DNA modification, gene cloning and rDNA technology

Deependra Dhakal

GAASC, Baitadi

Tribhuwan University

Academic year 2019-2020

Section 1

Genetic Structure

History

- 1972, two researchers (Herbert W. Boyer, UCSD and Stanley N. Cohen, Stanford) met at a conference in Hawaii to discuss *plasmids*.
- One studied Plasmids
- Other studied restriction and modification enzymes (EcoRI)
- After talks, ideas combined.

Structure of DNA

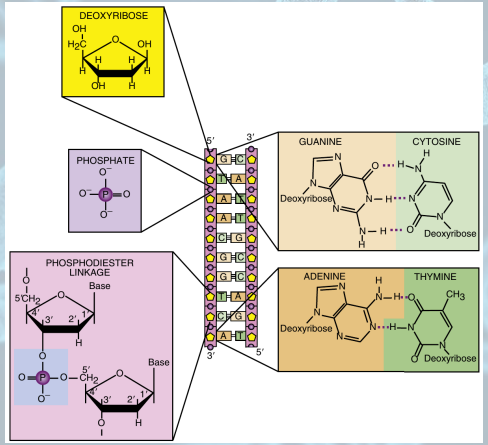


Figure 1: DNA has two strands antiparallel to each other. The structure of the subcomponents is shown to the sides.

Structure of RNA

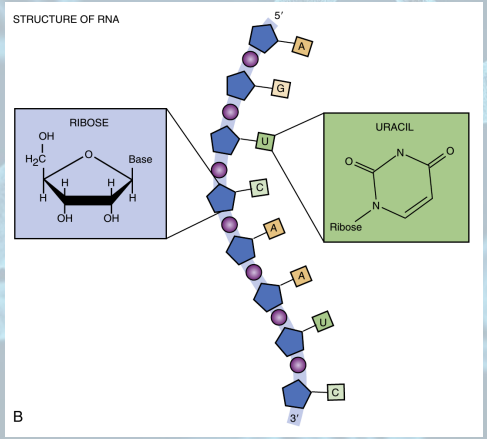


Figure 2: RNA is usually single-stranded and has two chemical differences from DNA. First, an extra hydroxyl group (-OH) is found at the 2 prime position of ribose, and second, thymine is replaced by uracil.

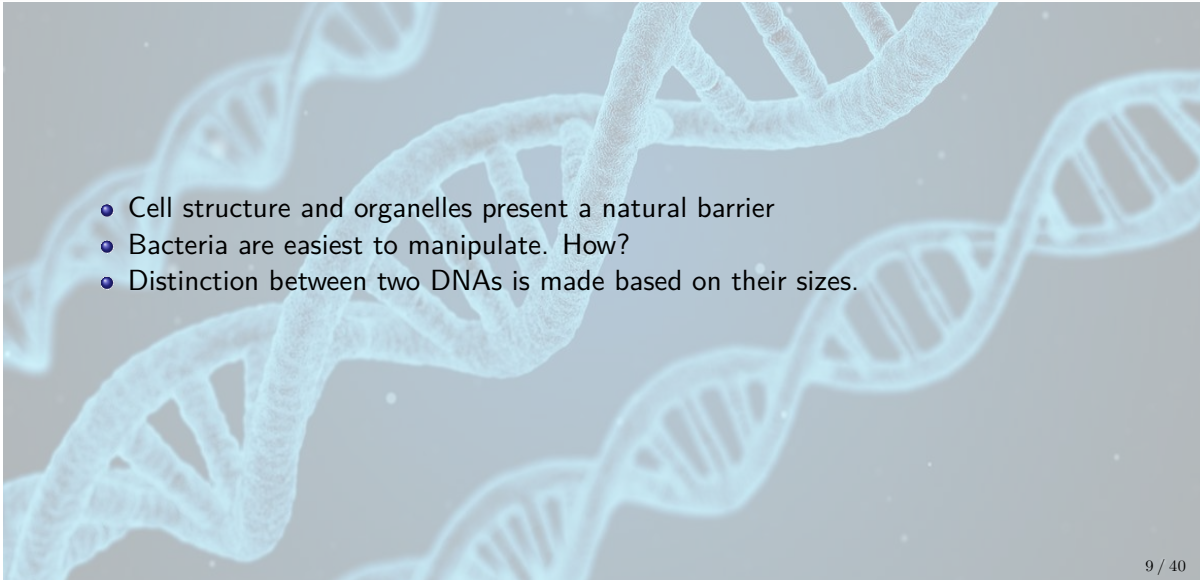
DNA modification: simplified

- Identification and isolation of the desired gene/DNA fragment to be cloned from different animals, other bacteria, and viruses. Restriction endonucleases are used for producing desired fragments of a given genome.
- Insertion of the gene/DNA segment in a vector; plasmid, virus, cosmid, etc.
- Introduction of the recombinant vector into a suitable host (transformation).
- Integration of the desired gene into the nuclear/organeller genome of the host cell.
- Selection of the transformed host cells.
- Expression of the cloned genes.
- Regeneration of the cloned genes.
- Transmission to the progenies.

Section 2

DNA isolation and purification

Overview



- Cell structure and organelles present a natural barrier
- Bacteria are easiest to manipulate. How?
- Distinction between two DNAs is made based on their sizes.

Isolation steps

1. Destruction of cell membrane. (Lysozyme digestion of peptidoglycan in bacteria)
2. Bursting of cell membrane by destruction of lipid bilayer (by detergent such as sodium dodecyl sulfate (SDS)). In plants and animals, tissue samples are generally ground up to release the intracellular components.
3. Separation of intracellular components from the insoluble remains (cellular membranes, bones, cartilage, etc.) by either centrifugation or chemical extraction.
4. Extraction of unwanted proteins from DNA with phenol (Dissolves 60%-70% of all living matter).

Purification procedure

- The two phases (one that of proteins dissolved in phenol and the other of nucleic acids in the aqueous layer) are separated by centrifugation, and the aqueous DNA layer is removed form the phenol
- Use enzyme ribonuclease (RNase) to digests RNA into small ribonucleotide fragments
- Treat this solution with alcohol to precipitate large DNA out of the aqueous phase, while ribonucleotides stay soluble, thus favoring centrifugation extraction of DNA

Section 3

DNA fragment separation using Electrophoresis

Initial preparation

- Gel electrophoresis is used to separate DNA fragments by size
- Gel consists of agarose, a polysaccharide extracted from seaweed that behaves like gelatin (commercial powder).
- For visualizing DNA agarose gel is solidified, after subsequent cooling once the powder-water mixture is heated, into a rectangular slab about 1/4 inch thick by casting the molten liquid into a special tray.
- To make small wells or holes at one end of the gel, comb is inserted before the gel hardens.

Process

Gel electrophoresis uses electric current to separate DNA molecules by size. The agarose slab is immersed in a buffer-filled tank that has a positive electrode at one end and a negative electrode at the other. DNA samples are loaded into the wells, and when an electrical field is applied, the DNA migrates through the gel. The phosphate backbone of DNA is negatively charged, so it moves away from the negative electrode and toward the positive electrode. Polymerized agarose acts as a sieve with small holes between the tangled chains of agarose. The DNA must migrate through these gaps. Agarose separates the DNA by size because larger pieces of DNA are slowed down more by the agarose.

Visualization

- The agarose gel is removed from the tank and immersed into a solution of ethidium bromide (mutagenic).
- This dye intercalates between the bases of DNA or RNA, although less dye binds to RNA because it is single-stranded. When the gel is exposed to ultraviolet light, it fluoresces bright orange.
- In Figure 3, the DNA fragments are visualized by a positively charged dye from the thiazin family. The dye interacts with the negatively charged backbone of the DNA and is a nontoxic alternative that does not require ultraviolet light sources.

Notes

- Size of DNA being examined affects what type of gel is used.
- Size of DNA molecules can be determined by comparing to a set of molecular weight standards run in a different well.
- Because the standards are of known size, the experimental DNA fragment can be compared directly.
- Resolvability of some gel matrix is different than other.

Fragments of DNA are separated by size using gel electrophoresis. A current causes the DNA fragments to move away from the negative electrode and toward the positive. As the DNA travels through agarose, the larger fragments get stuck in the gel pores more than the smaller DNA fragments. Pulsed field gel electrophoresis separates large pieces of DNA by alternating the electric current at right angles.

Electrophoresis apparatus and visualization

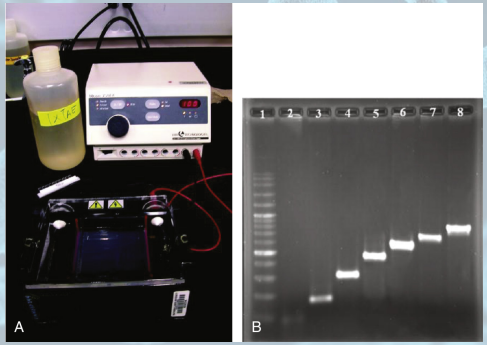


Figure 3: (A) Photo of electrophoresis supplies. Electrophoresis chamber holds an agarose gel in the center portion, and the rest of the tank is filled with buffer solution. The red and black leads are then attached to an electrical source. FisherBiotech Horizontal Electrophoresis Systems, Midigel System; Standard; 13 × 16-cm gel size; 800 mL buffer volume; Model No. FB-SB-1316. (B) Agarose gel separation of DNA. The size of the fragments can be calculated by comparing them to the standard DNA marker in lane 1. The brighter bands in the marker are 1000 base pairs and 500 base pairs, with the 1000 base-pair marker closer to the wells (marked with numbers 1–8).

Section 4

DNA modification

DNA cutters: Restriction enzymes

- DNA should be cut to fragments of different sizes because entire set of nuclear DNA is not of particular interest in visualization
- Naturally occuring *restriction enzymes* or *restriction endonucleases* come into rescue in this operation.
- These bacterial enzymes bind to specific recognition sites on DNA and cut the backbone of both strands.
- These enzymes are methylation sensitive.
- Bacteria produce *modification enzymes* that recognize the same sequence as the corresponding restriction enzyme. These methylate each recognition site in the bacterial genome.

DNA cutters: Restriction enzymes

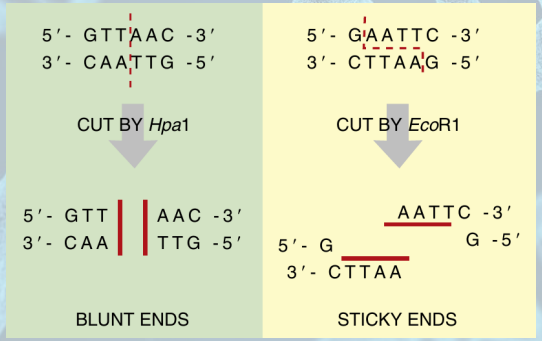


Figure 4: **Type II Restriction Enzymes – Blunt versus Sticky ends**
HpaI is a bunt-end restriction enzyme; that is, it cuts both strands of DNA in exactly the same position. extitEcoRI is a sticky-end restriction enzyme. The enzyme cuts between the G and A on both strands, which generates four base-pair overhangs on the ends of the DNA. Since these ends may base pair with complementary sequences, they are considered 'sticky'.

DNA cutters: Restriction enzymes

- Restriction enzymes have been exploited to cut DNA at specific sites, since each restriction enzyme has a particular recognition sequence. Difference in cleavage site determine the type of restriction enzyme.
  - Type I restriction enzymes cut the DNA strand 1000 or more base pairs from the recognition sequence.
  - Type II restriction enzymes cut in the middle of the recognition sequence and are the most useful in genetic engineering. The Type II restriction enzymes can form *both* sticky or *blunt* ends.
- The recognition sequences of Type II restriction enzymes are usually inverted repeats so that the enzymes cut between the same bases on the both strands.

DNA cutters: Restriction enzymes

Enzyme	Source Organism	Recognition Sequence
*Hpa*II	*Haemophilus parainfluenzae*	C/CGG GGC/C
*Mbo*I	*Moraxella bovis*	/GATC GATC/
*Nde*II	*Neisseria denitrificans*	/GATC GATC/
*Eco*RI	*Escherichia coli* RY13	G/AATTC CTTAA/G
*Eco*RII	*Escherichia coli* RY13	/CCWGG GGWCC/
*Eco*RV	*Escherichia coli* J62/pGL74	GAT/ATC CTA/TAG
*Bam*HI	*Bacillus amyloliquefaciens*	G/GATCC CCTAG/G
*Sau*I	*Staphylococcus aureus*	CC/TNAGG GGANT/CC
*Bgl*I	*Bacillus globigii*	GCCNNNN/NGGC CGGN/NNNNCCG
*Not*I	*Nocardia otitidis-caviarum*	GC/GGCCGC CGCCGG/CG
*Dra*II	*Deinococcus radiophilus*	RG/GNCCY YCCNG/GR

/, position where enzyme cuts.
N, any base; R, any purine; Y, any pyrimidine; W, A, or T.

Figure 5: Common restriction enzymes

DNA cutters: Restriction enzymes

- The number of base pairs in the recognition sequence determines the likelihood of cutting.
- So to generate fewer, longer fragments, restriction enzymes with six or more base-pair recognition sequences are used.

DNA joiners: Ligase – Overview

- When two different DNA samples are cut with the same sticky-end restriction enzyme, all fragments will have identical overhangs.
- This allows DNA fragments from two sources (e.g., two different organisms) to be linked together (Figure 6).
- Fragments are linked or ligated using DNA ligase
- The efficiency of ligases depends on the total concentration of the substrates and also on the closeness of the ends to be ligated.

DNA joiners: Ligase – Types

- There are 3 kinds of mammalian DNA ligases.
- Joining of Okazaki fragments and sealing of excision repair nicks, repair of double-strand breaks and nonhomologous end joining, joining of recombining plasmid vectors.

DNA joiners: Ligase

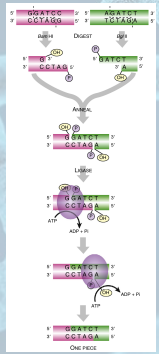


Figure 6: **Compatible overhangs are linked using DNA Ligase**
BamHI and Bgl II generate the same overhanging or sticky ends: a 3′-CTAG-5′ overhang plus a 5′-GATC-3′ overhang. These are complementary and base pair by hydrogen bonding. The breaks in the DNA backbones are sealed by T4 DNA ligase, which hydrolyzes ATP to energize the reaction.

Section 5

DNA detection

Background

- In 1969, Mary Lou Pardue and Joseph Gall developed a procedure by which they could hybridize radioactive single strands of DNA with complementary strands of DNA in chromosomes on glass slides.
- This procedure, called in situ hybridization, Pardue and Gall were able to determine the chromosomal locations of repetitive DNA sequences.
- Classical in situ hybridization involved spreading mitotic chromosomes on a glass slide, denaturing the DNA in the chromosomes by exposure to alkali (0.07 N NaOH) for a few minutes, rinsing with buffer to remove the alkaline solution, incubating the slide in hybridization solution containing radioactive copies of the nucleotide sequence of interest, washing off the radioactive strands that have not hybridized with complementary sequences in the chromosomes, exposing the slide to a photographic emulsion that is sensitive to low-energy radioactivity, developing the autoradiograph, and superimposing the autoradiograph on a photograph of the chromosomes.

In situ hybridization

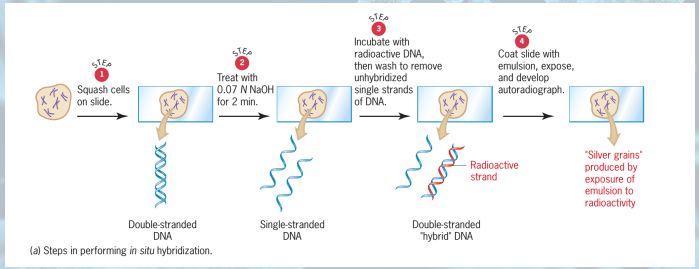


Figure 7: Steps in in-situ hybridization

Fluorescent In Sity Hybridization

- A repetitive DNA sequence can be identified as satellite DNA (generally non-transcribing) only if the sequence has a base composition sufficiently different from that of main-band DNA to produce a distinct band during density-gradient centrifugation. Therefore, centrifugation cannot be used to identify all repetitive DNA sequences.
- Today, in situ hybridization experiments are often done by using hybridization probes that are linked to fluorescent dyes or antibodies tagged with fluorescent compounds 8.

Fluorescent In Sity Hybridization

- In one protocol, DNA or RNA hybridization probes are linked to the vitamin biotin, which is bound with high affinity by the egg protein avidin 8.
- By using avidin covalently linked to a fluorescent dye, the chromosomal location of the hybridized probe can be detected by the fluorescence of the dye.
- This procedure, called FISH (Fluorescent In Situ Hybridization), has been used to demonstrate the presence of the repetitive sequence TTAGGG in the telomeres of human chromosomes 9.
- The FISH procedure is very sensitive and can be used to detect the locations of single-copy sequences in human mitotic and interphase chromosomes.

FISH: Procedure

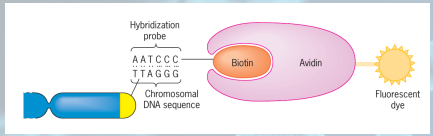


Figure 8: Localization of repeated DNA sequences in chromosomes by in situ hybridization performed with fluorescent probes

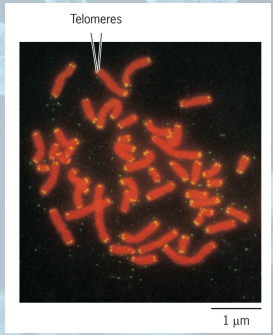


Figure 9: The use of fluorescent dyes to localize the TTAGGG repeat sequence to the telomeres of human chromosomes and a photomicrograph demonstrating its telomeric location is shown in.

Section 6

PCR

Background

- An in vitro technique that enables DNA fragments to be copied in a process that is referred to as *amplification*.
- Usefulness is because sufficient DNA can be generated to characterize and analyze it.
  - e.g.: tracking disease processes by characterizing genetic fingerprints of pathogenic organisms
- The polymerase chain reaction makes copies of dissociated DNA or single - stranded copies of mRNA.

PCR steps

- Denaturing of the target sequence so that the polynucleotide is in a single stranded, unwound form. For DNA, this requires a temperature of $94 - 96^{\circ}$ C for 5 min.
- Hybridization so that the primers bind to complementary bases that flank the regions on either side of the polynucleotide of interest. This step requires about 30 s and is carried out at $30 - 65^{\circ}$.
- Polymerization (i.e., polynucleotide or DNA synthesis) by DNA polymerase. The enzyme reads the template strand between the flanking primers, and matches the bases with complementary ribonucleotides, which are then incorporated into the primer strand. This is carried out at $72 - 80^{\circ}$C for several minutes at pH $= 8.4$ in Tris buffer containing ribonucleotides representing all 4 bases (A, G, C, T).

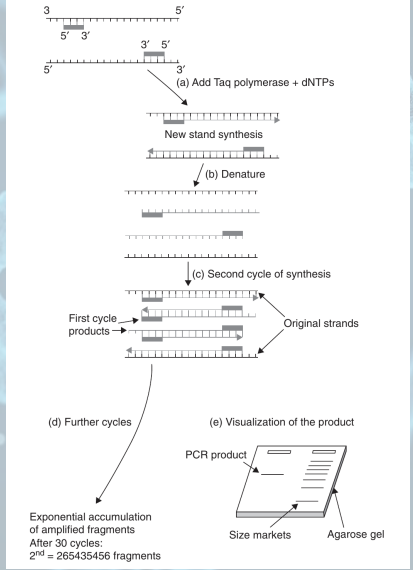


Figure 10: Graphical representation of amplifying a target DNA sequence through the polymerase chain reaction (PCR) [reproduced from Brown (2006), Fig. 9.2]

PCR ingredient: Thermally tolerant DNA polymerase

- Polymerase from the thermophilic bacterium *Thermus aquaticus* resolved issues of specificity and speed.
- *T. aquaticus* (abbreviated *Taq*) is found in hotsprings and grows at 75°C. Its enzyme has an optimum temperature of 72°C and is sufficiently stable at 96°C so that fresh Taq enzyme does not need to be added after each cycle.
- Taq polymerase allows primer annealing and extension to be carried out at temperatures significantly higher than those for E. coli polymerase. This reduces imperfect annealing and subsequent amplification of an imperfect (nonspecific) product.
- This enabled researchers to design and build automated PCR machines, and thereby accelerated the rapid application of this technology.

PCR: Classic protocol

Watson et al. (1992, p. 8) state:

The standard PCR is typically done in a 50 - or 100 μl volume and, in addition to the sample DNA, contains 50 mM KCl, 10 mM Tris/HCl (pH 8.4 at room temp.), 1.5 mM MgCl 2, 100 μg/ml gelatin, 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.5 units of Taq polymerase. The type of the DNA sample will be variable but it will usually have between 10^2 to 10^5 copies of the template (e.g., 0.1 μg DNA). A few drops of mineral oil are often added to seal the reaction and prevent condensation ... The amplification can be conveniently performed in a DNA Thermal Cycler (Perkin -Elmer Instruments) using the "Step-Cycle" program set to denature at 94°C for 20 sec, anneal at 55°C for 20 sec, and extend at 72°C for 30 sec for a total of 30 cycles. (The "Step-Cycle" program causes the instrument to heat and cool to the target temperatures as quickly as possible. In the current instrument, this results in a heating rate of about 0.3°C per sec and a cooling rate of about 1°C per sec, for an overall single cycle time of approximately 3.75 min).

PCR: Detailed methodology

- A sequence of a small stretch of nucleotides on either side of the target must be known, these flanking sequences are then used to specify and synthesize single-stranded oligonucleotide fragments, or primers (~20 bp).
- A mixture of the template sequence (primers), Taq polymerase, triphosphorylated deoxyribonucleotides, and buffer then doubles the amount of DNA each cycle.
- The amount of DNA is doubled during each cycle, with n cycles producing 2^n as present to begin with.
- Rate of misincorporation: 1 base out of 10,000 - 1 base out of 200,000 per PCR cycle.
- This limitation was overcome for sequencing long fragments of genomic DNA through use of in vivo DNA amplification using yeast or BACs.

Applications

- Detection of pathogenic microorganisms (*H. pylori*, tuberculosis, chlamydia, viral meningitis, viral hepatitis, AIDs, etc.)
- Disease epidemiology and forcasting.
- Crime detection and forensic application.
- Crop variety characterization by DNA fingerprinting.