CHAPTER 20
DNA TECHNOLOGY

OUTLINE

I. DNA Cloning
   A. DNA technology makes it possible to clone genes for basic research and commercial applications: *an overview*
   B. Restriction enzymes are used to make recombinant DNA
   C. Genes can be cloned in recombinant DNA vectors: *a closer look*
   D. Cloned genes are stored in DNA libraries
   E. The polymerase chain reaction (PCR) clones DNA entirely *in vitro*

II. Analysis of Cloned DNA
   A. Restriction fragment analysis detects DNA differences that affect restriction sites
   B. Entire genomes can be mapped at the DNA level

III. Practical Applications of DNA Technology
   A. DNA technology is reshaping medicine and the pharmaceutical industry
   B. DNA technology offers forensic, environmental, and agricultural applications
   C. DNA technology raises important safety and ethical questions

OBJECTIVES

After reading this chapter and attending lecture, the student should be able to:

1. Explain how advances in recombinant DNA technology have helped scientists study the eukaryotic genome.
2. Describe the natural function of restriction enzymes.
3. Describe how restriction enzymes and gel electrophoresis are used to isolate DNA fragments.
4. Explain how the creation of sticky ends by restriction enzymes is useful in producing a recombinant DNA molecule.
5. Outline the procedures for producing plasmid and phage vectors.
6. Explain how vectors are used in recombinant DNA technology.
7. List and describe the two major sources of genes for cloning.
8. Describe the function of reverse transcriptase in retroviruses and explain how they are useful in recombinant DNA technology.
9. Describe how "genes of interest" can be identified with the use of a probe.
10. Explain the importance of DNA synthesis and sequencing to modern studies of eukaryotic genomes.
11. Describe how bacteria can be induced to produce eukaryotic gene products.
12. List some advantages for using yeast in the production of gene products.
13. List and describe four complementary approaches used to map the human genome.

14. Explain how RFLP analysis and PCR can be applied to the Human Genome Project.
15. Describe how recombinant DNA technology can have medical applications such as diagnosis of genetic disease, development of gene therapy, vaccine production, and development of pharmaceutical products.
16. Describe how gene manipulation has practical applications for agriculture.
17. Describe how plant genes can be manipulated using the Ti plasmid carried by *Agrobacterium* as a vector.
18. Explain how foreign DNA may be transferred into monocotyledonous plants.
19. Describe how recombinant DNA studies and the biotechnology industry are regulated with regards to safety and policy matters.

KEY TERMS

genetic engineering
recombinant DNA
biotechnology
nucleic acid probe
gene cloning
restriction enzymes
antisense nucleic acid
restriction fragments length polymorphisms (RFLPs)
cloning vector
nucleic acid hybridization
denaturation
expression vector
restriction site
complementary DNA (cDNA)
sticky ends
DNA ligase
electroporation
genomic library
cDNA library
polymerase chain reaction (PCR)
in vitro mutagenesis
gel electrophoresis
Southern blotting
restriction fragment
artificial chromosomes
in situ hybridization
Ti plasmid
Human Genome Project
chromosome walking
DNA microarray assays
vaccine
DNA fingerprint
simple tandem repeats (STRs)

LECTURE NOTES

Recombinant DNA technology refers to the set of techniques for recombining genes from different sources *in vitro* and transferring this recombinant DNA into a cell where it may be expressed.

- These techniques were first developed around 1975 for basic research in bacterial molecular biology, but this technology has also led to many important discoveries in basic eukaryotic molecular biology.
- Such discoveries resulted in the appearance of the *biotechnology* industry. Biotechnology refers to the use of living organisms or their components to do practical tasks such as:
  - ⇒ The use of microorganisms to make wine and cheese
  - ⇒ Selective breeding of livestock and crops
  - ⇒ Production of antibiotics from microorganisms
  - ⇒ Production of monoclonal antibodies

The use of recombinant DNA techniques allows modern biotechnology to be a more precise and systematic process than earlier research methods.

- It is also a powerful tool since it allows genes to be moved across the species barrier.
- Using these techniques, scientists have advanced our understanding of eukaryotic molecular biology.
- The *Human Genome Project* is an important application of this technology. This project's goal is to transcribe and translate the entire human genome in order to better understand the human organism.
- A variety of applications are possible for this technology, and the practical goal is the improvement of human health and food production.

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I. DNA Cloning

A. DNA technology makes it possible to clone genes for basic research and commercial applications: *an overview*

Prior to the discovery of recombinant DNA techniques, procedures for altering the genes of organisms were constrained by the need to find and propagate desirable mutants.

- Geneticists relied on either natural processes, mutagenic radiation, or chemicals to induce mutations.
- In a laborious process, each organism's phenotype was checked to determine the presence of the desired mutation.
- Microbial geneticists developed techniques for screening mutants. For example, bacteria was cultured on media containing an antibiotic to isolate mutants which were antibiotic resistant.

Before 1975, transferring genes between organisms was accomplished by cumbersome and nonspecific breeding procedures. The only exception to this was the use of bacteria and their phages.

- Genes can be transferred from one bacterial strain to another by the natural processes of transformation, conjugation or transduction.
- Geneticists used these processes to carry out detailed molecular studies on the structure and functioning of prokaryotic and phage genes.
- Bacteria and phages are ideal for laboratory experiments because they are relatively small, have simple genomes, and are easily propagated.
- Although the technique was available to grow plant and animal cells in culture, the workings of their genomes could not be examined using existing methods.

Campbell Figure 20.1 provides an overview of how bacterial plasmids are used to clone genes for biotechnology.

Recombinant DNA technology now makes it possible for scientists to examine the structure and function of the eukaryotic genome, because it contains several key components:

- Biochemical tools that allow construction of recombinant DNA
- Methods for purifying DNA molecules and proteins of interest
- Vectors for carrying recombinant DNA into cells and replicating it
- Techniques for determining nucleotide sequences of DNA molecules.

B. Restriction enzymes are used to make recombinant DNA

Restriction enzymes are major tools in recombinant DNA technology.

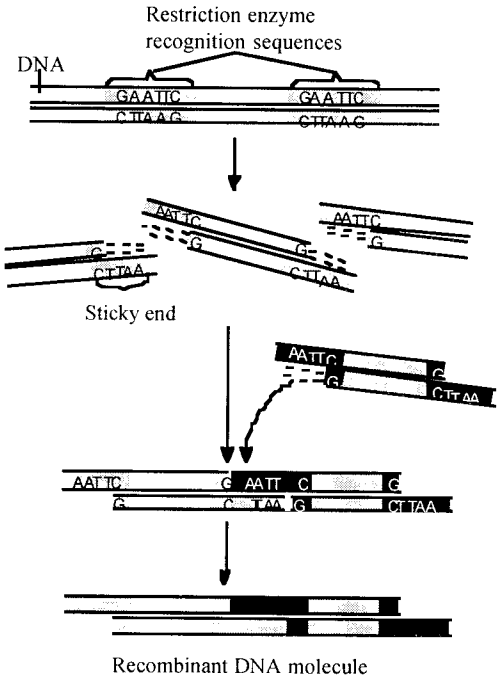
- First discovered in the late 1960s, these enzymes occur naturally in bacteria where they protect the bacterium against intruding DNA from other organisms.
- This protection involves *restriction*, a process in which the foreign DNA is cut into small segments.
- Most restriction enzymes only recognize short, specific nucleotide sequences called *recognition sequences* or restriction sites. They only cut at specific points within those sequences.

Bacterial cells protect their own DNA from restriction through *modification* or methylation of DNA.

- Methyl groups are added to nucleotides within the recognition sequences.

There are several hundred restriction enzymes and about a hundred different specific recognition sequences.

- Recognition sequences are symmetric in that the same sequence of four to eight nucleotides is found on both strands, but run in opposite directions.
- Restriction enzymes usually cut phosphodiester bonds of both strands in a staggered manner, so that the resulting double-stranded DNA fragments have single-stranded ends, called *sticky ends*.
- The single-stranded short extensions form hydrogen-bonded base pairs with complementary single-stranded stretches on other DNA molecules.



Sticky ends of *restriction fragments* are used in the laboratory to join DNA pieces from different sources (cells or even different organisms).

- These unions are temporary since they are only held by a few hydrogen bonds.
- These unions can be made permanent by adding the enzyme *DNA ligase*, which catalyzes formation of covalent phosphodiester bonds.

The outcome of this process is the same as natural genetic recombination, the production of recombinant DNA – a DNA molecule carrying a new combination of genes (see Campbell, Figure 20.2)..

C. Gene can be cloned in recombinant DNA vectors: *a closer look*

Most DNA technology procedures use carriers or vectors for moving DNA from test tubes back into cells.

Cloning vector = A DNA molecule that can carry foreign DNA into a cell and replicate there

- Two most often used types of vectors are bacterial plasmids and viruses.
- Restriction fragments of foreign DNA can be spliced into a bacterial plasmid without interfering with its ability to replicate within the bacterial cell. Isolated recombinant plasmids can be introduced into bacterial cells by transformation.

Bacteriophages, such as lambda phage, can also be used as vectors.

- The middle of the linear genome, which contains nonessential genes, is deleted by using restriction enzymes.
- Restriction fragments of foreign DNA are then inserted to replace the deleted area.
- The recombinant phage DNA is introduced into an *E. coli* cell.
- The phage replicates itself inside the bacterial cell.
- Each new phage particle carries the foreign DNA "passenger."

Sometimes it is necessary to clone DNA in eukaryotic cells rather than in bacteria. Under the right conditions, yeast and animal cells growing in culture can also take up foreign DNA from the medium.

- If the new DNA becomes incorporated into chromosomal DNA or *can* replicate itself, it can be cloned with the cell.
- Since yeast cells have plasmids, scientists can construct recombinant plasmids that combine yeast and bacterial DNA and that can replicate in either cell type.
- Viruses can also be used as vectors with eukaryotic cells. For example, retroviruses used as vectors in animal cells can integrate DNA directly into the chromosome.

1. Procedure for cloning a eukaryotic gene in a bacterial plasmid

Recombinant DNA molecules are only useful if they can be made to replicate and produce a large number of copies. A typical gene-cloning procedure includes the following steps (see Campbell, Figure 20.3):

Step 1: Isolation of vector and gene-source DNA

- Bacterial plasmids and foreign DNA containing the gene of interest are isolated.
- In this example, the foreign DNA is human, and the plasmid is from *E. coli* and has two genes:
  - amp^R which confers antibiotic resistance to ampicillin
  - *lacZ* which codes for β-galactosidase, the enzyme that catalyzes the hydrolysis of lactose
- Note that the recognition sequence for the restriction enzyme used in this example is *within* the *lacZ* gene.

Step 2: Insertion of gene-source DNA into the vector

a. Digestion
   - The restriction enzyme cuts plasmid DNA at the *restriction site*, disrupting the *lacZ* gene.
   - The foreign DNA is cut into thousands of fragments by the same restriction enzyme; one of the fragments contains the gene of interest.
   - When the restriction enzyme cuts, it produces *sticky ends* on both the foreign DNA fragments and the plasmid.

b. Mixture of foreign DNA fragments with clipped plasmids
   - Sticky ends of the plasmid base pair with complementary sticky ends of foreign DNA fragments.

c. Addition of DNA ligase
   - DNA ligase catalyzes the formation of covalent bonds, joining the two DNA molecules and forming a new plasmid with recombinant DNA.

Step 3: Introduction of cloning vector into bacterial cells

- The naked DNA is added to a bacterial culture.
- Some bacteria will take up the plasmid DNA by transformation.

Step 4: Cloning of cells (and foreign DNA)

- Bacteria with the recombinant plasmid are allowed to reproduce, cloning the inserted gene in the process.
- Recombinant plasmids can be identified by the fact that they are ampicillin resistant and will grow in the presence of ampicillin.

Step 5: Identification of cell clones carrying the gene of interest

- X-gal, a modified sugar added to the culture medium, turns blue when hydrolyzed by β-galactosidase. It is used as an indicator that cells have been transformed by plasmids containing the foreign insert.

- Since the foreign DNA insert disrupts the *lacZ* gene, bacterial colonies that have successfully acquired the foreign DNA fragment will be white. Those bacterial colonies lacking the DNA insert will have a complete *lacZ* gene that produces β-galactosidase and will turn blue in the presence of X-gal.
    - The methods for detecting the DNA of a gene depend directly on base pairing between the gene of interest and a complementary sequence on another nucleic acid molecule, a process called *nucleic acid hybridization*. The complementary molecule, a short piece of RNA or DNA is called a *nucleic acid probe* (see Campbell, Fig. 20.4).

2. Cloning and expressing eukaryotic genes: problems and solutions

Problem: Getting a cloned eukaryotic gene to function in a prokaryotic setting can be difficult because certain details of gene expression are different in the two kinds of cells.

Solution: Expression vectors allow the synthesis of many eukaryotic proteins in bacterial cells.

- Expression vectors contain a prokaryotic promoter just upstream of a restriction site where the eukaryotic gene can be inserted.
- The bacterial host cell recognizes the promoter and proceeds to express the foreign gene that has been linked to it.

Problem: Eukaryotic genes of interest may be too large to clone easily because they contain long noncoding regions (introns), which prevent correct expression of the gene by bacterial cells, which lack RNA-splicing machinery..

Solution: Scientists can make artificial eukaryotic genes that lack introns (see Campbell, Figure 20.5).

Solution: Artificial chromosomes, which combine the essentials of a eukaryotic chromosome with foreign DNA, can carry much more DNA than plasmid vectors, thereby enabling very long pieces of DNA to be cloned.

Bacteria are commonly used hosts in genetic engineering because:

- DNA can be easily isolated from and reintroduced into bacterial cells
- Bacterial cultures grow quickly, rapidly cloning the inserted foreign genes.

Some disadvantages to using bacterial host cells are that bacterial cells:

- May not be able to use the information in a eukaryotic gene, since eukaryotes and prokaryotes use different enzymes and regulatory mechanisms during transcription and translation.
- Cannot make the posttranslational modifications required to produce some eukaryotic proteins (e.g., addition of lipid or carbohydrate groups)

Using eukaryotic cells as hosts can avoid the eukaryotic-prokaryotic incompatibility issue.

- Yeast cells are as easy to grow as bacteria and contain plasmids.
- Some recombinant plasmids combine yeast and bacterial DNA and can replicate in either.
- Posttranslational modifications required to produce some eukaryotic proteins (e.g., addition of lipid or carbohydrate groups) can occur

There are more aggressive techniques for inserting foreign DNA into eukaryotic cells:

- In *electroporation*, a brief electric pulse applied to a cell solution causes temporary holes in the plasma membrane, through which DNA can enter.
- With thin needles, DNA can be injected directly into a eukaryotic cell.

- DNA attached to microscopic metal particles can be fired into plant cells with a gun (see Campbell, Figure 38.13).

Bacteria and yeast are not suitable for every purpose. For certain applications, plant or animal cell cultures must be used.

- Cells of more complex eukaryotes carry out certain biochemical processes not found in yeast (e.g. only animal cells produce antibodies).

D. Cloned genes are stored in DNA libraries

There are two major sources of DNA which can be inserted into vectors and clones:

1. DNA isolated directly from an organism
2. Complementary DNA made in the laboratory from mRNA templates

DNA isolated directly from an organism contains all genes including the gene of interest.

- Restriction enzymes are used to cut this DNA into thousands of pieces which are slightly larger than a gene.
- All of these pieces are then inserted into plasmids or viral DNA.
- These vectors containing the foreign DNA are introduced into bacteria.
- This produces the *genomic library,* the complete set of thousands of recombinant-plasmid clones, each carrying copies of a particular segment from the initial genome (see Campbell, Figure 20.6).
- Libraries can be saved and used as a source of other genes of interest or for genome mapping.

The cDNA method produces a more limited kind of gene library, a cDNA library. A cDNA library represents only part of the cell's genome because it contains only the genes that were transcribed in the starting cells (recall that cDNA is derived from isolated RNA).

- By using cells from specialized tissues or a cell culture used exclusively for making one gene product, the majority of mRNA produced is for the gene of interest.
- For example, most of the mRNA in precursors of mammalian erythrocytes is for the protein hemoglobin.

E. The polymerase chain reaction (PCR) clones DNA entirely in vitro

PCR is a technique that allows any piece of DNA to be quickly amplified (copied many times) in vitro (see Campbell, Methods Box)

- DNA is incubated under appropriate conditions with special primers and DNA polymerase molecules.
- Billions of copies of the DNA are produced in just a few hours.
- PCR is highly specific; primers determine the sequence to be amplified.
- Only minute amounts of DNA are needed.

PCR is presently being applied in many ways for analysis of DNA from a wide variety of sources:

- Ancient DNA fragments from a woolly mammoth; DNA is a stable molecule and can be amplified by PCR from sources thousands, even millions, of years old.
- DNA from tiny amounts of tissue or semen found at crime scenes
- DNA from single embryonic cells for prenatal diagnosis
- DNA of viral genes from cells infected with difficult to detect viruses such as HIV

Amplification of DNA by PCR is being used in the Human Genome Project to produce linkage maps without the need for large family pedigree analysis.

- DNA from sperm of a single donor can be amplified to analyze the immediate products of meiotic recombination.
- This process eliminates the need to rely on the chance that offspring will be produced with a particular type of recombinant chromosome.
- It makes it possible to study genetic markers that are extremely close together.

II. Analysis of Cloned DNA

Once a gene is cloned, scientists can then analyze the cloned DNA to address numerous questions, such as:

- Does a gene differ in different organisms?
- Are there certain alleles associated with a hereditary disorder?
- Where in the organism is the gene expressed?
- What control the pattern of expression?
- What is the location of the gene within the genome?

A. Restriction fragment analysis detects DNA differences that affect restriction sites

Gel electrophoresis (see Campbell's Methods box) is used to separate either nucleic acids or proteins based upon molecular size, charge, and other physical properties. Using this technique:

- Viral DNA, plasmid DNA, and segments of chromosomal DNA can be identified by their characteristic banding patterns after being cut with various restriction enzymes.
  - Each band corresponds to a DNA restriction fragment of a certain length.
  - DNA segments carrying different alleles of a gene can result in dissimilar banding patterns, since numbers and locations of restriction sites may not be the same in the different nucleotide sequences (see Campbell, Figure 20.7).
  - Similar differences in banding patterns result when *noncoding* segments of DNA are used as starting material.
- DNA fragments containing genes of interest can be isolated, purified, and then recovered from the gel with full biological activity.

The technique of hybridization is used to determine the presence of a specific nucleotide sequence (see Campbell's Methods Box on *Southern blotting* for an explanation of the entire procedure and a demonstration of how it can be used to compare DNA from three individuals).

- Labeled probes complementary to the gene of interest are allowed to bind to DNA from cells being tested (see Campbell, Fig. 20.4).
- Variations of this technique allows researchers to determine whether a:
  - Gene is present in various organisms
  - Sequence is present, how many sequences there are, and the size of the restriction fragments containing these sequences
  - Gene is made into mRNA, how much of that mRNA is present, and whether the amount of that mRNA changes at different stages of development or in response to certain regulatory signals (*Northern blotting*)

Differences in restriction fragment length that reflect variations in homologous DNA sequences are called *restriction fragment length polymorphisms* (*RFLPs*).

- DNA sequence differences on homologous chromosomes that result in RFLPs are scattered abundantly throughout genomes, including the human genome.
- RFLPs are not only abundant, but can easily be detected as to whether they affect the organism's phenotype; they can be located in an exon, intron, or any noncoding part of the genome.
- RFLP are detected and analyzed by Southern blotting.
- Because RFLPs can be readily detected, they are extremely useful as *genetic markers* for making linkage maps.
- A RFLP marker is often found in numerous variants in a population. RFLPs have provided many markers for mapping the human genome since geneticists are no longer limited to genetic variations that lead to phenotypic differences or protein products.

RFLPs are proving useful in several areas.

- Disease genes are being located by examining known RFLPs for linkage to them. RFLP markers inherited at a high frequency with a disease are probably located close to the defective gene on the chromosome.
- An individual's RFLP markers provide a "genetic fingerprint" which can be used in forensics, since there is a very low probability that two people would have the same set of RFLP markers.

B. Entire genomes can be mapped at the DNA level

1. Locating genes by *in situ* hybridization

DNA probes can be used to help map genes on eukaryotic chromosomes.

- *In situ hybridization* uses a radioactive DNA probe that base pairs with complementary sequences in the denatured DNA of intact chromosomes.
- Autoradiography and chromosome staining reveal to which band of which chromosome the probe has attached. Alternatively, the probe is labeled with fluorescent dye.

2. The mapping of entire genomes

The Human Genome Project, begun in 1990, is an international effort to map the entire human genome.

Scientists are also mapping the genomes of species that are particularly useful for genetic research, including *E. coli*, *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (nematode), *Drosophila melanogaster* (fruit fly), and *Mus musculus* (mouse).

Several complementary approaches are being used to map the precise locations of all of an organism's genes:

a. Genetic (linkage) mapping

- The first step in mapping a large genome is to construct a linkage map of several thousand genetic markers, which can be genes, RFLPs, or microsatellites.
- Relying primarily on microsatellites, researchers have completed a human genetic map with over 5000 markers.
- This map will enable researchers to locate other markers by testing for genetic linkage to the known markers.

b. Physical mapping: ordering the DNA fragments

- A physical map is made by cutting the DNA of each chromosome into a number of identifiable fragments.

- The key is to make fragments that overlap and to find the overlapping ends
- Campbell, Figure 20.8 shows *chromosome walking*, a method which uses probes to find the overlapping ends.
- Researchers carry out several rounds of DNA cutting, cloning, and mapping in order to prepare supplies of DNA fragments to map large genomes.

- The goal is to find the original order of the fragments in the chromosomal DNA.

c. Sequencing DNA

- The complete nucleotide sequence of a genome is the ultimate map.
- This will be the most time consuming part of the Human Genome Project as each haploid set of human chromosomes contains about 3 *billion* nucleotide pairs.

In addition to chromosome walking, Human Genome Project researchers are using PCR amplification.

- PCR can amplify specific portions of DNA from individual sperm cells—the immediate products of meiotic recombination.
- Researchers can amplify DNA in amounts sufficient for study, and analyze samples as large as thousands of sperm.
- Based on the crossover frequencies between genes, researchers can deduce human linkage maps without having to find large families for pedigree analysis.

These approaches will be used to completely map genomes and provide an understanding of how the human genome compares to those of other organisms. Potential benefits include:

- Identification and mapping of genes responsible for genetic diseases will aid diagnosis, treatment and prevention.
- Detailed knowledge of the genomes of humans and other species will give insight into genome organization, control of gene expression, cellular growth and differentiation, and evolutionary biology.

Achieving the goals of the Human Genome Project in a timely way will come from advances in automation and utilization of the latest electronic technology.

3. Genome analysis

Geneticists are also trying to determine phenotype from genotype, or identify genes within a long DNA sequence and determine their function.

a. Analyzing DNA sequences

Many researchers are studying the structure and organization of genes. This type of genome analysis relies of DNA sequencing (see Campbell's Methods box) and other mapping approaches outlined above.

DNA sequencing techniques have enabled scientists to collect thousands of DNA sequences in computer data banks.

- Using a computer, scientists can scan sequences for protein-coding genes and gene-control sequences.
- A list of nucleotide sequences for putative genes is assembled and compared to sequences of known genes.

- In the sequences compiled to date, many putative genes have been found to be entirely new to science; for example, 38% of the genes of E. Coli, the best studied research organism.

DNA sequences confirm the evolutionary connections between distantly related organisms and the relevance of research on simpler organisms to understanding human biology.

b. Studying gene expression

Other researchers are studying patterns of gene expression and how such patterns act to produce and maintain a functioning organism. This type of genome analysis can be performed without knowledge of the complete DNA sequence of an organism.

- One strategy for evaluating gene expression is to isolate the mRNA made in particular cells, use these molecules as templates for making a cDNA library by reverse transcription, and then compare this cDNA with other collections of DNA by hybridization.
  - This approach reveals which genes are active at different stages of development, in different tissues, or in different physiological conditions (or states of health).

Another approach uses DNA microarray assays to detect and measure the expression of thousands of genes at one time (see Campbell, Figure 20.9).

- This method is being used to compare cancerous and noncancerous tissues.
- Studying the differences in gene expression may lead researchers to new diagnostic techniques and biochemically targeted treatments

c. Determining gene function

Still other researchers are studying the function of genes.

In vitro mutagenesis is a technique that can be used to determine the function of a protein product from cDNA cloning of an mRNA.

- Mutations are induced into the sequence of a cloned gene.
- The mutated gene is returned to the host cell.
- If the mutation alters the function of the protein product, it may be possible to determine the function of the protein by examining what changes occur in cell physiology or developmental pattern.

III. Practical Applications of DNA Technology

A. DNA technology is reshaping medicine and the pharmaceutical industry

Modern biotechnology has resulted in significant advances in many areas of medicine.

1. Diagnosis of diseases

Medical scientists currently use DNA technology to diagnose hundreds of human genetic disorders.

- This allows early disease detection and identification of carriers for potentially harmful recessive mutations – even before the onset of symptoms.
- Genes have been cloned for many genetic disorders including hemophilia, phenylketonuria, cystic fibrosis, and Duchenne muscular dystrophy.

- Gene cloning permits direct detection of gene mutations. A cloned normal gene can be used as a probe to find the corresponding gene in cells being tested; the alleles are compared with normal and mutant standards usually by RFLP analysis.

When the normal gene has not been cloned, a closely linked RFLP marker may indicate the presence of an abnormal allele if the RFLP marker is frequently co-inherited with the disease.

- Blood samples from relatives can be used to determine which RFLP marker is linked to the abnormal allele and which is linked to the normal allele.
- The RFLP markers must be different for the normal and abnormal alleles.
- Under these conditions, the RFLP marker variant found in the person being tested can reveal whether the normal or abnormal allele is likely to be present.
- Alleles for cystic fibrosis and Huntington's disease can be detected in this manner.

2. Human gene therapy

Traceable genetic disorders in individuals may eventually be correctable.

- Theoretically, it should be possible to replace or supplement defective genes with functional normal genes using recombinant DNA techniques.
- Correcting somatic cells of individuals with well-defined, life-threatening genetic defects will be the starting place.

The principle behind human gene therapy is that normal genes are introduced into a patient's own somatic cells.

- For this therapy to be permanent, the cells receiving the normal allele must actively reproduce, so the normal allele will be replicated and continually expressed (see Campbell, Figure 20.11).
- Bone marrow cells are prime candidates.

Attempts at human gene therapy have not yet produced any proven benefits to patients, contrary to claims in the popular media.

- The most promising gene therapy trials under way are ones that involve bone marrow cells but are not necessarily aimed at correcting genetic defects; for example, improving the abilities of immune cells to fight off cancer and resist HIV
- Most experiments to date have been designed to test the safety and feasibility of a procedure rather than attempt a cure.

Many technical questions are posed by gene therapy.

- Can the proper genetic control mechanisms be made to operate on the transferred gene so that cells make appropriate amounts of the gene product at the right time and in the right place?
- How can we be sure that the inserted therapeutic gene does not harm some other necessary cell function?

Gene therapy raises difficult social and ethical questions:

- Is it advisable under any circumstances to alter the genomes of human germ lines (eggs) or embryos in hope of correcting the defect in future generations?
  - Some critics believe tampering with human genes in any way is wrong and may lead to eugenics.
  - Others say that genetic engineering of somatic cells is no different than other conventional medical interventions used to save lives.

Treating germ cells is possible and has been used in mice for some time.

- Mice have been created with sickle cell anemia to further the study of the disease.
- Recipient mice and their descendants contain the active human gene not only in the proper location (erythrocytes) but also during the proper stage of development.

3. Pharmaceutical products

DNA technology has been used to create many useful pharmaceutical products, mostly proteins.

- Highly active promoters and other gene control elements are put into vector DNA to create expression vectors that enable the host cell to make large amounts of the product of a gene inserted into the vector.
- Host cells can be engineered to secrete a protein as it is made, thereby simplifying the task of purifying it.

Human insulin and growth hormone are early applications of gene splicing..

- Two million individuals with diabetes in the United States have benefited from genetically engineered human insulin.
- Insulin produced this way is chemically identical to that made by the human pancreas, and it causes fewer adverse reactions than insulin extracted from pig and cattle pancreas.
- Human growth hormone has been a boon to children with hypopituitarism (pituitary dwarfism).
  - The growth hormone molecule is much larger than insulin (almost 200 amino acids long) and more species specific.
  - Thus, growth hormone from other animals is not an effective growth stimulator in humans.
  - Previously, these individuals were treated with growth hormone obtained from human cadavers.

Another important product produced by genetic engineering is tissue plasminogen activator (TPA).

- This protein helps dissolve blood clots and reduces the risk of later heart attacks if administered very shortly after an initial heart attack
- TPA illustrates a drawback to genetically engineered products. Because the development costs were high and the market relatively limited, the product has been very expensive.

Recent developments include novel ways to fight diseases that don't respond to traditional drug treatments.

- Antisense nucleic acid is used to base-pair with mRNA molecules and block their translation.
  - This could prevent the spread of diseases by interfering with viral replication or the transformation of cells into a cancerous state.
- Genetically engineered proteins block or mimic surface receptors on cell membranes.
  - For example, an experimental drug mimics a receptor protein that HIV binds to when it attacks white blood cells. The HIV binds to the drug molecules instead and fails to enter the blood cells.

Prevention by vaccine is the only way to fight many viral diseases for which no treatment exists.

Vaccine = A harmless variant or derivative of a pathogen that stimulates the immune system to fight the pathogen

Traditional vaccines for viral diseases are of two types:

- Particles of virulent virus that have been inactivated by chemical or physical means.
- Active virus particles of an attenuated (nonpathogenic) viral strain.

Since the particles in both cases are similar to active virus, both types of vaccine will trigger an animal's immune system to produce antibodies, which react very specifically against invading pathogens.

Biotechnology is being used in several ways to modify current vaccines and to produce new ones. Recombinant DNA techniques can be used to:

- Produce large amounts of specific protein molecules (*subunits*) from the surface of a pathogen. If these protein subunits cause immune responses to the pathogens, they can be used as vaccines.
- Modify genomes of pathogens to directly attenuate them. Vaccination with live, attenuated organisms is more effective than a subunit vaccine. Small amounts of material trigger greater immune response, and pathogens attenuated by gene-splicing may also be safer than using natural mutants.

B. DNA technology offers forensic, environmental, and agricultural applications

1. Forensic uses of DNA technology

Forensic labs can determine blood or tissue type from blood, small fragments of other tissue, or semen left at the scene of violent crime. These tests, however, have limitations:

- They require fresh tissue in sufficient amounts for testing.
- This approach can exclude a suspect but is not evidence of guilt, because many people have the same blood type or tissue type.

DNA testing can identify an individual with a much higher degree of certainty, since everyone's DNA base sequence is unique (except for identical twins).

- RFLP analysis by Southern blotting is a powerful method for the forensic detection of similarities and differences in DNA samples (see Campbell, Figure 20.12).
  - This method is used to compare DNA samples from the suspect, the victim, and a small amount of semen, blood or other tissue found at the scene of the crime.
  - Restriction fragments from the DNA samples are separated by electrophoresis; radioactive probes mark the bands containing RFLP markers.
  - Usually the forensic scientist tests for five markers.
  - Even a small set of RFLP markers from an individual can provide a *DNA fingerprint* that is of forensic use; the probability that two individuals would have the same RFLP markers is quite low.
- Increasingly, variations in the lengths of satellite DNA are used instead of RFLPs in DNA fingerprinting.
  - The most useful satellite sequences for forensic purposes are microsatellites, which are 10 - 100 base pairs long, have repeating units of only 1 - 4 base pairs, and are highly variable from person to person.
  - Individuals have different numbers of repeats at genome loci (*simple tandem repeats (STRs)*)

- Restriction fragments containing STRs vary in size among individuals because of differences in STR lengths rather than because of different numbers of restriction sites within that region of the genome, as in RFLP analysis.
- The greater the number of markers examined in a sample, the more likely it is that the DNA fingerprint is unique to one individual.

- PCR is often used to selectively simplify particular STRs or other markers before electrophoresis. This is especially useful when the DNA is in poor condition or only available in minute quantities (only 20 cells are needed!).

How reliable is DNA fingerprinting?

- Though each individual's DNA fingerprint is unique, most forensic tests do not analyze the entire genome but focus on tiny regions known to be highly variable from one person to another.
- The probability is minute (between on in 100,000 and one in a billion) that two people will have matching DNA fingerprints.
  - The exact figure depends on the number of markers compared and on the frequency of those markers in the population.
  - The frequency of markers varies by ethnic group, which allows forensic scientists to make extremely accurate statistical calculations.
- Problems can arise from insufficient statistical data, human error, or flawed evidence.
- DNA fingerprints are now accepted as compelling evidence by legal and scientific experts.

As with most new technology, forensic applications of DNA fingerprinting raises important ethical questions such as:

- Once collected, what happens to the DNA data?
- Should DNA fingerprints be filed or destroyed? Some states now save DNA data from convicted criminals.

2. Environmental uses of DNA technology

Scientists are engineering metabolic capabilities of organisms so they can transform chemicals and thus help solve environmental problems. For example:

- Some microorganisms can extract heavy metals (e.g., copper, lead, and nickel) from their environments and incorporate them into recoverable compounds such as copper sulfate or lead sulfate.
- As metal reserves are depleted, genetically engineered microbes may perhaps be used in mining and in cleaning up mining waste.

Metabolic diversity of microbes is used in the recycling of wastes and detoxification of toxic chemicals.

- Sewage treatment plants use microorganisms to degrade organic compounds into non-toxic forms.
- Biologically and chemically active compounds that cannot be easily degraded are often released into the environment.
- The intent is to engineer microorganisms that can degrade these compounds and that can be used in waste water treatment plants.
- Such microbes might be incorporated directly into the manufacturing process, preventing toxic chemicals from ever being released as waste in the first place.
- Bacterial strains have been developed to detoxify specific toxic wastes found in spills and waste dumps.

3. Agricultural uses of DNA technology

Recombinant DNA techniques are being used to study plants and animals of agricultural importance to improve their productivity.

a. Animal husbandry

Products produced by recombinant DNA methods, such as vaccines, antibodies and growth hormone, are already used in animal husbandry. For example,

- *Bovine growth hormone* (*bGH*). Made by *E. coli*, bGH is injected into milk cows to enhance milk production and into beef cattle to increase weight gain.
- *Cellulase*. Also produced by *E. coli*, this enzyme hydrolyzes cellulose making all plant parts useable for animal feed.

Transgenic animals, those that contain DNA from other species, have been commercially produced by injecting foreign DNA into egg nuclei or early embryos.

- Transgenic beef and dairy cattle, hogs, sheep and several species of commercially raised fish have been produced for potential agricultural use.

b. Genetic engineering in plants

Plant cells are easier to engineer than animal cells, because an adult plant can be produced from a single cell growing in tissue culture.

- This is important since many types of genetic manipulation are easier to perform and assess on single cells than on whole organisms.
- Asparagus, cabbage, citrus fruits, sunflowers, carrots, alfalfa, millet, tomatoes, potatoes, and tobacco are all commercial plants that can be grown from single somatic cells.

The best developed DNA vector for plant cells is the *Ti plasmid* (tumor inducing), carried by the normally pathogenic bacterium *Agrobacterium tumefaciens*.

- Ti plasmid usually induces tumor formation in infected plants by integrating a segment of its DNA (called T DNA) into the chromosomes of the host plant cell.
- Researchers have turned this plasmid into a useful vector by eliminating its disease-causing ability without interfering with its potential to move genetic material into infected plants.

With recombinant DNA methods, foreign genes can be inserted into Ti plasmid.

- The recombinant plasmid can either be put back into *Agrobacterium tumefaciens*, which is used to infect plant cells in culture, or it can be introduced directly into plant cells.
- Individual modified plant cells are then used to regenerate whole plants that contain, express and pass on to their progeny the foreign gene (see Campbell, Figure 20.13).

Using Ti plasmid as a vector has one major drawback, only dicotyledons are susceptible to infection by *Agrobacterium*; important commercial plants such as corn and wheat are monocotyledons and cannot be infected.

- Newer methods that allow researchers to overcome this limitation are *electroporation* and use of the *DNA particle gun*.
- Electroporation uses high-voltage jolts of electricity to open temporary pores in the cell membrane; foreign DNA can enter the cells through these pores.

- The DNA gun shoots tiny DNA-coated metal pellets through the cell walls into the cytoplasm, where the foreign DNA becomes integrated into the host cell DNA.

Though cloning plant DNA is straightforward, plant molecular geneticists still face several technical problems:

- Identifying genes of interest may be difficult.
- Many important plant traits (such as crop yield) are polygenic.

Genetic engineering of plants has yielded positive results in cases where useful traits are determined by single genes. For example:

- Of the genetically engineered plants now in field trials, over 40% have received genes for herbicide resistance
- A bacterial gene that makes plants resistant to glyphosate (a powerful herbicide) has been successfully introduced into several crop plants; glyphosate-resistant plants makes it easier to grow crops and destroy weeds simultaneously.
- The first gene-spliced fruits approved by the FDA for human consumption were tomatoes engineered with antisense RNA to suppress ripening and retard spoilage. The general process is outlined below:
  1. Researchers clone the tomato gene coding for enzymes responsible for ripening.
  2. The complementary (antisense) gene is cloned.
  3. The antisense gene is spliced into the tomato plant's DNA, where it transcribes mRNA complementary to the ripening genes' mRNA.
  4. When the ripening gene produces a normal mRNA transcript, the antisense mRNA binds to it, blocking synthesis of the ripening enzyme.

Crop plants are being engineered to resist pathogens and pest insects. For example,

- Tomato and tobacco plants can be engineered to carry certain genes of viruses that normally infect and damage plants; expression of these versions of viral genes confer resistance to viral attack.
- Some plants have been engineered to resist insect attack, reducing the need to apply chemical insecticides to crops. Genes for an insecticidal protein have been successfully transferred from the bacterium, *Bacillus thuringiensis*, to corn, cotton and potatoes; field tests show that these engineered plants are insect resistant.

In the near future, crop plants developed with recombinant DNA techniques are likely to:

- Be made more productive by enlarging agriculturally valuable parts - whether they be roots, leaves, flowers, or stems.
- Have an enhanced food value. For example, corn and wheat might be engineered to produce mixes of amino acids optimal for the human diet.

c. The nitrogen-fixation challenge

Nitrogen fixation is the conversion of atmospheric, gaseous nitrogen (N_2) into nitrogen-containing compounds.

- Gaseous nitrogen is useless to plants.
- Nitrogen-fixing bacteria live in the soil or are symbiotic within plant roots.

- The nitrogen-containing compounds produced by nitrogen fixation are taken up from the soil by plants and used to make organic molecules such as amino acids and nucleotides.
- A major limiting factor in plant growth and crop yield is availability of useable nitrogen compounds. This is why nitrogenous fertilizers are used in agriculture.

Recombinant DNA technology can possibly be used to increase biological nitrogen fixation of bacterial species living in the soil or in association with plants.

C. DNA technology raises important safety and ethical questions

When scientists realized the potential power of DNA technology, they also became concerned that recombinant microorganisms could create hazardous new pathogens, which might escape from the laboratory.

- In response to these concerns, scientists developed and agreed upon a self-monitoring approach, which was soon formalized into federal regulatory programs.
- Today, governments and regulatory agencies worldwide are monitoring the biotechnology industry - promoting potential industrial, medical, and agricultural benefits, while ensuring that new products are safe.
- In the U.S., the FDA, National Institutes of Health (NIH) Recombinant DNA Advisory Committee, Department of Agriculture (USDA), and Environmental Protection Agency (EPA) set policies and regulate new developments in genetic engineering.

While genetic engineering holds enormous potential for improving human health and increasing agricultural productivity, new developments in DNA technology raise ethical concerns. For example, mapping the human genome will contribute to significant advances in gene therapy, but:

- Who should have the right to examine someone else's genes?
- Should a person's genome be a factor in their suitability for a job?
- Should insurance companies have the right to examine an applicant's genes?
- How do we weigh the benefits of gene therapy against assurances that the gene vectors are safe?

For environmental problems such as oil spills, genetically engineered organisms may be part of the solution, but what is their potential impact on native species?

For new medical products, what is the potential for harmful side effects, both short-term and long-term?

- New medical products must pass exhaustive tests before the FDA approves it for general marketing.
- Currently awaiting federal approval are hundreds of new genetically engineered diagnostic products, vaccines, and drugs - including some to treat AIDS and cancer.

For agricultural products, what are the potential dangers of introducing new genetically engineered organisms into the environment?

- Some argue that producing transgenic organisms is only an extension of traditional hybridization and should not be treated differently from the production of other hybrid crops or animals. The FDA holds that if products of genetic engineering are not significantly different from products already on the market, testing is not required.

- Others argue that creating transgenic organisms by splicing genes from one species to another is radically different from hybridizing closely related species of plants or animals.
- Some concerns are that genetically altered food products may contain new proteins that are toxic or cause severe allergies; genetically engineered crop plants could become superweeds resistant to herbicides, disease and insect pests; and engineered crop plants may hybridize with native plants and pass their new genes to closely related plants in the wild.

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