MOLECULAR CELL PHYSIOLOGY - Recombinant DNA Technology

I. What is recombinant DNA technology?
- A. A set of techniques used to combine genes from different sources and transfer those genes into an organism where they may be expressed
  - 1. A targeted and potentially permanent manipulation of organisms

II. Why is recombinant DNA technology important?
- A. It provides knowledge about how organisms "work"
- B. It offers the potential to make organisms "new and improved"
- C. It enables understanding and circumvention of disease
- D. It provides a new (and "natural") way of making commercial products

III. Technologies used to understand genes
- A. Biochemical purification and analysis of a protein based on function (old)
- B. Classical genetic analysis by mapping of genes defined by mutations (old)
- C. Recombinant DNA technology (new)
  - 1. Enabled sequencing of human genome (6×10^9 base pairs)

IV. From protein to gene
- A. Isolate protein on basis of molecular function
- B. Determine partial amino acid sequence of protein
- C. Synthesize oligonucleotides that correspond to amino acid sequence
- D. Use oligonucleotides as probes to screen genomic library
- E. Sequence isolated gene

V. From gene to protein
- A. Isolate genomic clone corresponding to desired trait (often a mutant or altered gene)
- B. Use genomic DNA to isolate cDNA for the mRNA coded by the gene
- C. Sequence the cDNA and then determine amino acid sequence
- D. Compare amino acid sequence to known similar proteins to learn about function
- E. Use expression vector to produce the encoded protein

VI. What is the basis of recombinant DNA technology?
- A. Variation occurs naturally
  - 1. In meiosis, crossing over (prophase I) and recombination (metaphase I)
- B. Bacterial genes can transfer from cell to cell
  - 1. Transformation - Incorporation of new DNA from stuff around the cell (Griffith)
  - 2. Transduction - Transfer of DNA into bacteria by virus (bacteriophage)
  - 3. Conjugation - Reproduction or "mating" of bacterial cells

VII. Example of how small amounts of recombinant DNA are “manufactured” through use of plasmids
- A. Plasmids carrying recombinant DNA can be used to infect bacteria such as *E. coli*
- B. These plasmids contain a small, circular piece of DNA
  - 1. Plasmid can replicate itself within a cell or simply carry DNA (vector)
  - 2. Plasmid usually consists of replication origin, section that encodes for selection (like antibiotic resistance), and the section of DNA (20 kb) to be cloned
- C. The plasmid can be modified by inserting a gene of interest into it
  - 1. Once modified, the plasmid is said to carry the recombinant DNA
    - a) Plasmids are modified by "cutting" and "pasting" pieces of DNA
      - 1) Restriction enzymes (endonucleases) cut old DNA at special sequences
      - 2) Polylinkers, with several restriction sites, can be inserted into plasmid to help make “sticky ends”
      - 3) New DNA with "sticky ends" (complementary DNA) "fits in"
      - 4) DNA ligase pastes the new DNA into the old DNA to make recombinant DNA in the plasmid
- D. Once the recombinant plasmid is made, it can be taken up by transformation into a cell
- E. The cell then integrates the DNA, replicates it, and continues to make copies of the recombinant DNA or protein products

MOLECULAR CELL PHYSIOLOGY - Recombinant DNA Technology (continued)

VIII. Larger amounts of recombinant DNA are "manufactured" through use of other techniques

- **A. λ phage: cut up genomic DNA (25 kb pieces) and allow modified λ phage to take up pieces; phage then infects *E. coli;* this colonies which, essentially, constitute the genomic library**
- **B. Cosmids (45 kb pieces): generate pieces using λ phage and then insert, under special conditions into a plasmid**
- **C. P1 phage (100 kb pieces): takes advantage of the *E. coli* virus, bacteriophage P1, whose head can accommodate larger DNA molecules than the λ phage**
- **D. Bacterial artificial chromosome (300 kb pieces): make use of a large *E. coli* plasmid called the F-factor**
- **E. Yeast artificial chromosome (1000 kb pieces): in essence, eucaryotic cells are bigger**

IX. How is a gene of interest located?

- **A. The genomic "library" (two techniques)**
  1. **Start with DNA and cut it up into pieces by restriction enzymes**
  2. **Use reverse transcriptase to transform mRNA into DNA - then cut into pieces**
     - **a) Very useful for finding only the DNA that is expressed in a certain cell also referred to as ccomplementary "cDNA"**
- **B. Screen the library with a radioactive probe from another source**
  1. **Denature genomic DNA to make single-stranded DNA and dry on to a membrane such as nitrocellulose - this leaves most base pairs available for hybridization**
     - **a) Can use petri dishes containing recombinant λ virion present in plaques on A lawn of *E. coli* – place a piece of nitrocellulose on dish to allow some plaques to absorb**
     - **b) Store original petri dishes (marked for orientation) in refrigerator**
     - **c) Incubate nitrocellulose membrane in alkaline to disrupt virions and release encapsulated DNA, then dry filter**
  2. **Incubate the nitrocellulose with a (radioactive) probe containing unique DNA (or RNA) that encodes for the gene of interest – at least 20 nucleotides in length**
     - **a) Sometimes many probes are needed because the amino acid sequence in question can be encoded by numerous nucleotide sequences (this is called a degenerate "probe")**
     - **b) A database (expressed sequence tag) is available that contains specific DNA sequences in other organisms that correspond to amino acid sequences**
  3. **The probe adheres to one or more pieces of genomic DNA that probably contains the gene of interest**
  4. **Go back to the original DNA source (petri dishes) to get more purification, sequencing, and further analysis**
  5. **Obtain DNA from vector using restriction enzymes and isolate / purify with electrophoresis (polyacrylamide for up to 2000 bp; agarose for 500 to 20,000 bp; pulse-field electrophoresis for 20,000 to 10,000,000 bp)**
  6. **Visualize using ethidium bromide or radioactivity**
  7. **Determine DNA sequence by Maxam-Gilbert (cut and incubate with G, A+G, C+T, C) or Sanger (dideoxynucldeotides used) method – both followed by electrophoresis**

X. Analyzing specific nucleic acids

- **A. Southern blotting (named after Edwin Southern)**
  1. **Digest DNA with restriction enzyme and then use gel electophoresis to separate**
  2. **Denature with alkali and then transfer to nitrocellulose filter**
  3. **Incubate filter with specific radiolabeled DNA probe**
- **B. Northern blotting (named as such to make it different from Southern blotting)**
  1. **Denature RNA to make an unfolded, linear molecule**
  2. **Use gel electrophoresis to separate and transfer to nitrocellulose filter**
  3. **Incubate filter with radiolabeled DNA probe (quantitative method for RNAs)**
     - **a) Can also use endonucleases that act only on single-stranded polynucleotides for quantifying specific RNAs**

MOLECULAR CELL PHYSIOLOGY - Recombinant DNA Technology (continued)

XI. "Mass" producing protein from cloned DNA
- **A. Plasmid expression vectors carrying a strong, regulated promoter**
- **B. Plasmid expression vectors carrying the T7 late promoter (T7 RNA polymerase of bacteriophage T7)**
- **C. Because eukaryotes may encode post-translational modifications, eukaryotic expression vectors are sometimes needed**
- **D. Examples**
  1. **Vaccines**
  2. **Insulin**
  3. **Granulocyte colony-stimulating factor (stimulates production of phagocytic white blood cells)**
  4. **Human growth hormone**
  5. **Factor VIII for blood clotting (hemophiliacs need it)**

XII. Polymerase chain reaction
- **A. Directly amplifies specific DNA sequences in a complex mixture where the ends of the sequence are known**
  1. **Synthetic oligonucleoides complementary to desired DNA are added in excess to total denatured DNA and temperature is lowered**
  2. **Increase temperature (along with addition of *Thermus aquaticus* DNA polymerase) to 95 °C to melt strands and then cool to 60 °C to anneal primers**
  3. **Proceed with heating and cooling cycles to continue doubling desired DNA**
- **B. Applications of polymerase chain reaction**
  1. **Allows recovery and rapid amplification of DNA**
     - **a) Newly synthesized DNA can then be used for analytical purposes**
     - **b) Newly synthesized DNA improves specificity of probes and screening of DNA libraries**

XIII. Are there problems or risks of recombinant DNA technology?
- **A. Special labs and precautions are used to prevent harmful organisms from getting into the environment**
- **B. There are bioethics committees that oversee what is being done with recombinant DNA**
- **C. Use of information derived from DNA analysis and gene mapping can be debated (insurance company policies, for example)**