OVERVIEW OF LABORATORY MOLECULAR METHODS

Prof. Arjumand S. Warsy
Department of Biochemistry
College of Science, King Saud University, Riyadh

Introduction

Recombinant DNA technology has had a profound effect on medical genetics and on many other branches of medicine, as well as in animal and plant breeding and diagnostic microbiology.

Principles of Recombinant DNA Technology

- Recombinant DNA technology can be split into two main areas. These are:
  - A. DNA Cloning
  - B. Methods of DNA analysis

A. DNA Cloning is an in vitro technique used to produce multiple identical copies of the DNA fragment (i.e. clones). Thus the genes or DNA fragments of interest are amplified.

Essential step in the process of DNA cloning:

(i) Generation of DNA fragments.
- From genomic DNA using restriction endonucleases. DNA can be extracted from any nucleated tissue and the lymphocytes from 20 ml venous blood ~ 600g DNA can be obtained. Other sources of DNA are:
  - . Cultured skin fibroblast.
  - . Amniotic fluid
  - . Chorionic villus, etc.
- From mRNA using reverse transcriptase.
- Synthesis of DNA fragments, using automated DNA synthesisers.

Restriction Endonucleases

Restriction endonucleases (RE) are enzymes produced by different bacterial cells, that do not cleave the host DNA, but cleave other DNA molecules at a specific restriction site. Each RE recognizes a specific sequence of 4-6 nucleotides in length, that is usually palindromic. Over 300 RE have been isolated and the nomenclature depends on the organism from which they are derived e.g. Bam HI from Bacillus amyloliquefaciens H, ECoR1 from E coli etc. They may produce blunt or staggered end in the DNA.

- G AATTC - ECoR1
- CTTAA G

CCC GGG - Sma I
GGG CCC

Digestion of DNA from any source by the RE always produces the same ends in the DNA fragments.

(ii) Recombination of DNA fragments into a cloning vector:

Cloning vectors may be:

- Plasmids (bacterial, cytoplasmic, double stranded DNA e.g. pSC 101, pBR322 and can take insert upto 10 kb).
- Bacteriophages (virus which infect bacteria e.g. lambda (λ) phage and can take inserts upto 20 kb).
- Cosmids (a larger plasmid and can take insert upto 50 kb).
- Yeast artificial chromosomes (YAC) are plasmids with DNA sequences necessary for accurate replication within yeast. Can take upto 1000 kb insert.

The DNA fragment for cloning and the vector are cut by the same RE and by the help of a ligase reaction the two are joined together to form a 'Chimeric DNA' i.e. a recombinant DNA.

(iii) Transformation of the host organism
The recombinant vector is introduced into the host cell, by making cell membrane permeable by various methods e.g. exposing to calcium salts. Thus the host cell is said to be 'transformed'.

(iv) Cloning and screening for recombinant vector.
The transformed host cells are grown in culture medium to produce multiple identical copies of the various individual recombinant DNA i.e. clones.

Formation of DNA Libraries

Genomic DNA fragment or cDNA can be used to make "recombinant DNA libraries". DNA libraries using plasmids consists of several hundred thousand clones to be large enough to contain the whole human genome. YAC libraries are more useful as 13-14000 clones may contain the whole library.

- Screening is carried out by growing the host cells in special media, such as antibiotic sensitive colonies.
- Other screening methods have been used to detect the presence of clones with specific DNA sequence insert such as "nucleic acid hybridization".
- Such DNA sequences which are complementary to specific DNA sequences on the genome or cDNA are called "DNA probes".

Probes may be genomic DNA sequences, specific genes, cDNA sequences and synthetically prepared DNA sequences. These probes may be radio-labelled and detected by autoradiography. Fluorescently labelled DNA probes are non-radioactive, but less sensitive than the radio-labelled probes.

The polymerase chain reaction (PCR)

- The PCR is one of the most revolutionary developments in recombinant DNA technology.

- It is a method to amplify genes or DNA fragments to several thousand times by a simple technique involving four main steps per cycle i.e. denaturation primer matching, primer extension and denaturation to start the cycle again. (The method will be discussed in detail in a later lecture).

B. Methods for DNA analysis

Once the DNA is obtained in sufficient quantity several techniques are now available to analyze it. These include:

(i) Southern blotting
(ii) Restriction mapping
(iii) DNA sequencing
(iv) Special techniques for mutation detection

(a) Denaturing gradient gel electrophoresis (DGGE)
(b) Single stranded conformation polymorphism.
(c) Dot blot analysis
(d) Amplification mutation refractory system (ARMS)
(e) Reverse dot blot
(f) Cold SSCP
(g) Chemical cleavage mismatch.

(i) Southern Blotting

- Developed in mid 1970
- Standard means of analyzing structure of DNA cleaved by RE.
- The technique involves:
  (a) Obtaining DNA from an accessible source.
  (b) Treatment with RE under specific conditions of temperature and P^H.
  (c) Separation of the DNA fragments, generated by RE treatment on electrophoresis. Mobility of fragments is inversely proportional to size.
  (d) Denaturation of DNA in the gel by alkali treatment.
  (e) Transferring to a nitrocellulose sheet or hybond N by Southern blotting.

(f) Treatment with ^{32}P radiolabelled probe to localize the DNA fragment of interest on the Southern blot. The probes may be oligonucleotide probes, referred to as allele specific oligonucleotide (ASO), which hybridize to specific alleles of the gene e.g. the normal or the mutated gene.

(g) Visualization by autoradiography.

(ii) Restriction Mapping

By using different REs alone and in combination, allows the construction of restriction maps of a DNA region.

(iii) DNA Sequencing

Once DNA has been amplified or cloned and is available in sufficient quantity, then it is possible to sequence it i.e. determine the nucleotide sequence. Two methods are available and widely used:

- Sangers dideoxy method
- Chemical method

The dideoxy chain termination method is the most commonly used method for DNA sequencing. It involves:

- Making single stranded DNA template of the DNA to be sequenced (denaturation).
- An aliquote of this template is added to 4 different reaction mixture, which includes DNA polymerase and all 4 deoxynucleotides (one in each reaction is radioactivity labelled) and short primers.
- The dideoxynucleotide (ddATP, ddCTP, ddGTP and dd TTP) is added, one in each reaction to compete with the deoxynucleotide. This inhibits the DNA polymerase resulting in the production of DNA fragment of different lengths which terminate in their respective dideoxynucleotide.
- The reaction products of all 4 experiments are obtained and run next to each other in a gel. A ladder of deoxynucleoides is produced and direct reading on the gel gives the DNA sequence. (The mobility is inversely proportional to the size of DNA fragment).
- The DNA sequence complementary to the single stranded DNA template can be read directly from an autoradiograph of the gel.

(iv) Special method for detecting mutations.

Several methods have been developed and are so sensitive as to detect single base changes in the DNA. In addition the new techniques can be run in a short period of time and require a small amount of DNA. These

methods are very useful in prenatal diagnosis, carrier detection study of disease pathogenesis etc.
These methods are discussed separately in the lectures that follow.