Recombinant DNA Technology and Genomics

Introduction to Recombinant DNA Technology and DNA Cloning

Introduction

- 1970s gene cloning became a reality
  - **Clone** – a molecule, cell, or organism that was produced from another single entity
- Made possible by the discovery of
  - **Restriction Enzymes** – DNA cutting enzymes
  - **Plasmid DNA Vectors** – circular form of self-replicating DNA
    - Can be manipulated to carry and clone other pieces of DNA

Recombinant DNA Technology

- Human Genome -> 6 X 10^9 bp in 23 pairs of chromosomes
- Cleavage with restriction enzymes (produce about 1 cut for every 3000 bp) yields 2 million fragments
- 2 million fragments far too many to separate
- This obstacle of obtaining pure DNA from large genomes is overcome by **recombinant DNA technology**

Recombinant DNA Technology

- Preparation of large numbers of identical DNA molecules
- Definition of recombinant DNA
  - Production of a unique DNA molecule by joining together two or more DNA fragments not normally associated with each other
  - DNA fragments are usually derived from different biological sources

Common steps involved in isolating a particular DNA fragment from a complex mixture of DNA fragments or molecules

1. DNA molecules are digested with enzymes called restriction endonucleases which reduces the size of the fragments → Renders them more manageable for cloning purposes

2. These products of digestion are inserted into a DNA molecule called a vector → Enables desired fragment to be replicated in cell culture to very high levels in a given cell (copy #)

3. Introduction of recombinant DNA molecule into an appropriate host cell
   - Transformation or transfection
   - Each cell receiving rDNA = CLONE
   - May have thousands of copies of rDNA molecules/cell after DNA replication
   - As host cell divides, rDNA partitioned into daughter cells

4. Population of cells of a given clone is expanded, and therefore so is the rDNA.
   - Amplification
   - DNA can be extracted, purified and used for molecular analyses
     - Investigate organization of genes
     - Structure/function
     - Activation
     - Processing
   - Gene product encoded by that rDNA can be characterized or modified through mutational experiments

Vectors for Gene Cloning

Requirements of a vector to serve as a carrier molecule

- "vectors", because they can take DNA from one organism to the next
- The choice of a vector depends on the design of the experimental system and how the cloned gene will be screened or utilized subsequently

What Makes a Good Vector?

- Practical Features of DNA Cloning Vectors
  - Small Size
  - Origin of replication (ori)
  - Multiple cloning site (MCS)
  - Selectable marker genes
  - RNA polymerase promoter sequences
  - DNA sequencing primers

Vectors

- Vectors must be relatively small molecules for convenience of manipulation.
- They must be capable of prolific replication in a living cell, thereby enabling the amplification of the inserted donor fragment.
- Most vectors contain a prokaryotic origin of replication allowing maintenance in bacterial cells.

Vectors

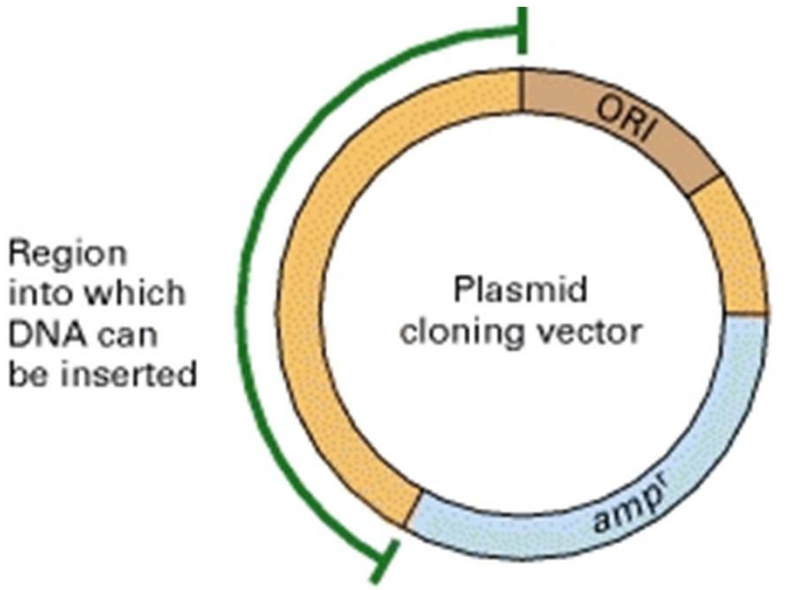
- Another important requirement is that there must be convenient restriction sites that can be used for insertion of the DNA to be cloned.
- Multiple unique cloning sites are often included for versatility and easier library construction.
- Antibiotic resistance genes and/or other selectable markers enable identification of cells that have acquired the vector construct.

Plasmids as Vectors

- Plasmids are circular, double-stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA.
- Occur naturally in bacteria, yeast, and some higher eukaryotic cells, exist in a parasitic or symbiotic relationship with their host cell.
- During cell division, at least one copy of the plasmid DNA is segregated to each daughter cell.
- Some bacterial plasmids encode enzymes that inactivate antibiotics

Plasmids - ORI

- Since a plasmid is (by definition) an extrachromosomal element, it cannot make use of any origin of DNA replication in a chromosome.
- That is, DNA synthesis within (i.e. copying of) a plasmid depends on its having an origin of DNA synthesis of its own.



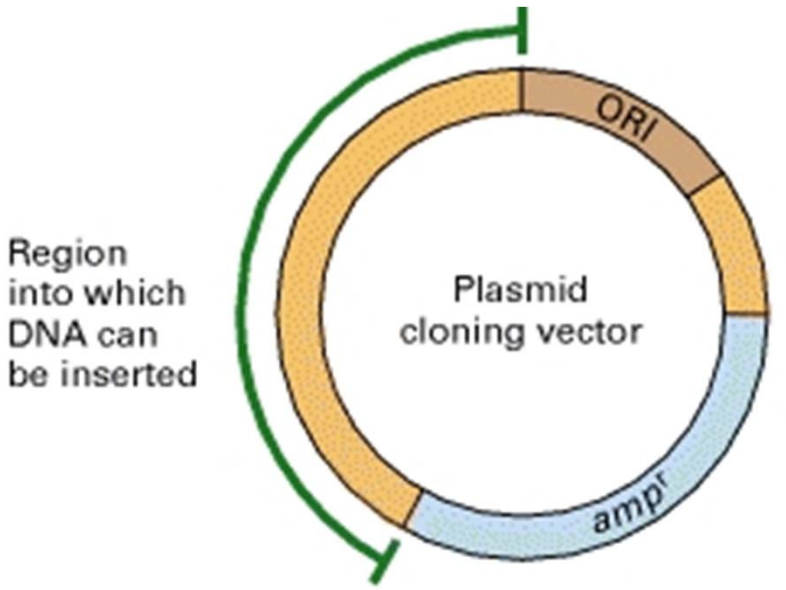
selectable marker

- A selectable marker is not actually a required element of a plasmid, but it makes it possible for us to maintain stocks of cells that contain the plasmid uniformly.
- Sometimes, carrying a plasmid puts a cell at a *selective disadvantage* compared to its plasmid-free neighbors, so the cells with plasmids grow more slowly.

- Cells that happen to "kick out" their plasmid during division may be "rewarded" by having a higher rate of growth, and so these plasmid-free (sometimes referred to as "cured") cells may take over a population.
- If a plasmid contains a gene that the cell needs to survive (for example, a gene encoding an enzyme that destroys an antibiotic), then cells that happen to kick out a plasmid are "punished" (by subsequent death) rather than "rewarded" (as in the previous scenario).

Multiple cloning sites

- A cloning site is a place where the DNA can be digested by specific restriction enzymes - a point of entry or analysis for genetic engineering work.



- For now, think of the following example: Suppose you are really thirsty and you buy a can of soda. Does it occur to you that one end of the can (the "top") is designed so that you can open it easily? If you bought a can of soda with two bottom ends and no top, you would have a hard time drinking it! It's the same way with plasmids. You can have a plasmid with lots of terrific features, but you might lack an easy way of "getting it open" with restriction enzymes

Approximate Maximum Length of DNA That Can Be Cloned in Vectors

Vector Type	Cloned DNA (kb)
Plasmid	20
λ phage	25
Cosmid	45
P1 phage	100
BAC (bacterial artificial chromosome)	300
YAC (yeast artificial chromosome)	1000

Restriction Enzymes

- Bacterial origin = enzymes that cleave foreign DNA
- Named after the organism from which they were derived
  - EcoRI from *Escherichia coli*
  - BamHI from *Bacillus amyloliquefaciens*
- Protect bacteria from bacteriophage infection
  - Restricts viral replication
- Bacterium protects it's own DNA by methylating those specific sequence motifs

Restriction Enzymes

- Recognition sequences are *palindromes*
  - A *palindrome* is a word, phrase, number or other sequence of units that can be read the same way in either direction
  - *GAATTC*
  - *Reverse Comp -> CTTAAG*
- Cohesive (sticky) ends – overhanging single-stranded ends
- Blunt ends – double-stranded, non-overhanging ends

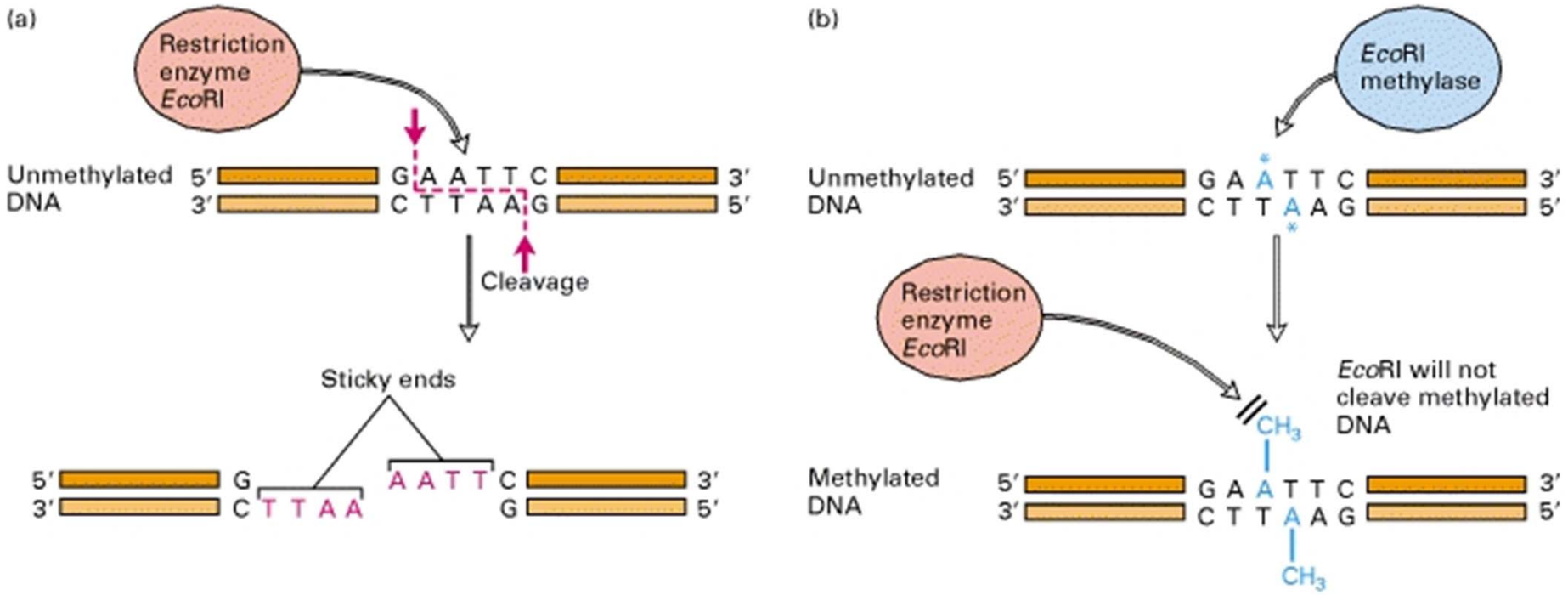
Classes

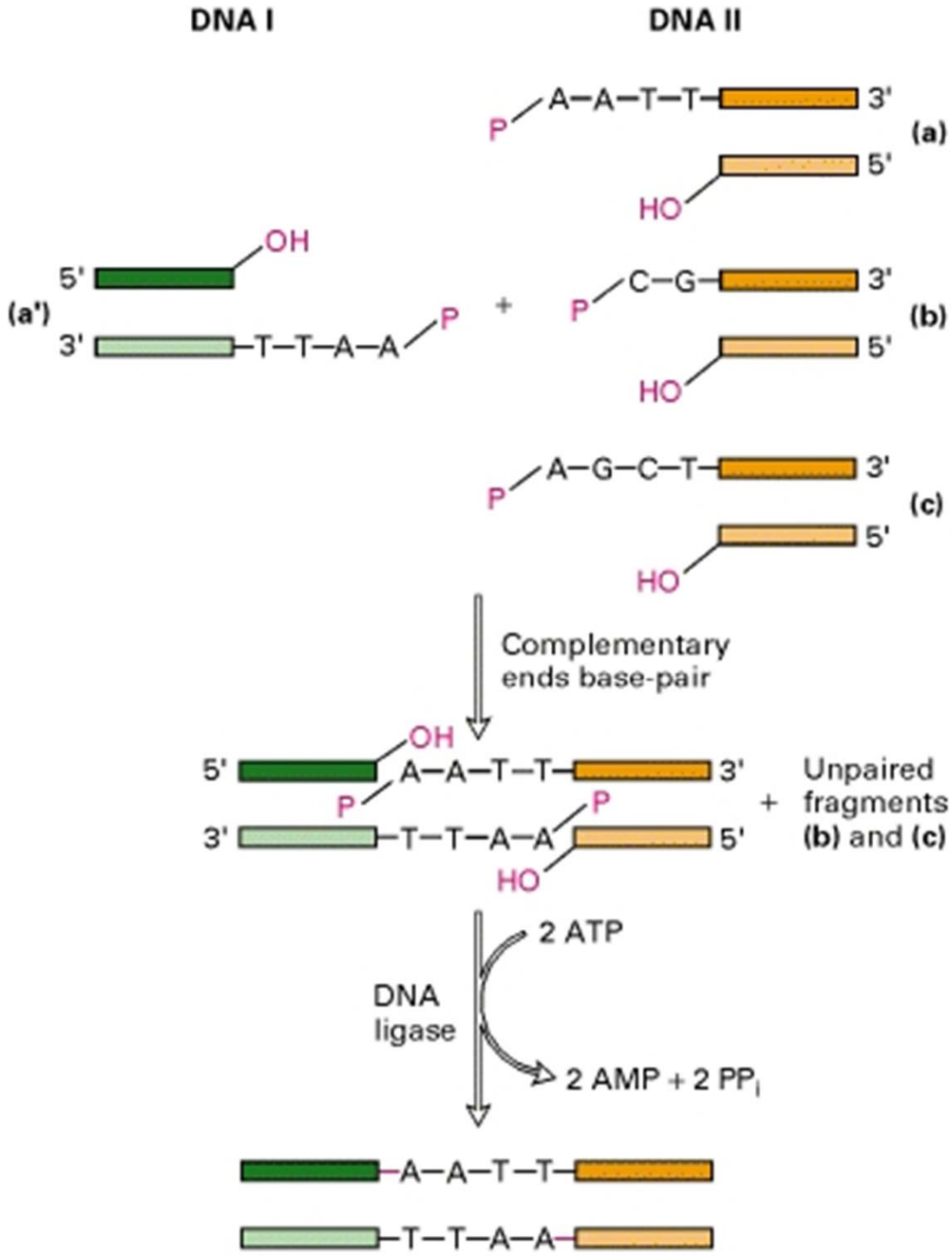
- Type I
  - Cuts the DNA on both strands but at a non-specific location at varying distances from the particular sequence that is recognized by the restriction enzyme
  - Therefore random/imprecise cuts
  - Not very useful for rDNA applications

- Type II
  - Cuts both strands of DNA within the particular sequence known as **restriction site** recognized by the restriction enzyme
  - Used widely for molecular biology procedures
  - DNA sequence = symmetrical

- Reads the same in the 5’→ 3’ direction on both strands = Palindromic Sequence
- Some enzymes generate “blunt ends” (cut in middle)
- Others generate “sticky ends” (staggered cuts)
  - H-bonding possible with complementary tails
  - DNA ligase covalently links the two fragments together by forming phosphodiester bonds of the phosphate-sugar backbones

Restriction-recognition sites are short DNA sequences recognized and cleaved by various restriction endonucleases





Restriction Fragments with Complementary "Sticky Ends" Are Ligated Easily

- Transformation of Bacterial Cells
  - A process for inserting foreign DNA into bacteria
    - Treat bacterial cells with ice-cold calcium chloride
    - Add plasmid DNA to cells chilled on ice
    - Heat the cell and DNA mixture to 42^{o}C
    - Membrane becomes fluid and plasmid DNA enters bacterial cells and is replicated and expressed

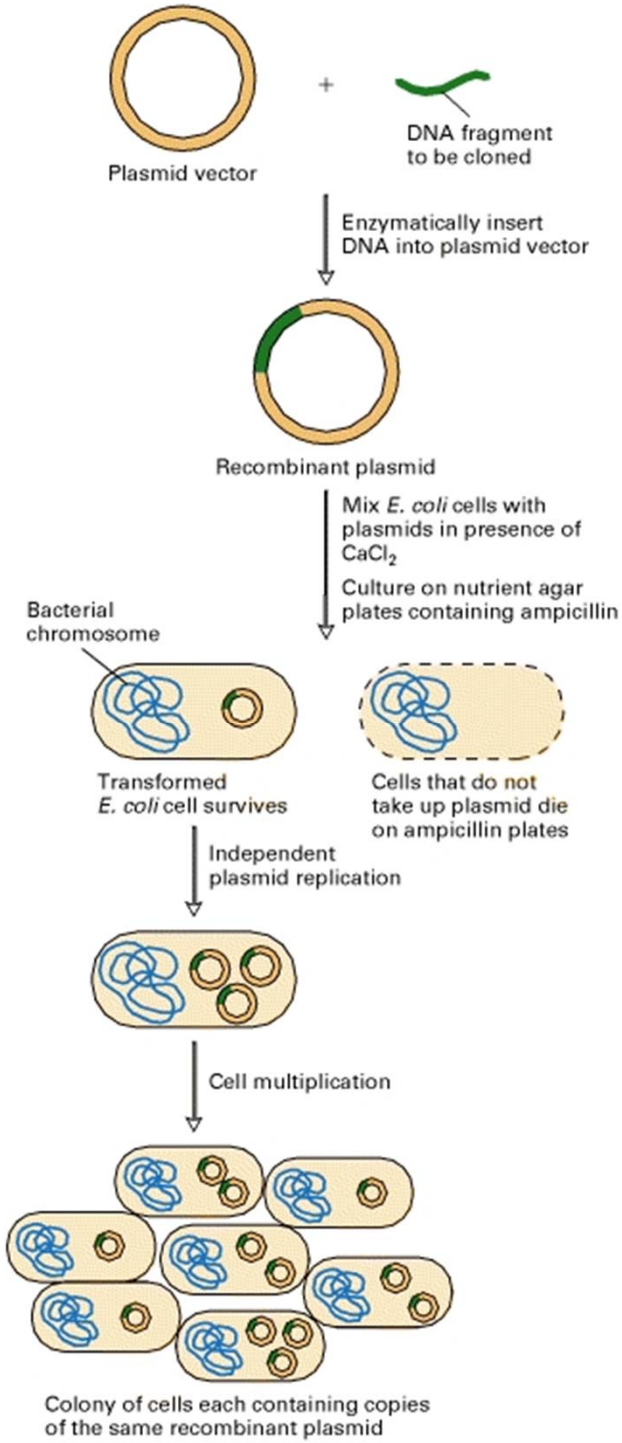
Electroporation

– More modern method of transformation is *electroporation* which delivers an electric shock to the cell, releasing a charged capacitor with a field strength in the sample of approximately 1200 volts per millimeter. The DNA is swept into the cells as the membranes are temporarily breached.

- After transformation, we challenge the bacteria with an antibiotic (such as ampicillin). If the E. coli have taken up and expressed an ampicillin resistance gene on a plasmid, they will live - otherwise they will die. This process is called selection, because we are selecting which bacteria may survive.

- Selection of Recombinant Bacteria
  - **Selection** is a process designed to facilitate the identification of recombinant bacteria while preventing the growth of non-transformed bacteria
    - Antibiotic selection
    - Blue-white selection

General procedure for cloning a DNA fragment in a plasmid vector.



Isolation of DNA fragments from a mixture by cloning in a plasmid vector