Gene Cloning

an introduction

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The cover shows an electron micrograph of two molecules of the plasmid cloning vector pBR322. The plasmids have been coated with cytochrome c, stained with uranyl acetate and then shadowed to make them visible with the electron microscope. Preparation and micrograph by Mr S. Butler, Department of Biochemistry and Applied Molecular Biology, UMIST.

Preface

This book is intended to introduce gene cloning and recombinant DNA technology to undergraduates who have no previous experience of the subject. As such, it assumes very little background knowledge on the part of the reader — just the fundamental details of DNA and genes that would be expected of an average sixth-former capable of a university entrance grade at A-level biology. I have tried to explain all the important concepts from first principles, to define all unfamiliar terms either in the text or in the glossary, to avoid the less-helpful jargon words, and to reinforce the text with as many figures as are commensurate with a book of reasonable price.

Although aimed specifically at first- and second-year undergraduates in biochemistry and related degree courses, I hope that this book will also prove useful to some experienced researchers. I have been struck over the last few years by the number of biologists, expert in other aspects of the science, who have realized that gene cloning may have a role in their own research projects. Possibly this text can act as a painless introduction to the complexities of recombinant DNA technology for those of my colleagues wishing to branch out into this new discipline.

I would like to make it clear that this book is not intended as competition for the two excellent gene cloning texts already on the market. I have considerable regard for the books by Drs Old and Primrose and by Professor Glover, but believe that both texts are aimed primarily at advanced undergraduates who have had some previous exposure to the subject. It is this 'previous exposure' that I aim to provide. My greatest satisfaction will come if this book is accepted as a primer for Old and Primrose or for Glover.

I underestimated the effort needed to produce such a book and must thank several people for their help. The publishers provided the initial push to get the project under way. I am indebted to Don Grierson at Nottingham University and Paul Sims at UMIST for reading the text and suggesting improvements; all errors and naïveties are, however, mine. Finally, my wife Keri typed most of the manuscript and came to my rescue on several occasions with the right word or turn of phrase. This would never have been finished without her encouragement.

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Contents

PART I

The basic principles of gene cloning

1 Why gene cloning is important

Just over a century ago, Gregor Mendel formulated a set of rules to explain the inheritance of biological characteristics. The basic assumption of these rules is that each heritable property of an organism is controlled by a factor, called a **gene**, that is a physical particle present somewhere in the cell. The rediscovery of Mendel's laws in 1900 marks the birth of **genetics**, the science aimed at understanding what these genes are and exactly how they work.

The early development of genetics

For the first 30 years of its life this new science grew at an astonishing rate. The idea that genes reside on **chromosomes** was proposed by W. Sutton in 1903, and received experimental backing from T. H. Morgan in 1910. Morgan and his colleagues then developed the techniques for **gene mapping**, and by 1922 had produced a comprehensive analysis of the relative positions of over 2000 genes on the four chromosomes of the fruit fly, *Drosophila melanogaster*.

Despite the brilliance of these classical genetic studies, there was no real understanding of the molecular nature of the gene until the 1940s. Indeed, it was not until the experiments of Avery, MacLeod and McCarty in 1944, and of Hershey and Chase in 1952, that anyone believed DNA to be the genetic material; up to then it was widely thought that genes were made of protein. The discovery of the role of DNA was a tremendous stimulus to genetic research, and many famous biologists (Delbruck, Chargaff, Crick and Monod were among the most influential) contributed to the second great age of genetics. In the fourteen years between 1952 and 1966 the structure of DNA was elucidated, the genetic code cracked, and the processes of transcription and translation described.

The advent of gene cloning

These years of activity and discovery were followed by a lull, a period of anti-climax when it seemed to some molecular biologists (as the new generation of geneticists styled themselves) that there was little of fundamental importance that was not understood. In truth there was a frustration that the experimental techniques of the late 1960s were not sophisticated enough to allow the gene to be studied in any greater detail.

Then, in the years 1971–73 genetic research was thrown back into gear by what can only be described as a revolution in modern biology. A whole new methodology was developed, allowing previously impossible experiments to be planned and carried out, if not with ease, then at least with success. These methods, referred to as **recombinant DNA technology** or **genetic engineering**, and having at their core the process of **gene cloning**, sparked the third great age of genetics. We are still in the midst of the boom caused by this revolution and there is no end to the excitement in sight.

What is gene cloning?

The basic steps in a gene cloning experiment are as follows (Fig. 1.1):

(1) A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a **vector**, to produce a **chimaera** or **recombinant DNA molecule**.
(2) The vector acts as a **vehicle** that transports the gene into a host cell, which is usually a bacterium, although other types of living cell can be used.
(3) Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries.
(4) When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.
(5) After a large number of cell divisions, a colony, or **clone** of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

Gene cloning requires specialized tools and techniques

Vehicles

The central component of a gene cloning experiment is the vehicle, which transports the gene into the host cell and is responsible for its replication. To act as a cloning vehicle a DNA molecule must be capable of entering a host cell and, once inside, replicating to produce multiple copies of itself. Two naturally-occurring types of DNA molecule satisfy these requirements:

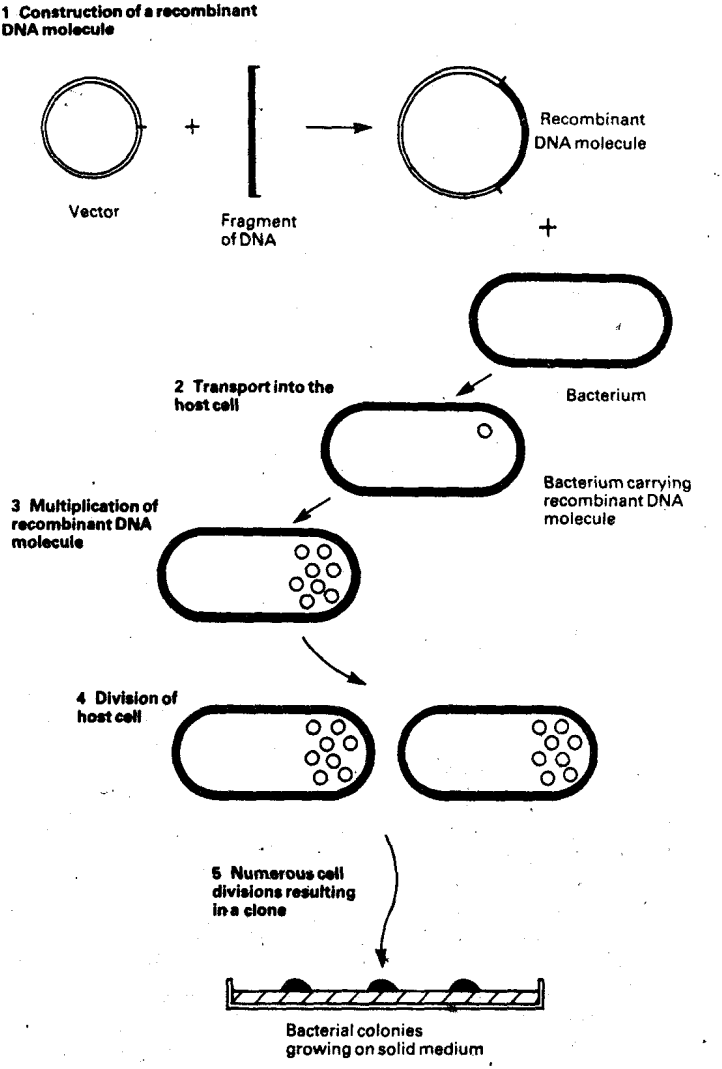

Figure1.1 The basic steps in gene cloning.

(1) **Plasmids,** which are small circles of DNA found in bacteria and some other organisms. Plasmids can replicate independently of the host cell chromosome.

(2) **Virus chromosomes,** in particular the chromosomes of **bacteriophages**, which are viruses that specifically infect bacteria. During infection the bacteriophage DNA molecule is injected into the host cell where it undergoes replication.

Chapter 2 covers the basic features of plasmids and bacteriophage chromo-

somes, providing the necessary background for an understanding of how these molecules are used as cloning vehicles.

Techniques for handling DNA

Plasmids and bacteriophage DNA molecules display the basic properties required of potential cloning vehicles. But this potential would be wasted without experimental techniques for handling DNA molecules in the laboratory. The fundamental steps in gene cloning, as described on page 4 and in Fig. 1.1, require several manipulative skills (Table 1.1). First, pure samples of DNA must be available, both of the cloning vehicle and of the gene to be cloned. The methods used to purify DNA from living cells are outlined in Chapter 3.

Table 1.1 Basic skills needed to carry out a simple gene cloning experiment

(1) Preparation of pure samples of DNA	(see Chapter 3).
(2) Cutting DNA molecules	(Chapter 4, pp. 50–58).
(3) Analysis of DNA fragment sizes	(Chapter 4, pp. 58–66).
(4) Joining DNA molecules together	(Chapter 4, pp. 66–73).
(5) Introduction of DNA into host cells	(Chapter 5, pp. 77–80).
(6) Identification of cells that contain recombinant DNA molecules	(Chapter 5, pp. 80–88, and Chapter 8).

Having prepared samples of DNA, construction of a recombinant DNA molecule requires that the vector be cut at a specific point and then repaired in such a way that the gene is inserted into the vehicle. The ability to manipulate DNA in this way is an offshoot of basic research into DNA synthesis and modification within living cells. The discovery of enzymes that can cut or join DNA molecules in the cell has led to the purification of **restriction endonucleases** and **ligases**, which are now used to construct recombinant DNA molecules in the test tube. The properties of these enzymes, and the way they are used in gene cloning experiments, are described in Chapter 4.

Once a recombinant DNA molecule has been constructed, it must be introduced into the host cell so that replication can take place. Transport into the host cell makes use of natural processes for uptake of plasmid and viral DNA molecules. These processes, and the ways they are utilized in gene cloning, are described in Chapter 5.

The diversity of cloning vectors

Although gene cloning is relatively new, it has nevertheless developed into a very sophisticated technology. Today a wide variety of different cloning vectors are available. All are derived from naturally-occurring plasmids or viruses, but most have been modified in various ways to suit each for a particular type of cloning experiment. In Chapters 6 and 7 the most important types of vector are described, and their uses examined.

Gene cloning is of central importance in research

Cloning has acted as a spur to fundamental biological research as it allows an individual gene to be selected and purified in large amounts free of contamination by other DNA sequences. Cloning is virtually the only procedure by which a DNA fragment containing a specific gene can be prepared, and is therefore in most cases the only means of obtaining material with which to make a direct study of the structure of genes and the control of gene expression.

In practice, the key to the success or failure of a cloning experiment is the

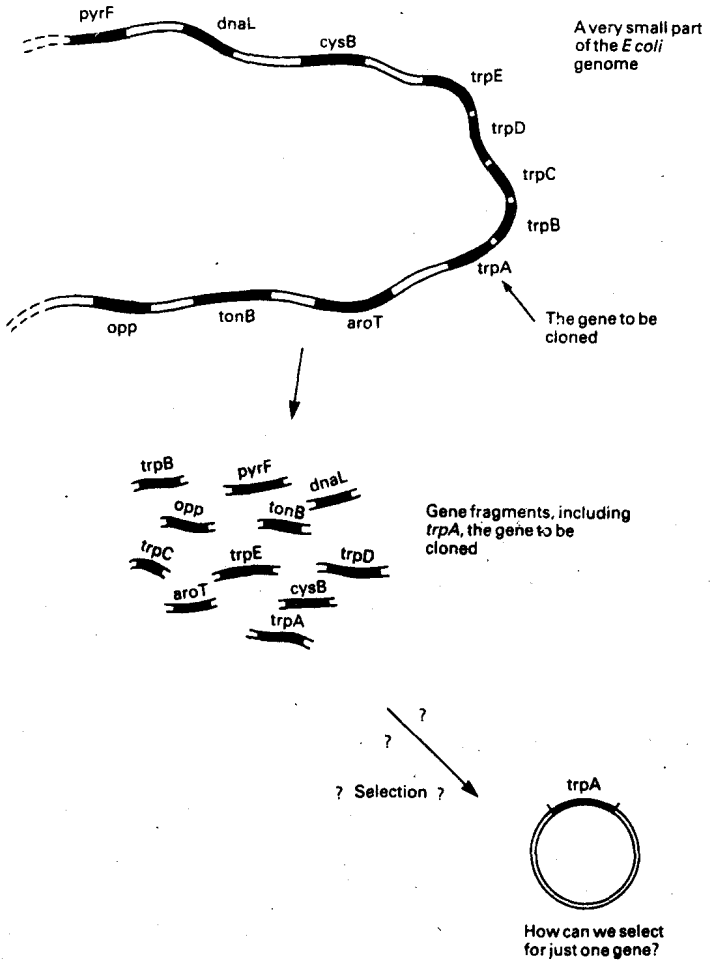

Figure 1.2 The problem of selection.

ability to select for the particular gene of interest. If we consider the **genome** of the bacterium *Escherichia coli*, which contains something in the region of 2000 different genes, we might at first despair at being able to clone selectively just one particular gene from all these hundreds (Fig. 1.2). However, as explained in Chapter 8, a variety of different strategies can be used to ensure that the correct gene is obtained at the end of the cloning experiment. Nevertheless, **selection** is still the major difficulty in many research projects involving gene cloning.

Once a gene has been cloned there is almost no limit to the information that can be obtained about the structure, function and expression of that gene. The availability of cloned material has stimulated the development of analytical methods for studying genes, and the last decade has seen remarkable improvements in techniques such as **DNA sequencing** and ***in vitro* mutagenesis**. These and other important methods for studying cloned genes are described in Chapter 9.

Not surprisingly, the ability to clone genes has resulted in rapid advances in our understanding of gene structure and the control of gene activity. An exciting parallel to these advances is the realization by biologists not specializing in molecular biology that gene cloning can help to answer questions posed by their own research. This has led to cloning being applied in recent months to such diverse subjects as premature ageing (Alzheimer's disease), the nutritional quality of cereals, and heavy metal biotransformations in polluted rivers. In Chapter 10 the potential uses for gene cloning in biological research are explored.

Cloned genes are important in industry

For many years man has made use of microorganisms such as bacteria and fungi as living factories in which to produce useful compounds. Typical examples of the use of microbes in **biotechnology** are the production of antibiotics, such as penicillin, which is synthesized by a fungus called *Penicillium*, and streptomycin, produced by the bacterium *Streptomyces griseus*. Gene cloning is revolutionizing biotechnology in a number of ways, most notably in providing a way in which mammalian proteins can be produced in bacterial cells. A remarkable property of a cloned gene is that it can often be made to function in an organism totally unrelated to that in which it is normally found. For example, an animal gene can be transferred by cloning into a bacterium, and then induced by some careful modifications to carry on working as though nothing had happened (Fig. 1.3).

The implications of this are enormous. Genes controlling the synthesis of important pharmaceuticals, such as antibiotics, drugs and hormones, can be taken from the organism in which they occur naturally, but from which the gene products may be costly and difficult to prepare, and placed in a bacterium or other type of organism, from which the product can be recovered conveniently and in large quantities. Already biotechnologists have notched up several successes in this line (Table 1.2), with recombinant insulin being the

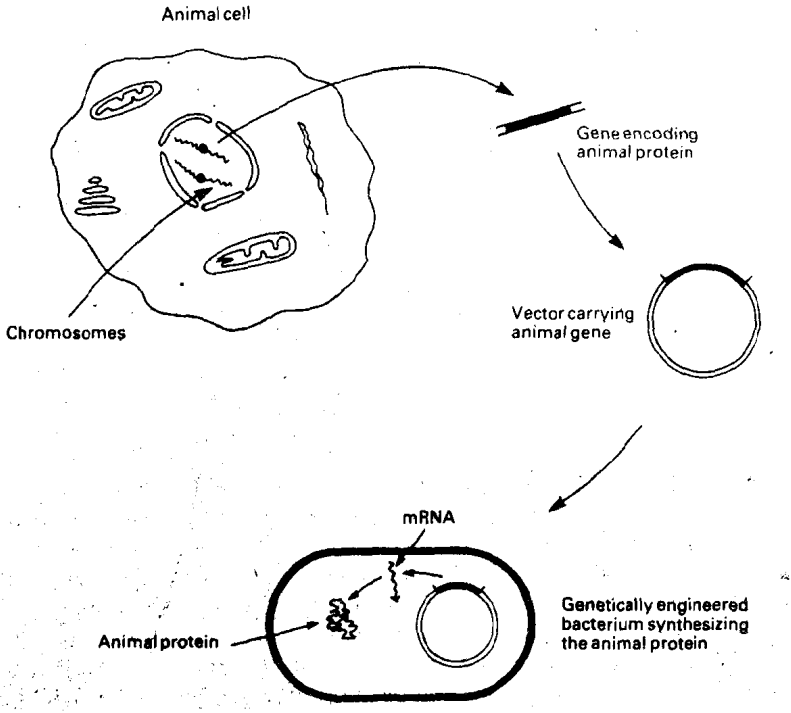

Figure 1.3 A possible scheme for the production of an animal protein by a bacterium.

most noteworthy achievement so far. Production of protein by cloned genes, and the applications of gene cloning in biotechnology, are described in the final two chapters of this book.

Table 1.2 *Some of the non-bacterial* proteins produced in *E. coli* as a result of gene cloning.

Protein	Use	Described on pages
Human insulin	Hormone — controls blood glucose levels	204–7
Human somatostatin	Hormone — regulates growth	208
Human somatotropin	Growth hormone — acts in conjunction with somatostatin	208–9
Human interferon	Anti-viral agent	209–10
Foot-and-mouth VP1 and VP3	Vaccines against foot-and-mouth virus	212
Hepatitis B core antigen	Diagnosis of hepatitis B	212–13

2 Vehicles: plasmids and bacteriophages

A DNA molecule needs to display several features to be able to act as a vehicle for gene cloning. Most important, it must be able to replicate within the host cell, so that numerous copies of the recombinant DNA molecule can be produced and passed to the daughter cells. A cloning vehicle also needs to be relatively small, ideally less than 10 kilobases (kb) in size, as large molecules tend to break down during purification, and are also more difficult to manipulate. Two kinds of DNA molecule that satisfy these criteria can be found in bacterial cells: plasmids and bacteriophage chromosomes. Although plasmids are very frequently employed as cloning vehicles, two of the most important types of vector in use today are derived from bacteriophages.

Plasmids

Basic features of plasmids

Plasmids are circular molecules of DNA that lead an independent existence in the bacterial cell (Fig. 2.1). Plasmids almost always carry one or more genes, and often these genes are responsible for a useful characteristic displayed by the host bacterium. For example, the ability to survive in normally-toxic concentrations of antibiotics such as chloramphenicol or ampicillin is often due to the presence in the bacterium of a plasmid carrying antibiotic-resistance genes. In the laboratory antibiotic resistance is often used as a **selectable marker** to ensure that bacteria in a culture contain a particular plasmid (Fig. 2.2).

All plasmids possess at least one DNA sequence that can act as an **origin of replication**, so they are able to multiply within the cell quite independently of the main bacterial chromosome (Fig. 2.3a). The smaller plasmids make use of the host cell's own DNA replicative enzymes in order to make copies of themselves, whereas some of the larger ones carry genes that code for special enzymes that are specific for plasmid replication.

A few types of plasmid are also able to replicate by inserting themselves into the bacterial chromosome (Fig. 2.3b). These integrative plasmids or **episomes**

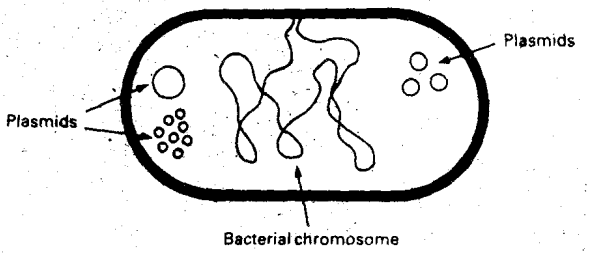

Figure 2.1 Plasmids: independent genetic elements found in bacterial cells.

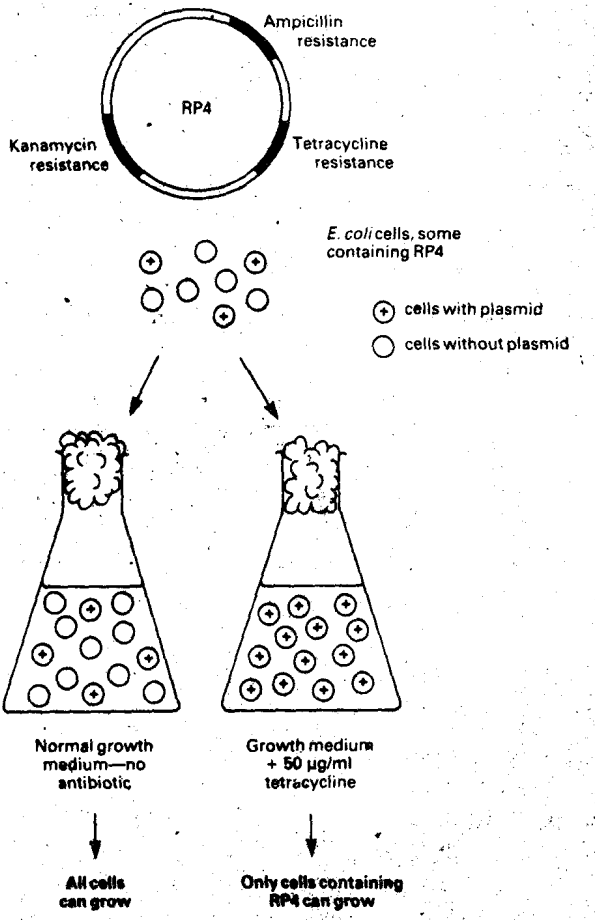

Figure 2.2 The use of antibiotic resistance as a selectable marker for a plasmid. RP4 (top) carries genes for resistance to ampicillin, tetracycline and kanamycin. Only those *E. coli* cells that contain RP4 (or a related plasmid) will be able to survive and grow in a medium that contains toxic amounts of one or more of these antibiotics.