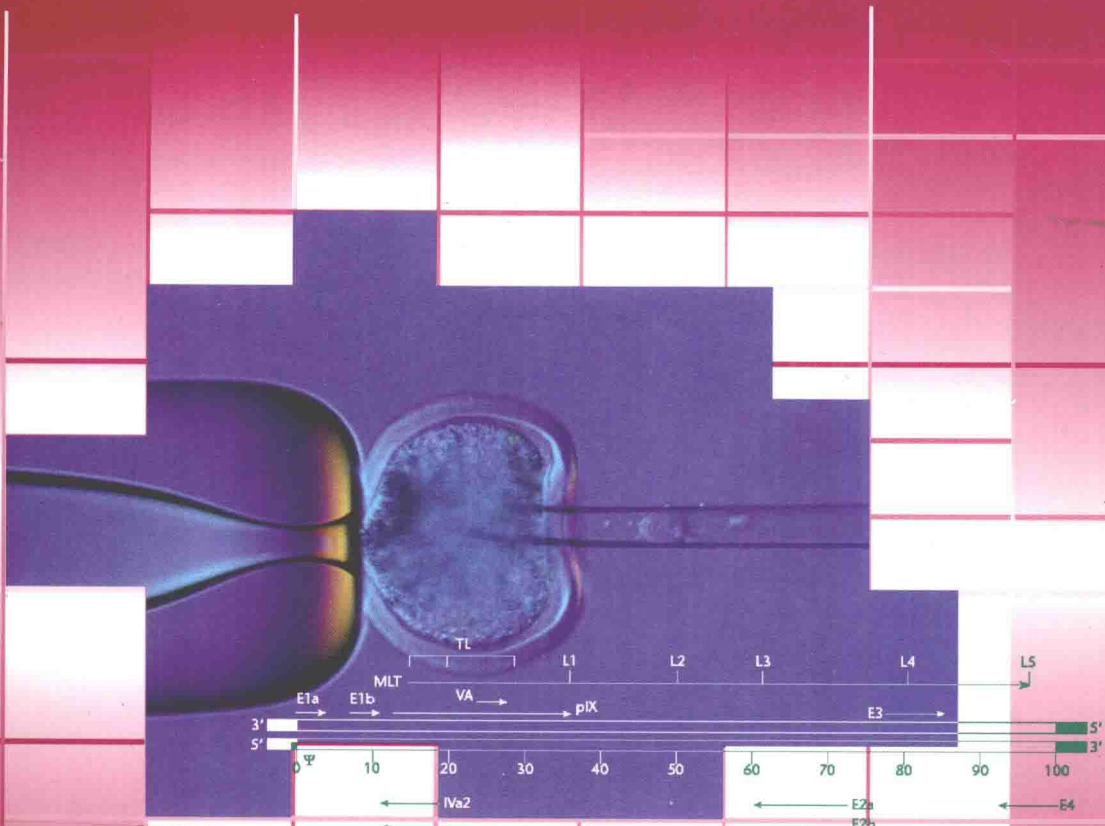
Gene Transfer to Animal Cells

R.M. Twyman



ADVANCED METHODS

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Abbreviations

AAV	adeno-associated virus
ADA	adenosine deaminase
AMP	adenosine 5′-monophosphate
ATT	adenosine 5′-triphosphate
BAC	bacterial artificial chromosome
BIV	bovine immunodeficiency virus
BPV	bovine papillomavirus
CAT	chloramphenicol acetyltransferase
CHO	Chinese hamster ovary
CID	chemically induced dimerization
CMV	cytomegalovirus
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
dTTP	deoxythymidine triphosphate
EBV	Epstein–Barr virus
EGC	embryonic germ cell
EIAV	equine infectious anemia virus
ES	embryonic stem
FIV	feline immunodeficiency virus
GFP	green fluorescent protein
GMP	good manufacturing practice
GMP	guanosine 5′-monophosphate
HIV	human immunodeficiency virus
HSV	herpes simplex virus
ICSI	intracytoplasmic sperm injection
IFN	interferon
IMAC	immobilized metal-affinity chromatography
IMP	inosine monophosphate
IRES	internal ribosome entry site
LAT	latency-associated transcript
LCR	locus control region
LTR	long terminal repeat
MLV	murine leukemia virus
MMTV	murine mammary tumor virus
NPV	nuclear polyhedrosis virus
PEI	polyethyleneimine
PEG	polyethylene glycol
PFU	plaque-forming unit
RAGE	recombinase activation of gene expression
REMI	restriction enzyme-mediated integration
RIGS	repeat-induced gene silencing
RSV	Rous sarcoma virus
SCID	severe combined immunodeficiency
SEAP	secreted alkaline phosphatase
SFV	Semliki Forest virus
SIN	Sindbis virus
SIV	simian immunodeficiency virus
TK	thymidine kinase
TNF	tumor necrosis factor
UTR	untranslated region
UV	ultraviolet
VSV	vesicular stomatitis virus
YAC	yeast artificial chromosome

Preface

Gene transfer methods for animal cells were first developed in the early 1960s, when researchers working with cultured mammalian cells sought ways to enhance the efficiency of infection with viral DNA and RNA. Since then, techniques for the introduction of exogenous genetic material have diversified and improved significantly, and they now underpin much of the current research in molecular and cellular biology as well as forming the basis of many applied aspects of biotechnology. A wide variety of methods has been described for gene transfer into animal cells, including chemical and physical delivery techniques, gene transfer using viral vectors and, most recently, gene transfer using bacterial vectors. These techniques are used not only to add new DNA to animal cells, they also form the basis of methods which allow random or targeted gene disruption and other forms of genome modification. In the last few years, the delivery of RNA into animal cells has become increasingly important as researchers have turned to RNA interference as a method for the inactivation of specific genes.

Although initially developed for the transformation of cells in culture, many gene transfer techniques have also been adapted to allow the transformation of cells in vivo, opening up new medical applications such as gene therapy and the use of DNA vaccines. Furthermore, gene transfer to germ cells, gametes or early embryos facilitates germline transformation and thus allows the production of transgenic animals, in which every cell is genetically modified. More recently, transgenic animals have also been produced through the manipulation of somatic cells followed by nuclear transfer into enucleated eggs. As gene transfer methods have matured, so we have come to understand in more detail the factors that influence the delivery, behavior and expression of exogenous genes, helping to increase the efficiency of gene transfer, enabling more control over the delivery system and facilitating the precise regulation of transgene expression. Over the last 40 years, gene transfer to animal cells has thus evolved from a rather hit-and-miss affair to an independent discipline in which precise goals can be achieved with high levels of efficiency.

To the researcher faced with the myriad of different delivery methods, host–vector systems and expression strategies, it can be difficult to make informed choices as to the most suitable strategy for any particular set of experimental goals. The aim of this *Advanced Methods* book is to provide an overview of these many different techniques, to summarize their advantages and disadvantages, and to provide guidance as to what can and cannot be achieved with each method. After a brief introductory chapter, which summarizes contemporary gene transfer technologies and provides an overview of the different selectable and scorable marker genes used with animal cells, Chapters 2–4 cover the different gene transfer methods in more detail. Chapter 2 considers chemical and physical transfection methods, Chapter 3 focuses on the relatively recent development of cell-based delivery systems and Chapter 4 provides an overview of the different viral vectors that are available for gene delivery. These chapters cover all the different vector/delivery systems and their applications in terms of transient vs stable expression, integration vs episomal maintenance, host range, vector construction and major applications. Chapter 5 extends the discussion of these vector/delivery systems in the context of

producing transgenic animals. Whereas the first half of the book focuses on gene transfer methods, the second half is more goal-orientated and considers the different ways in which gene transfer can be applied to achieve different aims. Chapter 6 covers the use of homologous recombination and site-specific recombination to disrupt or modify endogenous genes and genomes. Chapter 7 provides an overview of available strategies for controlling transgene expression, including the use of inducible expression systems. Chapter 8 briefly considers some of the principles of transgene behavior which can generate unexpected results in gene transfer experiments, such as the impact of position effects, dosage effects, genetic background and transgene structure. Chapter 9 focuses on the use of gene transfer to achieve the targeted or random inactivation of endogenous genes, e.g. with antisense RNA, ribozymes, RNA interference or specific recombinant antibodies. Finally, Chapter 10 briefly outlines some of the major applications of gene transfer in animal cells, including the study of gene function and regulation, the commercial synthesis of recombinant proteins, the improvement of domestic animals, disease modeling and gene therapy. Selected methods are included in some of the chapters to provide the reader with examples of how the range of methods discussed in the book could be applied, but this is not intended to be comprehensive or exhaustive coverage because the range of systems, methods and applications discussed in the main text would make this an impossible goal. For readers interested in the fine details of individual methods, there are many specialized texts available on particular expression systems or applications, and these are mentioned in the further reading section included at the end of each chapter.

This book would not have been possible without the help and support of the team at Garland-BIOS, particularly Chris Dixon and Nigel Farrar. I would like to thank all at the Fraunhofer Institute of Molecular Biology and Applied Ecology, the Technical University in Aachen and the Department of Biological Sciences, University of York. Very special thanks are due to Ricarda Finnern, RWTH Aachen, for patient editing and attention to detail.

This book is dedicated with love to my parents, Peter and Irene, to my children, Emily and Lucy, and to Paula who makes it all worthwhile.

Richard Twyman

Contents

Basic principles of gene transfer

1

1.1 Introduction

The delivery of DNA into animal cells is a fundamental and established procedure, which is used very widely both in basic research and applied biology. In research, the transfer of DNA into animal cells is an indispensable tool for gene cloning, the study of gene function and regulation, and the production of small amounts of recombinant proteins for analysis and verification. Although in many cases the aim of a gene transfer experiment is to express the introduced genetic construct (or *transgene*) in the recipient cells and provide those cells with a *gain of function*, gene transfer can also be used to disrupt or inactivate particular endogenous genes (resulting in a *loss of function*), or to perform specific genome modifications. The applications of gene transfer are manifold, and range from the use of mammalian and insect cell cultures for the large-scale commercial production of recombinant antibodies and vaccines, to the transfer of DNA into human patients for the correction or prevention of disease, a field known as *gene medicine* or *gene therapy*. Gene transfer to animal cells is also the first step in the creation of genetically modified whole animals, in which every cell or a specific target population of cells carries a particular alteration. Such animals are used to study gene function and expression, model human diseases, produce recombinant proteins in their milk and other fluids, and to improve the quality of livestock herds and other domestic species. In the last 5 years, animal gene transfer experiments have been conceived on an ever larger scale in an attempt to determine the functions of the many genes discovered in the genome projects (*functional genomics*). Examples of such experiments include systematic DNA-mediated mutagenesis and gene trap programs in the mouse and in the fruit fly, *Drosophila melanogaster*, genome-wide RNA interference experiments in the nematode, *Caenorhabditis elegans*, and novel protein interaction screens based on the yeast two-hybrid system but performed using mammalian cells.

1.2 Historical perspective and some definitions

The concept of gene transfer between cells was first demonstrated in bacteria, which are capable of at least four natural forms of genetic exchange. The first mechanism was discovered in 1928 by Frederick Griffith while working with two different strains of the bacterium *Streptococcus pneumoniae*. He found that the harmless R strain, characterized by rough-looking colonies, could be converted into the virulent S strain, characterized by smooth colonies, if living R cells were mixed with killed S cells before injecting them into mice (*Figure 1.1*). When this experiment was carried out, the

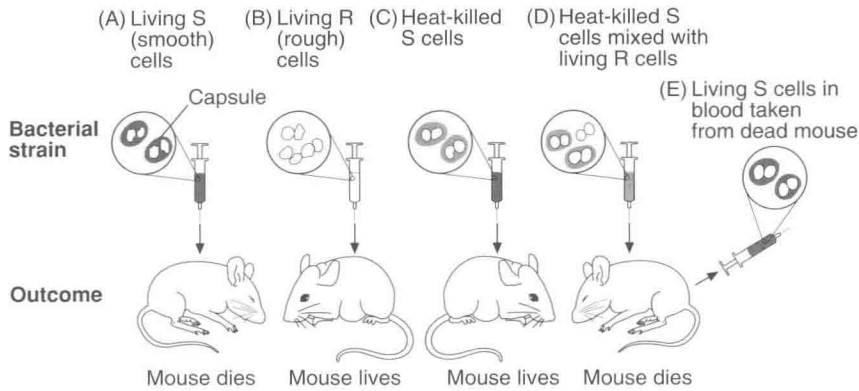

Figure 1.1

Griffith's experiment in 1928 was the first to demonstrate bacterial transformation, but it was over 15 years before the transforming principle was identified as DNA.

mice got sick and died, and living S cells could be recovered from the bodies. Griffith proposed that the genetic instructions to make virulent smooth colonies had somehow been transferred from the killed S cells to the living R cells, and he named this phenomenon *transformation*. However, he was unable to determine the nature of the transforming principle, and it was not until 1944 that Oswald Avery repeated the experiment and established that the substance transferred between cells was DNA. Later, it was discovered that the genetic information present in the S strain but absent from the R strain was a series of genes required to synthesize a proteinaceous capsule that helped the bacteria evade the host's immune response and also gave the colonies their smooth appearance.

A second form of gene transfer known as *conjugation* was discovered by Joshua Lederberg and Edward Tatum in 1946 while working with *Escherichia coli*. This process involved the transfer of DNA between bacterial cells following the establishment of a direct link between them, and in *E. coli* this conduit between cells took the form of a proteinaceous tube known as a pilus. The ability of the cells to construct the pilus and pass DNA through it was encoded on a large plasmid known as the F (for fertility) factor. In most cases, the act of conjugation involved transfer of the plasmid alone, which became established in the recipient cell thereby converting it from an F^- to an F^+ phenotype (*Figure 1.2*). In some cases, however, the F plasmid could integrate into the bacterial chromosome, and conjugation could result in the transfer of chromosomal genes. This process, which was used to construct the first genetic map of *E. coli*, was termed *sexduction* (*Figure 1.3*). Conjugation was soon found to occur in many species of bacteria, and often occurs *between* different species. In some cases it even occurs between bacteria and eukaryotes (see Chapter 3).

It was while studying gene transfer in *Salmonella* that Joshua Lederberg and Norton Zinder discovered in 1951 a new type of gene transfer mediated by bacteriophage. They found that newly formed phage could

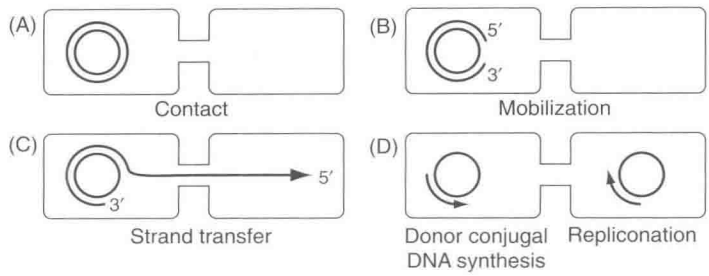

Figure 1.2

The four steps involved in gene transfer by conjugation. (A) Establishment of contact between cells (in *E. coli* and many other bacteria this is achieved by the formation of a conjugal tube known as a pilus). (B) Mobilization (nicking of the donor plasmid to initiate transfer). (C) Strand transfer into the recipient cell. (D) Donor conjugal DNA synthesis (replacement of the donated strand in the donor cell) and repliconation (synthesis of the complementary strand in the recipient cell).

occasionally package some of the host cell's DNA and then transfer it to a second host cell in a subsequent infection. This process was named *transduction*. Two forms of transduction could be distinguished – *generalized transduction*, where the phage head was mistakenly stuffed completely with host cell DNA (*Figure 1.4*), and *specialized transduction*, where the phage genome integrated into the bacterial chromosome and became linked to host DNA, and both types of DNA were packaged together. Specialized transduction in *E. coli* infected with bacteriophage λ is shown in *Figure 1.5*. The fourth mechanism of gene transfer in bacteria is mediated by complete cell fusion, and occurs in several genera of bacteria including *Bacillus* and *Streptomyces*.

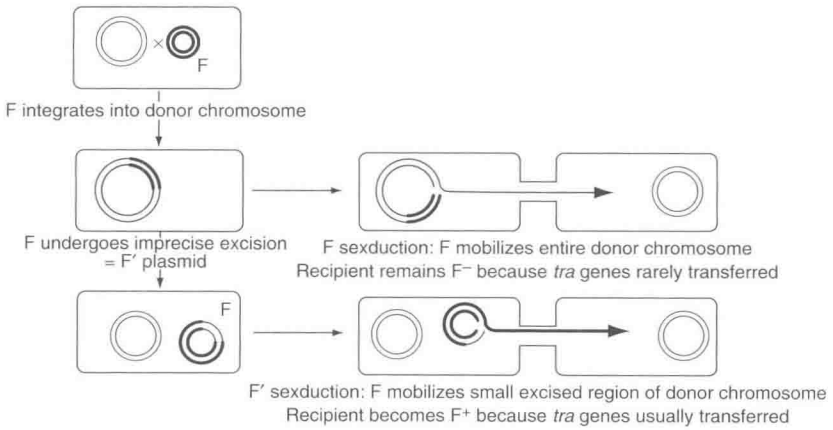

Figure 1.3

Sexduction: the transfer of chromosomal DNA during bacterial conjugation. The F plasmid can mediate sexduction either by conducting the chromosome into which it has integrated, or by exising imprecisely and conducting the chromosomal genes it has captured.

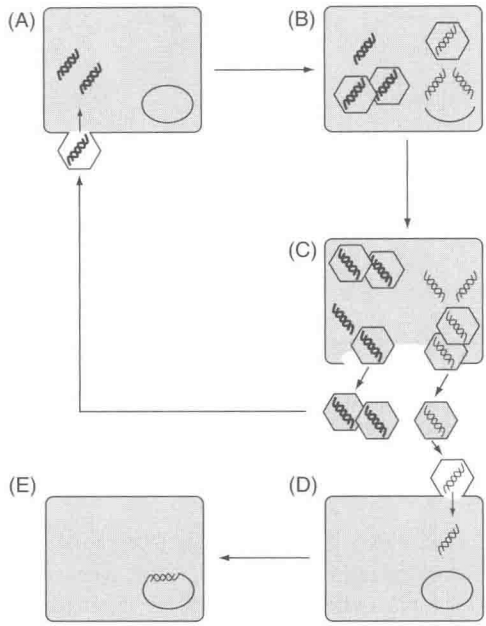

Figure 1.4

Generalized transduction in bacteria occurs when host DNA is accidentally packaged in the phage head and transferred to another cell in the subsequent round of infection. (A) Page infects a bacterial cell and injects its own DNA (thick lines). (B) As DNA is packaged into progeny phage, the bacterial genome breaks up and some is packaged into phage heads. (C) Phage are released from the cell. Those carrying phage DNA initiate new infections, while those carrying bacterial DNA can (D) transfer this to a new host. (E) In the new host, the transduced DNA can integrate or recombine with the host genome, changing the cell's phenotype.

The earliest experiments involving gene transfer to animal cells were reported shortly after the discovery of transduction in bacteria. Perhaps the first significant development was the study by Manker and colleagues in 1956 showing that embryonic chicken cells formed discrete clumps or *foci* when exposed to the genetic material of Rous sarcoma virus, a retrovirus that causes tumors in susceptible birds. The ability of this virus to induce tumors in birds and promote clumping in cultured cells reflects the presence of an *oncogene* (a gene that stimulates cell proliferation) within the viral genome. Under natural conditions, the viral capsid is needed to introduce the oncogene and the rest of the genome into an animal host cell. The unusual process of gene transfer without the viral capsid was named *transfection* to distinguish it from normal infection. A few years later, Ito and colleagues showed that tumors could be induced in rabbits injected with papillomavirus DNA, providing the first evidence for in vivo gene transfer in animals.

In the late 1960s and early 1970s, as the recombinant DNA era began, it became desirable to introduce exogenous DNA into cells artificially, usually

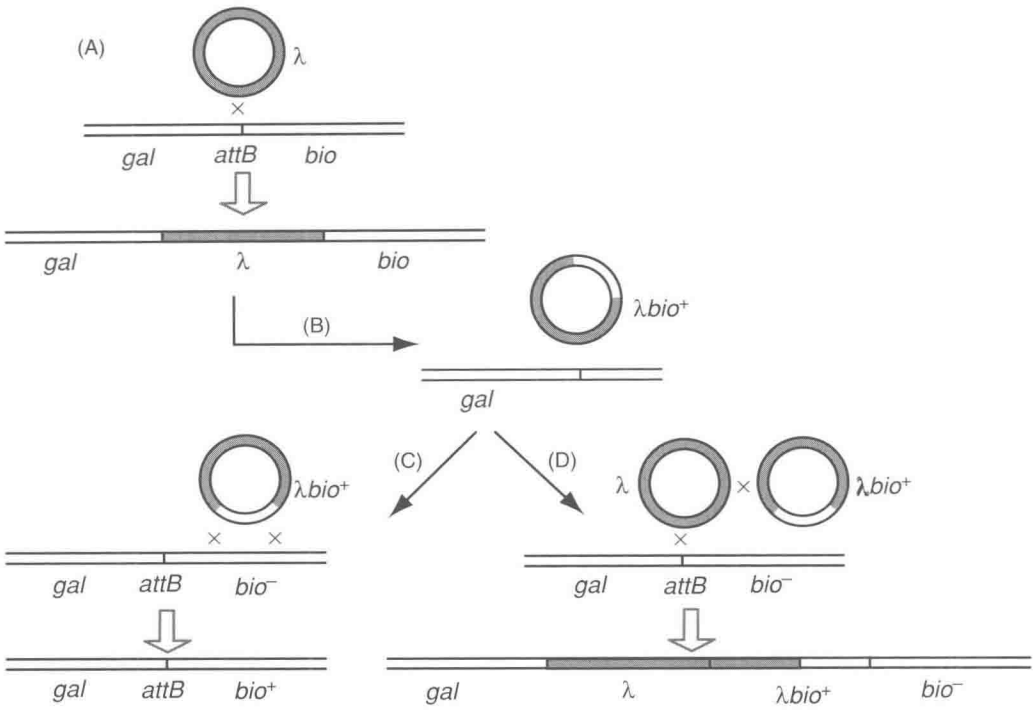

Figure 1.5

Specialized transduction in bacteriophage λ. (A) λ integrates at *attB* between the *gal* and *bio* loci of *E. coli*. (B) Aberrant excision generates a specialized transducing particle λ *bio*⁺ which carries the *bio* gene. Subsequent infection of *bio*⁻ host can lead to (C) replacement transduction by recombination, or (D) addition transduction by integration, the latter generating a λ:λ *bio*⁺ double lysogen which generates high-frequency transduction lysates. Similar events can occur which involve the *gal* locus.

in the form of a plasmid. The reason for this was simple: it was the most efficient way to deliver specific genes and study their effects; it allowed the use of very small plasmids, which were easy to manipulate in vitro. In bacterial genetics, the term *transformation* continued to be used to describe the uptake of naked plasmid or genomic DNA (essentially any DNA which had the potential to transform the phenotype of the recipient cell) while *transfection* was used specifically to describe the uptake of naked phage DNA (or RNA), i.e. nucleic acid which had the potential to initiate a phage replication cycle. For researchers working with animal cells, however, the term *transformation* had never been used in the gene transfer context and was already widely used to describe the cellular phenomenon discussed earlier, i.e. the conversion of normally growing cells to those with an oncogenic phenotype (growth transformation or oncogenic transformation). Therefore, the term *transfection* became generally accepted to mean the introduction of any sort of DNA – phage, plasmid, genomic or otherwise – into an animal cell, in the absence of a biological vector. The same term is also used when analogous methods are used to introduce RNA into cells. The transfection

of animal cells thus encompasses all chemical and physical means of nucleic acid delivery, as discussed in Chapter 2. Gene transfer to animal cells mediated by viruses is termed *transduction*, as it is in bacteria, and is the subject of Chapter 4. Chapter 3 considers a further category of gene transfer mechanism which uses bacterial vectors, and is sometimes termed *bactofection*. This can sometimes be analogous to bacterial conjugation, as in the case of HeLa cell transformation by *Agrobacterium tumefaciens*, but in most cases involves invasion of the animal host cell by the bacterium followed by the release of plasmid DNA, which is perhaps more analogous to bacterial cell fusion. The gene transfer methods used with animal cells are summarized in *Table 1.1*. (See references (1–5).)

1.3 Stages of gene transfer and the fate of exogenous nucleic acid

Regardless of the delivery method, gene transfer into animal cells must accomplish three distinct goals (*Figure 1.6*). First, the exogenous genetic

Table 1.1 Overview of gene transfer methods used with animal cells

Method	Advantages	Disadvantages
Transfection (chemical delivery methods)	Highly effective with cultured cells No limitations on transgene size Relatively simple Rapid and suitable for high throughput experiments	Limited in terms of clinical applications Challenge to prepare consistent formulations Some formulations are expensive Cell type dependency
Transfection (physical delivery methods)	High efficiency gene transfer No limitations on transgene size No cell type dependency Suitable for clinical applications	Low throughout Require specific instruments
Transduction (viral vectors)	High efficiency gene transfer Systemic in whole animals Cell-specific targeting possible	Complex cloning required More expensive than transfection methods Safety concerns regarding production of infectious viruses in humans May provoke immune response in mammals
Bactofection (bacterial vectors)	High-efficiency gene transfer	Safety concerns regarding production of infectious bacteria in humans May provoke immune response in mammals Not widely tested

material must be *transported across the cell membrane*. Such transfer is independent of the nature of the genetic material, which is inert and passive at this stage. In physical transfection methods, transport across the membrane is achieved by direct transfer, e.g. in microinjection or particle bombardment where the membrane is breached during delivery, or in electroporation where transient holes are formed, through which DNA and RNA can diffuse. In other delivery methods, the nucleic acid must form some sort of complex which binds to the cell surface before internalization. For example, in chemical transfection methods the complex is formed between nucleic acid and a synthetic compound, while in transduction methods the complex comprises nucleic acid packaged inside a viral capsid.

Once across the cell membrane, the genetic material must be released in the cell and transported to its site of expression or activity. Again, the nucleic acid is passive at this stage. In most transfection methods, DNA or RNA complexes are deposited in the cytoplasm, either directly under the plasma membrane or deeper in the cytosol following escape from the endosomal vesicle. DNA must be transported to the nucleus, a process which depends on the cell's poorly understood intrinsic DNA trafficking system, while RNA can function directly in the cytoplasm. In methods such as particle bombardment and microinjection, it is possible to deliver DNA directly into the nucleus, so intrinsic transport pathways are not required. Many viruses also deliver their nucleic acid cargo to the nucleus as part of the

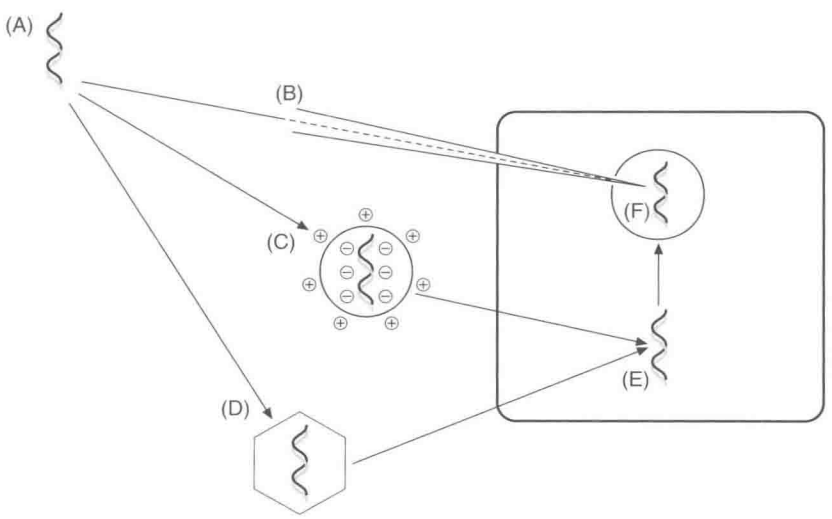

Figure 1.6

Summary of gene transfer mechanisms for introducing genetic material into animal cells. (A) DNA can be (B) introduced directly into the cell, and in some cases into the nucleus, by physical transfection methods. Alternatively (C) the DNA can form a chemical complex with a transfection reagent, resulting in its uptake into the cell, or may (D) be encapsulated within a viral particle (or a bacterial cell). The exogenous DNA first enters the cytoplasm (E) and then the nucleus (F).