I. Recombinant DNA technology (Chapter 14)

A. recombinant DNA technology = collection of methods used to perform genetic engineering

1. genetic engineering = deliberate modification of an organism’s genetic information

2. restriction endonucleases = cut double-stranded DNA at specific locations (ligases rejoin)
   a. usually used by cell to destroy foreign (phage) DNA
   b. own DNA protected by methylation patterns

3. Three general types of restriction enzymes
   a. Type I and III cleave DNA away from recognition sites
   b. Type II cleave DNA at the specific recognition site
      (1) can be used for genetic engineering
      (2) EcoRI: 5' GAATTC 3' (from E. coli)
      (3) HindIII: (Haemophilus influenzae d) 5' AAGCTT 3'

4. reverse transcriptase (RNA-directed DNA polymerase) from retroviruses can be used to synthesize complementary DNA (cDNA) from RNA

5. cloning vector = carrier of gene to be recombined

6. markers = selectable phenotypic traits

7. southern blot = DNA-DNA hybridization on nitrocellulose filter
   a. DNA fragments separated by AGE, denatured, transferred to NC filter
   b. filter soaked in solution with radioactive probe (labeled gene fragment)
   c. autoradiography reveals band containing the gene of interest

B. Synthetic DNA

1. oligonucleotides (≤30 nucleotides) are RNA or DNA sequences synthesized in the lab
   a. 3’ end of nucleotide is attached to a solid support (e.g., silica gel)
   b. next nucleotide is added to 5’ end
   c. each nucleotide addition takes about 40 minutes
   d. good for oligos up to about 100 bases
2. **site-directed mutagenesis** = insertion of constructed sequence into ss region of gene to produce a specific alteration in a protein
   a. oligo is allowed to bind to the gene
   b. DNA polymerase extends the oligo, using the gene as a template
   c. the constructed gene is introduced into a ssDNA phage
   d. a host cell is transduced with the modified gene

C. **polymerase-chain reaction (pcr)** = method for high volume synthesis of DNA fragment
   1. allows production of high quantities of a specific DNA sequence without having to clone the sequence
   2. primer sequences (oligos) are constructed to complement the sequences flanking the targeted sequence
   3. the reaction mix contains thermostable DNA polymerase, an excess of primers, the four dNTP
   4. each cycle results in doubling the number of targeted sequence and involves three steps
      a. heat denaturation to separate the DNA strands (94°C for 15 seconds)
      b. temperature is lowered to allow binding of the primers to the target sequence (68°C)
      c. DNA polymerase extends the primers and copies the target sequence (about 1 min)
   5. pcr can provide billions of copies in a very short time
      a. 30 cycles provide about a billion copies
      b. 25 cycles take less than one hour (about 57 minutes)
   6. high temperature for denaturation requires thermostable DNA polymerase (Taq from *Thermus aquaticus* or pol from *Thermococcus litoralis*)
   7. pcr allows detection of specific DNA sequences from very small samples
      a. clinical application for early detection of many diseases
      b. forensic application for DNA fingerprinting

D. Preparation of recombinant DNA
1. DNA fragments can be separated using **electrophoresis**
   a. electrophoresis = molecules move through a gel matrix in an electrical field
      (1) gel is typically agarose or polyacrylamide
      (2) molecules move from negative to positive electrodes
      (3) molecules are separated based on size and charge
   b. fragments can be made by passage through a hypodermic syringe (shearing) or with restriction enzymes

2. the DNA fragment that contains the sequence of interest can be identified with Southern blotting

3. the DNA fragment is ligated to an appropriate vector
   a. produce sticky ends with a restriction enzyme
   b. add fragment to vector (e.g., a plasmid) with complementary sticky ends
   c. join the fragment to the plasmid with ligase
   d. blunt ends can be joined by adding a polyA tail to the fragment and a polyT tail to the plasmid

4. the engineered genetic information (rDNA) is introduced to a host cell through transformation or transduction

5. the complete genome can by fragmented and added to vectors to create a genomic **library**

6. specific clones can be identified by selection for a trait or by using a **probe**
   a. to use a probe, cells are replica plated onto nitrocellulose and lysed with sodium hydroxide
   b. the membrane is treated with a radioactive oligonucleotide probe
   c. the radioactive spots (gene of interest) is located by autoradiography

7. probes can be created by working backwards from proteins or mRNA
   a. 20 oligo sequences from proteins
   b. reverse transcriptase (RNA directed DNA polymerase) with mRNA

E. Cloning vectors

1. four types
a. plasmids
b. bacteriophages
c. cosmids
d. artificial chromosomes

2. plasmids
a. easy to isolate and purify
b. introduced via transformation
c. often contain markers (selectable phenotypic traits) like antibiotic resistance
d. most useful with restriction site within a marker
e. chimera = recombinant plasmid

3. phage vectors
a. foreign DNA is inserted into a functioning phage, replacing non-functional phage genes
b. recombinant phages are useful for generating libraries
c. transfection = transformation with recombinant phage DNA

4. cosmids
a. cosmids = plasmids that contain lambda phage cos sites and can be packaged into phage capsids
b. cos sequence (cohesive end) is a recognition sequence that allows integration into the bacterial chromosome
c. typically contain restriction sites and antibiotic resistance genes

5. artificial chromosomes
a. can carry large amounts of genetic material
   (1) yac up to 2000 kilobases
   (2) bac up to 200 kb
b. yeast artificial chromosome is one of the most widely used
   (1) contains all elements required to propagate a chromosome in yeast
      (a) replication origin
      (b) centromere
(c) telomeres

(2) contain restriction sites and markers

c. **bacterial artificial chromosome** is based on the *E. coli* F-factor plasmid

F. Inserting genes into eukaryotic cells

1. **transgenic animal** = an animal whose genome contains foreign DNA
2. genetic material may be directly injected into animal cells like fertilized eggs
3. **electroporation** = introducing DNA through pores in the membrane created by an electric pulse
4. **gene gun** = DNA-coated pellets are shot into the cell

G. Expression of foreign genes in bacteria

1. hosts usually lack restriction enzymes and are *recA*- to prevent recombination
2. several considerations for expression of foreign DNA
   a. promoter recognized by host RNA polymerase
   b. mRNA that allows ribosome binding
   c. mRNA processing
3. **expression vectors** help reduce problems
   a. typically derivatives of pBR322 with appropriate transcription and translation start signals
   b. appropriate restriction sites
   c. may incorporate *lac* operon control region
4. cDNA prepared from processed mRNA is the easiest way to avoid introns

H. Applications of genetic engineering

1. medical applications
   a. include production of somatostatin, insulin, human growth hormone, interferon, blood-clotting factor VIII, and interleukin-2
   (1) replaces human tissues as source
   (2) compounds are much more readily available
   b. plants have been modified to produce monoclonal antibodies and oral vaccines
   c. mammalian systems (mice) are also producing monoclonals and vaccines
d. probes for diagnosis of disease

e. somatic cell gene therapy = replacement of defective genes

f. fusion toxins to control cell metabolism (e.g., treat cancers and rheumatoid arthritis)

g. transgenic animals for blood, enzymes, or organs

2. industrial applications

a. many potential applications in pharmaceutical, chemical, and food industries

b. production of enzymes or other products

c. bioremediation

3. agricultural applications

a. directly transfer traits and bypass breeding

b. Ti plasmid from Agrobacterium tumefaciens is a useful vector for transforming plants

c. increase yields, respond to stress more efficiently, prevent damage from frost or insects, and become resistant to viral diseases