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Gene Targeting Protocols

Edited by

Eric B. Kmiec

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METHODS IN MOLECULAR BIOLOGY™

Gene Targeting Protocols

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Dedication

For my wife, Jennifer

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Cover illustration: The cover depicts the presence of active Green Fluorescent Protein being expressed in Chinese Hamster Ovary (CHO) cells. The phenotypic change is a result of the activity of the chimeric oligonucleotide on CHO cells that have integrated copies of mutated (inactive) GFP genes. Cells that have not been targeted or corrected to wild-type appear in the background. The technique known as gene repair or chimeraplasty was pioneered by the editor who supplied this picture.
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Preface

The potential now exists in many experimental systems to transfer a cloned, modified gene back into the genome of the host organism. In the ideal situation, the cloned gene is returned to its homologous location in the genome and becomes inserted at the target locus. This process is a controlled means for the repair of DNA damage and ensures accurate chromosome disjunction during meiosis. The paradigm for thinking about the mechanism of this process has emerged primarily from two sources: (1) The principles of reaction mechanics have come from detailed biochemical analyses of the RecA protein purified from *Escherichia coli*; and (2) the principles of information transfer have been derived from genetic studies carried out in bacteriophage and fungi. A compelling picture of the process of homologous pairing and DNA strand exchange has been influential in directing investigators interested in gene targeting experiments.

The ability to find and pair homologous DNA molecules enables accurate gene targeting and is the central phenomenon underlying genetic recombination. Biochemically, the overall process can be thought of as a series of steps in a reaction pathway whereby DNA molecules are brought into homologous register, the four-stranded Holliday structure intermediate is formed, heteroduplex DNA is extended, and DNA strands are exchanged. Not much is known about the biochemical pathway leading to homologous recombination in eukaryotes. Nevertheless, in *Saccharomyces cerevisiae*, a great deal of information has accumulated about the genetic control of recombination and the molecular events leading to integration of plasmid DNA into homologous sequences within the genome during transformation. Substantial insight into the mechanism of recombination between plasmid DNA and the genome has come from studies using nonreplicating plasmids containing a cloned gene homologous to an endogenous genomic sequence. Transformation of *S. cerevisiae* at high frequency takes place when the plasmid DNA is cut within the cloned DNA sequence. Almost invariably, transformants contain plasmid DNA integrated into the yeast genome at the homologous site. Autonomously replicating plasmids containing gaps of several hundred nucleotide residues within the cloned gene also transform at high efficiency and are repaired by recombination using chromosomal information as a template.

What has emerged from these studies on transformation of *S. cerevisiae* is a body of observations that has helped shape strategies for gene targeting in higher organisms. Unfortunately, the limited biochemical data available from yeast, and the often confusing and sometimes contradictory results from the genetic studies, have not provided a thorough mechanistic foundation for experimentation. It is not completely clear from the transformation studies carried out that information on the genetic control of plasmid integration will be generally applicable to high eukaryotic systems under study by investigators interested in gene targeting. The significance of the functionally independent, yet structurally redundant, RecA-like *Rad51*, *Rad55*, *Rad57*, and *Dmc1* genes in *S. cerevisiae* is not clear. The virtual absence of the illegitimate integration events during plasmid transformation commonly observed in many other eukaryotic systems raises certain caveats about the generality of the recombination system in yeast. Nevertheless, structural homologs of *rad51* and/or *rad52* have been identified in several higher eukaryotes, providing some indication that fundamentally similar biological principles underlie the mechanism of homologous recombination from bacteria to higher animals and plants, as well as that rules of gene targeting learned from transformation analysis of lower eukaryotes will be widely applicable.

With respect to gene targeting in higher eukaryotes, the tantalizing carrot of gene replacement as gene therapy remains dangling. Though noble approaches are underway to incorporate this methodology in molecular medicine venues, it is unlikely that gene therapies will become elements of common practice in the near term. Hence, what we are left with is a powerful process and extension technique in which gene targeting protocols can be used to achieve equally important goals. That is what *Gene Targeting Protocols* is about—the use of gene targeting techniques to create experimental systems that help us understand biological processes at a genetic level.

We have requested chapter contributions from members of the scientific community who are at the forefront of those dealing with and/or overcoming many of the barriers caused by the low frequency of homologous recombination. Clearly, more techniques are under study than those represented here, but we have striven to present a wide range of approaches that may be intriguing and, we hope, useful to the reader.

One of the most important features of gene targeting is the delivery of the construct into the nucleus of the cell. Whereas viral vectors are naturally occurring delivery vehicles, naked DNA is taken up quite poorly by mammalian cells. To overcome this problem, a number of strategies have been employed, one of which is the use of cationic lipids. The field of liposome

delivery is rapidly expanding and the literature is often misleading and confusing. In addition, the choice of a particular liposome transfer vehicle for delivery into a particular cell type is viewed as crucial. In the chapter by Natasha Caplen, the variety of liposomes available to investigators and the criteria for choosing one to fit the experimental purpose is discussed in detail. Caplen surveys commercially available liposomes and outlines the advantages and disadvantages of each.

Along the same lines, Barbara Demeneix and colleagues discuss the use of polyethylenimine (PEI) as a gene transfer vehicle. One of the most appealing aspects of PEI is its nontoxicity in vivo. Details regarding the importance of determining the optimal ratio of PEI to DNA are outlined and a specific case study using brain cells is provided. In contrast, Xi Zhao discusses a relatively new approach to gene delivery using electronic pulse delivery (EPD). The EPD system differs from traditional electroporation in the use of selected pulse waves and the ultralow current. This technique provides a transfer efficiency of over 80% with a viability index of EPD-treated cells approaching 90%. It may be the most efficient physical delivery protocol currently in use. In the chapter by Greg May and colleagues, electroporation conditions for transfer of oligonucleotides into plant cells are outlined. Though the focus of many gene transfer protocols is mammalian cells, the capacity to alter plant genomes is of utmost importance.

Since many of the protocols outlined above discuss the virtues and drawbacks of the transfer vehicle, it is also imperative to understand the cell itself. Clearly, the state of the cell in terms of metabolism and cell cycle position upon becoming manipulated affects the efficiency of transfer. Nancy Smyth Templeton discusses the various parameters that affect vector uptake. In addition, she discusses the design of the DNA vector itself in a protocol aimed at gene targeting in mammalian cells. What has become apparent is that the amount of vector introduced relative to the cell culture conditions is critical in improving gene targeting frequency. The method of transfer for this protocol is electroporation, which complements the May chapter on plant cell electroporation. The chapter contributed by David Strayer outlines an important use of the viral vector SV40. Strayer's group has developed an efficient delivery system to assess cellular uptake and extended expression of marker genes after integration. The use of this vector is novel and will likely overcome significant delivery problems.

The next group of chapters outlines a series of protocols commonly used for gene targeting. Among the most successful is Cre-lox, developed by Brian Sauer and colleagues. In his chapter, he outlines the strategy for creat-

ing cell lines that express specific transgenes using the Cre recombinase. This system has been widely used because of its remarkable versatility. Perhaps the most important aspect of Cre-lox is its reversibility. A transgene can be inserted, expressed, studied for cellular effects, and then removed. Kaarin Goncz and Dieter Gruenert outline a similar approach in which small fragments of DNA are used to alter the genome by site-directed insertion. Such a technique enables gene replacement strategies that can lead to molecular therapy or improve knockout events. The simplicity of the vector itself is a key feature in using this approach to disrupt mammalian cells.

Several viral-based systems are also described, including the use of a modified adenoviral vector by Ichizo Kobayashi and colleagues to create a cell line that is amenable to high levels of homologous recombination events. This work pulls together several aspects of this volume including cell culture manipulation and vector design. The adenoviral vectors can allow for nearly 100% of the cells to acquire the transfer without influencing viability. In the same vein, Jude Samulski and colleagues provide a protocol for using adeno-associated virus (AAV) to introduce transgenes at a specific site in chromosome 19. The objective of this strategy is somewhat different from the others reported herein, since the site of integration is determined by the viral vector, not by the transgene. AAV has a predilection for integrating at a specific site on chromosome 19, and if one wishes to introduce a transgene permanently into the chromosome for inheritable expression, this technique is optimal. Richard Bartlett and Jesica McCue provide an excellent background on AAV biology, detailing targeted integration and studies of rAAV-based gene therapy vectors. They also provide an introduction to the studies using an AAV-based plasmid vector to express human insulin in skeletal muscle of diabetic animals.

Two chapters in *Gene Targeting Protocols* take a fundamentally different approach to gene targeting. The first by Sun Song and Wayne Marasco utilizes a fusion protein, attached to a plasmid, to deliver the vector to a target, in this case a virus. Although many protocols are aimed at targeting host chromosomal genes, the field of gene targeting also includes virus targeting. These authors outline a protocol that can deliver a therapeutic gene to a specific cell in animals. The target cell may be one that has been infected with a virus and the expression of the gene once transferred into the correct cell may have a therapeutic effect. The chapter by Jovan Mirkovitch and colleagues provides an interesting system for overcoming a serious barrier to therapeutic gene targeting. This problem is centered on the regulation of transgene expression. In many cases, the chromatin structure covering the transgene heavily influences its expression and may subvert even heroic efforts used to introduce the gene

into the chromosome. To avert this problem, these authors have designed an episomal-based Epstein-Barr viral vector that can modulate the chromatin assembly process. This contribution impacts the choices of integrative vectors and enables evaluation of gene therapy expression cassettes prior to introduction into mammalian cells.

The final section of *Gene Targeting Protocols* centers on the use of oligonucleotides in gene targeting. These types of molecules have been used in the antisense field for many years to block gene expression at the mRNA level. In most cases, the mechanism of inhibition involves the hybridization of the oligo with the complementary mRNA sequence and subsequent destruction of the hybrid by cellular enzymes. Clinical applications have received mixed reviews, but no one disputes the controlled environment in which synthetic molecules can be produced. The authors of these chapters are developing new strategies for the use of oligonucleotides in gene targeting. In all cases, the objective is to alter or manipulate the gene at the genomic level, in other words, within the coding region. Karen Vasquez and John Wilson employ specialized oligonucleotides capable of forming a third strand of the helix to block gene expression, while Howard Gamper and colleagues use modified single-stranded oligos to introduce an adduct at a specific site. Peter Kipp and colleagues use a novel chimeric RNA/DNA oligonucleotide to introduce a specific base mutation in the tobacco genome to render the target cells resistant to herbicide. Ryszard Kole and colleagues have developed an interesting strategy for altering the splice sites in pre-mRNA. Such changes are then translated into mRNA molecules that code for different proteins. The field of targeted gene manipulation by oligonucleotides is quite new and among all the areas of scientific endeavor, is likely to be one that revolutionizes the entire field. Even with such a promising future, the current targets are single bases only and larger conversions are likely to require futuristic designs.

In the past ten years, a number of genetic protocols aimed at improving the frequency of gene targeting have been developed. Some of them have been significantly limited in their applications, whereas others are still being evaluated. The scientific community is necessarily skeptical at the advent of new techniques until their validity can be irrevocably established. Clearly, politics often plays a role in the acceptance of new techniques, but even such opinions are ultimately rewritten by the accumulation of careful and rigorous scientific experimentation.

The authors who contributed to this volume, in our opinion, comprise a group of the most innovative and dedicated workers in the field. The majority of techniques presented here are described by the lab from which they origi-

nated. It is likely that many, if not all, of these protocols will become commonplace in future molecular genetics research. I wish to thank all of the authors for their contributions and their patience. I am indebted to Paul Dolgert from Humana Press and John Walker from the University of Hertfordshire for their continued support throughout this endeavor. Finally, I wish to thank my administrative assistant, Tony Rice, for his efforts on this project. He played a crucial role in organizing this volume and without his dedication it is unlikely this book would have been completed.

Eric B. Kmiec

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1

Nucleic Acid Transfer Using Cationic Lipids

Natasha J. Caplen

1. Introduction

The use of cationic lipids or cationic polymers to mediate the transfer of nucleic acids into mammalian cells has become a widely applied technology in recent years. The principal reasons for this have been the ease with which the methodology can be applied to a wide range of cell types; the relatively low cytotoxicity compared to other techniques; the high efficiency of nucleic acid transfer in comparison with methods such as calcium phosphate or diethyl-aminoethyl-dextran-mediated transfection; and the potential application of these systems to human gene therapy. The use of positively charged lipid-based macromolecules to deliver nucleic acids makes use of the fact that DNA, RNA, and oligonucleotides carry a negative charge caused by the phosphate groups that form the backbone of these molecules. The electrostatic interaction between the negatively charged nucleic acid and the positively charged macro-molecule induces a range of structural changes that vary, depending on the macro-molecule used. In general, however, the process results in condensation or compaction of the nucleic acid and physical association of the nucleic acid with the lipid. The interaction generates a complex that is more amenable to cellular uptake, protects sufficient nucleic acid molecules to allow trafficking to the nucleus, and, in at least some cases, may also facilitate transfer into the nucleus.

Cationic lipid- and polymer-mediated nucleic acid transfer have been used mostly for the transfection of plasmid DNA in applications in which transient gene expression is sufficient or required, but nucleic acids in all forms, ranging from small oligonucleotides to artificial chromosomes, can be transferred using these systems *(1–5)*. RNA has also been transfected using these techniques *(6,7)*, and, recently, hybrid molecules containing RNA and DNA residues have been transfected using both lipid- and polymer-based delivery systems *(8,9)*.

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