionein- [70], interferon- [71], ecdysone- [72], and cytochrome P-450-inducible [73] systems, but the most advanced systems are the tetracycline-inducible systems (see review 74). These systems require the interbreeding of two transgenic mouse lines, one carrying the gene of interest under the control of a tetracycline-inducible promoter (fusion of CMV minimal promoter and seven copies of the TetO sequence termed the tetracycline response element or TRE), and the other carrying the transcriptional activator (tTA for the Tet-off system or rtTA for the Tet-on system). In the absence of tTA or rtTA, the CMV minimal promoter is not enough to drive expression of the transgene. However, when tTA or rtTA binds to the TetO sequences of the tetracycline-inducible promoter, RNA polymerase can be recruited to the CMV minimum promoter to begin transcription. Both tTA and rtTA are created by fusion of a TetR protein and the herpes simplex virus VP16 protein, but they behave oppositely in terms of their requirements for tetracycline to activate the tetracycline-inducible promoter. tTA requires the absence of tetracycline for binding to TRE, whereas rtTA requires the presence of tetracycline to be able to bind to the promoter. Therefore, when a tetracycline analog such as doxycycline is administered through food, water, or IP injection, rtTA can turn on the transgene whereas tTA can turn it off.

Both the Tet-on and Tet-off systems have been used to obtain inducible transgene expression. There are dozens of ubiquitous or tissue-specific tTA and rtTA transgenic lines available at The Jackson Laboratory. One advantage of the rtTA over the tTA system is that induction of gene expression can be achieved quickly. This is because the added doxycycline can enter the tissues in a matter of minutes or hours, whereas clearing it out of the system usually takes days. It is worth mentioning that for brain tissues, it takes a longer period of time for the added doxycycline to take effect because of the blood–brain barrier. However, the disadvantage of the rtTA system is that basal level (leaky) expression appears to be higher [75, 76].

After generating the inducible transgenic lines, it is essential to carefully characterize them before conducting any experiments. A convenient approach for examining expression pattern and inducibility is to include a reporter gene in the same transgenic construct. This can be done by using a bidirectional inducible promoter, which contains two oppositely oriented CMV minimum promoters adjacent to the shared TRE sequences [77].

The conditional system described in the previous section and the inducible systems described here can be combined to create inducible conditional systems. Schonig et al. [78] reported that a tetracycline-inducible Cre transgene line can be successfully generated, but some other studies [79] have had trouble generating efficient tetracycline-inducible Cre lines. Because the tetracycline-inducible system itself requires two mouse lines, when they are further crossed to the conditional knockout/knockin mouse line, breeding and genotyping become very complicated. Therefore, the tamoxifen-inducible system is often adopted for achieving inducible recombinase transgene expression. In this case, the Cre protein is fused to a modified estrogen receptor (ER) protein. Depending on the presence or absence of estrogen analogs, particularly tamoxifen or 4-hydroxy (OH) tamoxifen, ER can drag the recombinase in and out of cell nuclei [80]. In the absence of tamoxifen, the Cre–ER fusion proteins stay in the cytoplasm. Upon tamoxifen administration, tamoxifen binds to ER to cause a conformational change in the receptor and result in transport of the Cre–ER fusion protein into the nucleus. Because Cre needs to be located in the cell nucleus to perform its function of mediating homologous recombination between two loxP sites, its activity can be effectively turned on and off by tamoxifen. Several versions of Cre–ER fusion proteins are currently available, and CreERT2 appears to be the most sensitive variant to date. Hunter et al. [81] created the FLPeERT2 fusion gene, which can be used for inducibly removing sequences flanked by FRTs. Besides ER, the progesterone receptor (PR) can also be fused to Cre recombinase to create a Cre–PR fusion protein, which can be induced by the synthetic steroid RU486 [82].

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