

## Chapter 17: Recombinant DNA and Biotechnology

### I Cleaving and Rejoining DNA

- Recombinant DNA technology is the manipulation and combination of DNA molecules from different sources.
- Recombinant DNA technology uses the techniques of sequencing, rejoining, amplifying, and locating DNA fragments, all of which use complementary base pairing of A with T (or U) and G with C.

#### A. Restriction endonucleases cleave DNA at specific sequences

- Restriction endonucleases are enzymes that hydrolyze two phosphodiester linkages on opposite strands.
- They recognize and cut specific sequences, generally short 4 to 6 base pairs in length.
- Bacteria evolved these enzymes as a defense against viruses. (*See Figure 17.1*)
- The site cut is called a recognition site.
- Bacteria avoid damaging their own DNA by modifying their DNA with methyl groups.
  - The enzyme *EcoRI* cuts double stranded DNA with the sequence 5'...GAATTC...3'.
  - The complementary strand reads the same 5'GAATTC3'. This type of sequence is called a palindromic sequence.
  - The likelihood of such a sequence is  $4^6$ , or 1 sequence per 4098 base pairs along a linear strand of double-stranded DNA molecules.
  - Using this enzyme on a long stretch of random DNA would create fragments with an average length of 4098 bases.

#### B. Gel electrophoresis identifies the sizes of DNA fragments

- Fragments of DNA can be separated using electrophoresis.
- DNA is an acid. When ionized in an aqueous solution at a relatively neutral pH, it has a net negative charge. (*See Figure 17.2*)
- When DNA is placed in a semi-solid gel and an electric field is applied, the molecules migrate toward the positive pole.
- The gel acts as a sieve and smaller molecules can migrate quicker.
- Separated molecules can be visualized by simple staining or by transferring the DNA to a nylon membrane denaturing the DNA and using a complementary labeled probe.
- *See Figure 17.3.*
- When double stranded DNA is heated it denatures or melts and becomes single stranded. When cooled it reanneals becoming double stranded. Complementary sequences reanneal.

#### C. Recombinant DNA can be made in a test tube

- Some restriction enzymes cut DNA bluntly, while others leave staggered ends of single-stranded DNA.

- *EcoRI* leaves staggered ends. If two different DNA's are cut so each has *EcoRI* staggered ends, they can be recombined via complementary ends and DNA ligase. (See Figure 17.4)

## II Cloning Genes

- The goal of recombinant DNA work is to produce many copies of a particular gene.
- If the DNA is to be used to make protein, it must get introduced into a host cell.

If the cells are prokaryotic, the cells will be transformed.
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- If eukaryotic, they are transfected.
- Cells are treated with DNA in special conditions.
- The cells that actually get the DNA must be identified from those that do not.

### A. Genes can be inserted into prokaryotic or eukaryotic cells

- Bacteria are useful as host for the DNA.
  - Bacteria are easy to grow, and divide quickly (20 to 60 minutes per division)
  - They have markers that make it easy to select or screen for insertion.
  - They have been intensely studied.
- Bacteria have some disadvantages as well.
  - Bacteria lack splicing machinery to excise introns.
  - Protein modifications, such as glycosylation and phosphorylations, fail to occur as they would in an appropriate eukaryotic cell.
  - Sometimes the organism is changed, becoming *transgenic*, by the DNA insertion, and this is the point of the transfer.
- *Saccharomyces*, baker's and brewer's yeast, are commonly used eukaryotic cells.
  - Yeast divide quickly for eukaryotic cells (2 to 8 hours).
  - They are easy to grow.
  - Yeast have small genome size ( $\approx 20$  million base pairs).
  - Yeast have the smallest genome for a eukaryotic cell.
- Plants are also made transgenic.
- Plant cells are relatively easy to regenerate from cells.
- The transgenic plant can then reproduce naturally in the field.

### B. Vectors can carry new DNA into host cells

- DNA can be introduced into the cell's genome by integration into a chromosome.
- DNA can be incorporated into a vector, which has its own origin of replication.
  - Vectors have a recognition sequence for a restriction enzyme, permitting it to form recombinant DNA.
  - Vectors must have genetic markers that will announce its presence in the host cell.
- Plasmids are commonly used as vectors.
  - A plasmid is a small circular DNA molecule, separate from the bacterial chromosome.

- They have their own origin of replication, and can divide separately from the host bacteria's chromosome.
- They are usually small, 2,000 - 6,000 base pairs.
- They often have just one restriction site, if any, for any given restriction enzyme.
- Cutting the plasmid at one site linearizes it.
- If the restriction enzyme leaves staggered "sticky" ends, and other DNA is cut with the same enzyme, it is easily possible to insert the DNA into the plasmid. (*See Figure 17.5*)
- Plasmids are isolated from bacteria, cut with restriction enzyme, the enzyme is inactivated, insert DNA is added and then ligase is added to seal the plasmids back together with the inserts.
- Plasmids are transferred to a live culture of bacteria that have no plasmids.
- These bacteria are treated so they will take up the plasmids.
- Plasmids often have selection genes such as antibiotic resistance genes.
- Bacteria are grown in the presence of the antibiotic countered by the plasmid, and the bacteria that take up the plasmid survive, while the others die. (*See Figure 17.6*)
- Viruses are also used as vectors.
  - Plasmids can fit about 5,000 base pairs additional to the DNA of the plasmid.
  - Both prokaryotic and eukaryotic viruses are often used as vectors.
  - *Bacteriophage lambda* which infects *E.coli*, can accommodate 20,000 base pairs by eliminating the genes that cause host death and lyses.
- A yeast artificial chromosome, or YAC has been made that has a yeast origin of replication, a centromere sequence and telomeres.
  - They have been engineered to include specialized single restriction sites and selectable markers.
  - YACs are 10,000 base pairs in size, but can accommodate up to 1.5 million base pairs.
- Human artificial chromosomes (*HAC*) have been constructed of a human centromere, telomeres and origins of replication.
- Plasmid vectors for plants include a plasmid that can transfer to plants from bacteria that causes crown gall, *Agrobacterium tumefaciens*.
  - The *Ti* plasmid is a transposon.
  - It produces copies of itself in the plant host chromosomes.
  - *T DNA*, tumor causing sequences, can be replaced by the desired DNA.
  - The plant cells that are purposely infected can be used to generate transgenic plants.

### **C. There are many ways to insert recombinant DNA into host cells**

- DNA usually cannot get across cell membranes.
- High  $\text{Ca}^{2+}$  causes membrane changes that reduce the barriers to DNA movement.
- Treatment of cells with high  $\text{Ca}^{2+}$  and DNA allows DNA uptake by cells.
- Other methods include:
  - Electroporation, exposure of cells to rapid pulses of high-voltage;
  - Microinjection, physical puncturing of a cell with a tiny pipette and transfer of the DNA through the bore;

- Lipofection, DNA is coated with lipid, which allows it to pass through the plasma membrane;
- Particle bombardments, tiny high-velocity particles of tungsten or gold are coated with DNA and then shot into cells.

#### **D. Genetic markers identify host cells that contain recombinant DNA**

- When a population of host cells is treated to introduce DNA, just a fraction actually incorporate and express it.
- Only a few vectors that move into cells actually contain the new DNA sequence.
- Therefore, a method for selecting for transfected cells and screening for inserts is needed.
- A classic example of how this was originally done is as follows:
  - The plasmid *pBR322* carries within its sequences an origin of replication and two antibiotic resistance genes: *amp<sup>r</sup>* (ampicillin resistance) and *tet<sup>r</sup>* (tetracycline resistance).
  - Within the antibiotic resistance genes are restriction enzyme recognition sites.
    - The *amp<sup>r</sup>* gene has a *PstI* recognition site.
    - The *tet<sup>r</sup>* gene has one *HindIII*, *BamHI* and *Sall*.
    - In each case these are the only sites for each enzyme in the plasmid.
  - If the foreign DNA is cut with *BamHI*, for example, and the plasmid is too, they can be recombined and sealed together with ligase.
  - If this site (*BamHI*) is used, the resistance to tetracycline is activated.
  - In practice, when plasmid and foreign DNA are placed together in the test tube several outcomes are possible:
    - The plasmid might just re-seal with no insert being incorporated.
    - The plasmid and foreign DNA get integrated.
- Bacteria treated with these plasmids might get just the foreign DNA, or a plasmid either with or without the insert.
- Bacteria are selected by growing on a medium that has the antibiotic ampicillin.
- Only those that take up a plasmid survive.
- The survivors might have a plasmid that either contains or does not contain an insert.
  - To determine the presence of the insert, the colonies on the plate containing ampicillin are copied over to a plate with tetracycline.
  - Those that *fail* to grow are the ones likely to have the insert. This is called screening.
  - The colonies that failed to grow on the tetracycline plate are selected from the ampicillin plate.
- Other methods have since been developed for screening.
  - Reporter genes, for example luciferase, have been used. Luciferase is the enzyme that makes fireflies glow in the dark when supplied with its substrate.
  - Green fluorescent protein, which is the product of a jellyfish gene, glows without any required substrate.
  - Cells with this gene in the plasmid grow on ampicillin and glow in the dark.

- Many vectors in common use have just a single resistance gene outside of the sites for foreign DNA insertion. (*See Figure 17.7*)

### III Sources of Genes Cloning

- DNA for insertion can be random fragments of the DNA from an organism (a DNA library).
- DNA can be generated from mRNA. This DNA is called cDNA (complementary DNA).
- DNA can be synthesized.

#### A. Gene libraries contain pieces of a genome

- The average size of the 23 human chromosomes found in a haploid set is 80 million base pairs. (*See Figure 17.8*)
- To study them, chromosomes are sorted and fragmented.
- Lambda phage are used to hold these random fragments. It takes about 250,000 different lambda phage to insure a copy of every sequence.
- Just one plate can hold as many as 80,000 phage colonies.

#### B. A DNA copy of mRNA can be made

- The poly A tail, found on many mRNA molecules from eukaryotes, makes it possible to make DNA from it. (*See Figure 17.9*)
  - Reverse transcriptase and poly T primer is added.
  - Reverse transcriptase is an enzyme that is an RNA template dependent DNA polymerase.
  - The DNA is complementary to the RNA and is called cDNA.
  - See Figure 17.9.
- cDNA's from certain cell types have been useful in discovering the nature of differential gene expression.

#### C. DNA can be synthesized chemically in the laboratory

- If the amino acid sequence of a protein is known, a DNA that can code for it can be easily synthesized.
- The knowledge of the genetic code is used.
- A promoter is added to the sequence to promote expression of the protein.
- Human insulin was cloned using this approach.

#### D. DNA can be mutated in the laboratory

- Mutational effects can be studied by creating specific mutations.
- Additions, deletions, and base-pair substitutions are all possible by manipulating isolated DNA or making DNA synthetically.
- The functional importance of certain amino acid sequences can be studied.
  - The signals that signal proteins to the ER membrane were discovered by site directed mutagenesis.
    - If removed, proteins fail to get transported across the ER membrane.
    - When added to a protein that normally doesn't get transported, it does.
- Rules for tertiary structure of proteins are being devised using site-directed mutagenesis.

- The effects of the tertiary structure on enzyme activation are being studied using site-specific mutagenesis.

#### IV Some Additional Tools for DNA Manipulation

##### A. Genes can be inactivated by homologous recombination

- Homologous recombination can be used to selectively inactivate genes.
- This is called a knockout experiment. (*See Figure 17.10*)
  - A gene of interest is cloned into a plasmid.
  - Additional DNA is added within the gene to disable it.
  - Mouse embryonic cells are transfected with the DNA.
  - Homologous recombination knocks out the normal functioning copy.
  - Transfected cells are identified by selecting or screening for a genetic marker included in the insert.
  - These transfected cells are included into an early mouse embryo. Some of the cells end up as germ cells and homozygous mice with a knocked out gene can be generated.

##### B. DNA chips can reveal DNA mutations and RNA expression

- There are a large number of genes in eukaryotic genomes.
- The pattern of expression between different tissues and at different times is quite distinctive.
  - Cells have unique mRNA's.
  - For example, early stage skin cancer have a unique mRNA "fingerprint".
- To find these patterns, DNA sequences can be arranged in an array on a solid support.
- DNA chip technology provides these large arrays. (*See Figure 17.11*)
- Merging DNA technology with the manufacturing technology of the semiconductor industry, large arrays are being produced.
  - DNA chips are glass slides onto which DNA sequences are attached in precise order.
  - The typical slide is divided into 24x24 uM squares.
  - Each contains about 10 million copies of a particular sequence, which is up to 20 nucleotides long.
  - A computer controls the additions of the nucleotides in predetermined pattern.
  - Up to 60,000 different sequences can be put on a single chip.
  - Cellular mRNA is isolated from cells and is used to make complementary DNA, which is called cDNA.
  - Reverse transcriptase and PCR are used together in a process called RT-PCR.
  - The amplified cDNA is coupled to a fluorescent dye. It is then hybridized to the chip.
  - A sensitive scanner detects the spots on the array that glow. The combinations of spots that light up differ with different types of cells or different physiological states.
- DNA chip technology can be used in detecting genetic variants.

- Twenty-nucleotide fragments of DNA sequences of all possible mutations are arranged.
- The person's DNA is hybridized to determine if any hybridize to a mutant sequence on the chip.

### C. Antisense RNA and ribozymes can prevent the expression of specific genes.

- Antisense RNA is RNA that is complementary to a sequence of mRNA. (*See Figure 17.12*)
- The formation of a double-stranded RNA hybrid prevents tRNA from binding to mRNA.
- These hybrids are broken down rapidly in the cytoplasm.
- In the laboratory, either antisense RNA or DNA that codes for it is introduced into cells.
- A tissue specific promoter makes it possible to have mRNA inactivation occur only in a certain type of cell.
- Some antisense RNA have been coupled with another RNA sequence, a ribozyme, which destroys the target mRNA by cleavage.
- Antisense RNA technology has been used to provide volumes of information on development, cancer and other important biological questions.

## V Biotechnology: Applications of DNA Manipulation

- Biotechnology is the use of microbial, plant and animal cells to produce materials useful to people.
- These products include foods, medicines, and chemicals.
- Beer and wine are examples of biotechnology, which have been used for a long time.
- Modern molecular biology has vastly increased the number of products, and will continue to do so.

### A. Expression vectors can turn cells into protein factories

- Expression vectors are typical vectors, but which also have bacterial promoters, which are used to make transcription of the foreign DNA happen.
- An expression vector might have an *inducible promoter*, which can be stimulated or not into expression.
- A *tissue-specific promoter* is expressed just in a certain tissue.
- *Targeting sequences* are sometimes added to direct the protein product to an appropriate destination.
- *See Figure 17.13.*

### B. Medically useful proteins can be made by DNA technology

- Many medical products have been made using recombinant DNA technology. Hundreds more are in various stages of development.
- *See Table 17.1.*
  - Tissue Plasminogen Activator:
    - This product converts plasminogen, found normally in the blood, into plasmin, a protein that dissolves clots. (*See Figure 17.14*)
    - Plasminogen activator is currently being produced in *E.coli*.

- This new drug has been useful in treating patients who have suffered heart attacks or strokes.
- The only drug available prior was streptokinase, which is a bacterial enzyme and causes immune reactions if used repeatedly on a patient.
- Erythropoietin:
  - This hormone stimulates the production of red blood cells and is produced by the kidneys.
  - Those with kidney disease often fail to produce enough.
  - Currently, transgenic bacteria now produce large amounts of the protein.
- Human Insulin:
  - People with certain forms of diabetes mellitus have a deficiency of pancreatic insulin.
  - In the past, insulin was obtained from pig pancreases. This required difficult purification procedures, and caused risks of infectious disease and allergic reactions.
  - Human insulin now is made in bacteria. This was the first human protein to be made for drug use.

### C. DNA manipulation is changing agriculture

- Selective breeding has been used for centuries to improve plant and animal species. (See Table 17.2)
- Progress is accelerating using molecular biology.
- Two major advantages are that specific genes can be affected and genes can be introduced from other organisms.
  - Nutritional properties have been modified.
  - Edible crops have been modified to make oral vaccines.
- Plants that make their own insecticides:
  - Insecticides tend to be nonspecific, killing pest and beneficial insects.
  - They are applied to the surface of plants and tend to be blown or washed away.
  - *Bacillus thuringiensis* produces a protein toxin that kills the insect larvae that prey on them.
  - The toxicity is 80,000 times that of the typical chemical insecticide.
  - Transgenic tomato, corn, potato and cotton plants have been made that produce a toxin from *Bacillus thuringiensis*. They are insect resistant.
- Cloned animals that express useful genes:
  - Dolly, the cloned sheep, is transgenic and produces human  $\alpha$ -1-antitrypsin in her milk. (See Figure 17.15)
  - This protein inhibits elastase, which hydrolyzes connective tissue.
    - Excess elastase is found on the surface of the lungs of people with cystic fibrosis.
    - It is partially responsible for their severe breathing problems.
  - The promoter for lactoglobulin, a protein secreted into milk, was attached to the gene for  $\alpha$ -IAT and this was introduced into a fertilized sheep egg.
  - The process of producing pharmaceuticals using agriculture is nicknamed "pharming".
  - Goats, sheep, and cows are all being used for the production of medically useful products in milk.

- These products include blood clotting factors and antibodies for treating colon cancer.
- Crops that are resistant to herbicides:
  - Glyphosate, trade marked Roundup, is a broad spectrum herbicide.
  - It inhibits an enzyme system in chloroplasts that is involved in the synthesis of amino acids.
  - A bacterial gene is able to break down glyphosate.
  - This gene, when inserted into plants, confers resistance to glyphosate, making these transgenic resistant.
- Grains with improved nutritional characteristics:
  - Rice has no  $\beta$ -carotene, a molecule which is converted to vitamin A in animals.
  - Genes from bacteria and daffodil plants were transferred to rice using the vector *Agrobacterium tumefaciens*.
  - Now a genetically modified strain of rice produced  $\beta$ -carotene.
  - See Figure 17.16.

#### **D. There is public concern about biotechnology**

- Strains of *E. coli* used in the lab have mutations that are said to make their survival in human intestine impossible.
- Medical products made by DNA technology are widely accepted.
- There is currently resistance to the introduction of genetically modified plants. The complaints are:
  - Genetic manipulation is an unnatural interference with nature.
  - Genetically altered foods are unsafe to eat.
  - Genetically altered plants are dangerous to the environment.
- In terms of safety, each new plant undergoes stringent and rigorous safety review.
- Most scientists believe we should proceed, however with caution.

#### **E. DNA fingerprinting uses the polymerase chain reaction.**

- Everyone, except identical twins, are genetically distinct. (See Figure 17.17)
- To develop a test that can find distinctions, scientists find DNA sequences that are highly polymorphic.
- Sequences called *VNTRs* (variable number of tandem repeats) are easily detectable, if they are between two restriction enzyme recognition sites.
- Different individuals have different numbers of repeats. Each gets two sequences of repeats, one from the mother and one from the father.
- Using PCR and gel electrophoresis, patterns for each individual can be determined. DNA from a single cell is sufficient to determine the DNA fingerprint. (See Figure 17.18)
- Tsar family members have been studied.
- The existence of an illegitimate child of Jefferson has been proven.
- California condors are tested to reduce inbreeding in breeding programs.
- Genetic disease are diagnosable prior to manifesting. New treatments are being developed based on genetic knowledge.