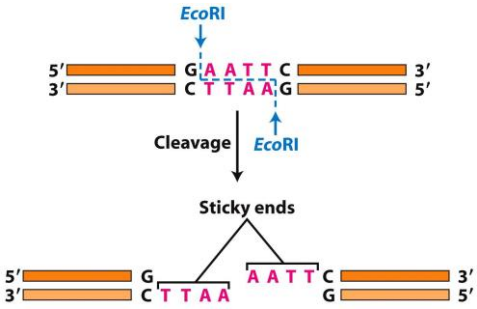
Restriction Enzymes

- Restriction endonucleases (RE) cut at specific sequences (restriction sites) within DNA molecules
  - Break phosphodiester bonds
  - Can have single stranded overhangs
  - Ends are cohesive (complementary, sticky)- they can re-anneal



Common Lab Procedure

- Complementary single stranded ends produced by restriction enzyme cleavage can be joined (ligated) together using DNA ligase to create recombinant DNA molecule

Nomenclature of Restriction Enzymes

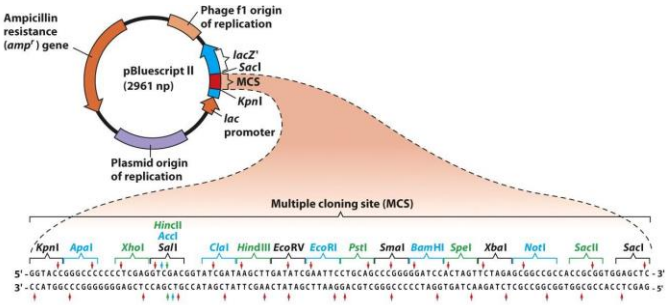
- RE named according to bacterial strain in which it was originally identified in
- RE generally recognize palindromic sequences and create either staggered (i.e. cohesive, sticky) or blunt ends
- Palindrome: sequence that reads the same in either direction (i.e. race car)
  - DNA is identical but inverted in complementary strand

Plasmid Cloning Vectors

- Cloning vectors: used for isolation and amplification of DNA sequences
  - Mostly originated from bacteria, some from yeast
  - Double-stranded circular DNA
  - Extra-chromosomal
  - Replicate independently of cell DNA
    - Plasmids can be multi-copy (i.e. >1 plasmid/cell)
    - Usually more than one copy due to independent replication
  - Small, up to ~10kb (easy for manipulation)
    - Max. insert size: ~15kb pairs
- ***3 essential components***__EXAM__***
  - Origin of DNA replication
    - For amplification in bacterial cells
  - Selectable marker
    - Antibiotic resistance gene
  - Unique RE cleavage
    - At least 1, outside of origin and resistance gene (many have multiple cloning sites: MCS)

Characteristics of the pBluescript Cloning Vector

1. Bacterial origin of replication to allow plasmid replication
2. Multiple cloning site (MCS) to allow insertion of foreign DNA
3. Antibiotic resistance gene for selection of bacteria transformed with the plasmid



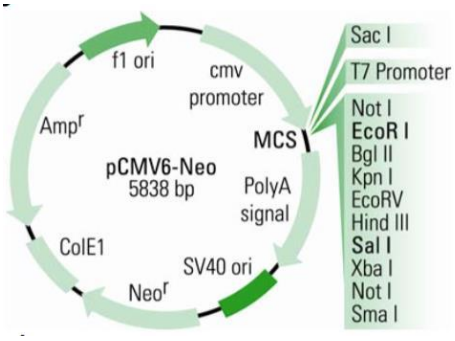
Collection of unique plasmid types- can be used in cloning process

Classic “blue white” selection for identifying bacteria containing <u>recombinant</u> plasmids

- Bluescript plasmid expresses the E. coli lacZ gene (encodes β-galactosidase)
  - MCS located within the plasmid encoded lacZ gene (“actually a portion of lacZ)
  - Insertion of foreign DNA into the MCS disrupts the reading frame of the lacZ gene
    - “insertion inactivation”

Expression Plasmids

- Designed for expression of proteins in bacteria, yeast, plant or animal cells
- Must be able to <u>replicate in bacteria</u> to amplify the plasmid and contain a <u>selectable marker</u> to identify bacteria containing the plasmid
- Must contain a <u>cloning site located downstream from a promoter appropriate for host cell</u> to allow the inserted gene to be expressed
- Usually contains a <u>selectable marker</u> (antibiotic resistance gene) appropriate for host cell to allow for selection of cells that have incorporated the plasmid in their genome



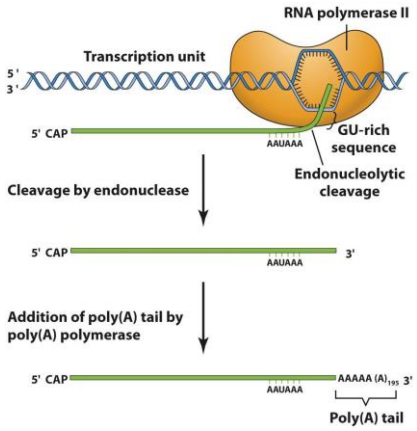
Mammalian promoter; PolyA signal is a necessary component

NOTE:

1. Promoters have directionality
2. Eukaryotic expression plasmids must contain a promoter upstream of the MCS and a polyA signal downstream of the MCS

Termination and 3' polyA tail addition

- Transcription proceeds past actual 3' end of mature mRNA
- Distal segment removed by endonucleolytic cleavage that occurs 10-30 nucleotides down from polyadenylation signal AAUAAA
- Following cleavage, polyA polymerase adds ~200 residues of adenosine monophosphate
- Polyadenylation protects the mRNA from degradation

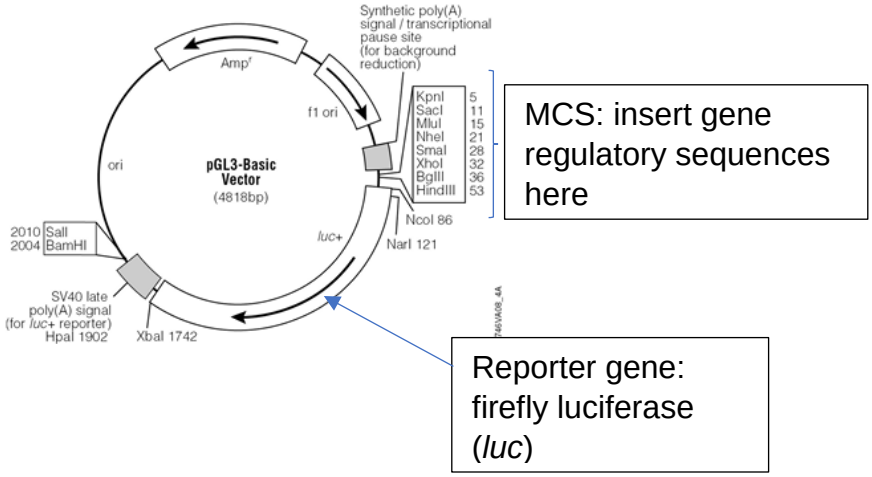


Reporter Plasmid

- MCS allows insertion of potential gene regulatory sequences
- Reporter gene encodes for a protein that can be visualized or measured
- Used to study gene regulatory sequences after transfection into cells

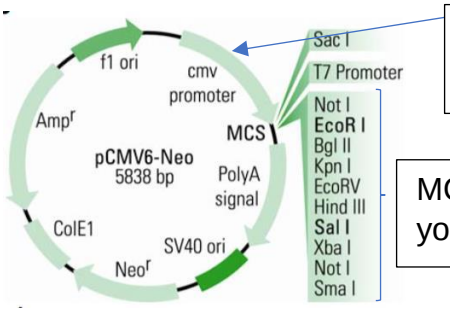
Luc+ = firefly luciferase gene

- Luciferase protein can be assayed (converts luciferin to light)
- Light can be measured in a luminometer
- [light] proportional to [luciferase protein] proportional [luciferase mRNA] which is proportional to transcriptional activity
- NOTE: reporter gene does not have a promoter to control its expression



Reporter gene: its expression level will tell you the efficiency of the regulatory sequences that you inserted

Expression Plasmid



Promoter to control expression of your gene of interest

MCS: insert gene of interest (the gene you want to express) here

NOTE: Similarities and differences between reporter and expression plasmid

Cloning Plasmids

- Isolation/ amplification of DNA sequences
- Contains:
  - Origin of replication
  - Selectable marker
  - MCS
- Ex. pBluescript

Expression Plasmids

- Expression of proteins in bacteria, yeast, plant or animal cells
- Contains:
  - Promoter appropriate for target cell located upstream of MCS
  - Elements found in cloning vector (for cloning/amplification in E. coli)
- Ex. pCMV, a mammalian cell expression vector

Reporter Plasmids

- Measurement of gene regulatory sequences
- Contains:
  - Reporter gene that when expressed can be quantitatively measured
  - Elements found in cloning vector
- MCS is located upstream of the reporter gene
- Ex. pGL3 (contains luciferase reporter gene)

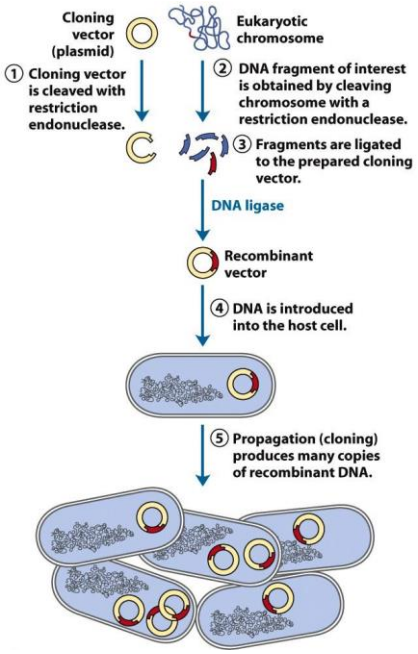
Questions :

1. The name of the part of a cloning vector that has several restriction sites is called: multiple cloning site (MCS)
2. In the Bluesprint cloning plasmid, the MCS is located within the reading frame of what gene? β-galactosidase (lacZ)
3. Bacteria containing the Bluescript plasmid that do not have foreign DNA inserted in the MCS of the plasmid will be what colour when grown on agar plates containing X-gal? Blue

Construction of DNA Libraries

Summary of major steps:

1. Digestion vector: cut vector with restriction enzymes within the multiple cloning site
2. Digest genomic DNA: cut with the same restriction enzyme used to cut vector
3. Annealing and ligation: allow the compatible sticky ends of vector and target DNAs to base pair with each other. Treat annealed DNA with ligase to seal the “nicks” (broken phosphodiester linkage between two adjacent nucleotides on the same DNA strand)
4. Transformation: of E. coli, transfer the DNA into E. coli cells
5. Selection: grow the transformed cells on selective media appropriate for the vector (e.g. selection media containing ampicillin)



Clone library: collection of DNA restriction enzyme fragments cloned into a plasmid, each separated into different bacteria, each isolated on an agar plate to produce colonies

see diagram slide 35

cDNA Libraries (copies of all mRNAs in a cell)

- Cannot clone mRNA directly (has to be in DNA form)
- cDNAs contain DNA sequences complementary to the mRNAs expressed in the cells used to construct the library

How?

- Isolate mRNA from cells
- Convert mRNA to cDNA
- Ligate cDNA into cloning vector
- Transform bacteria
    - cDNA= complementary DNA (i.e. complementary to mRNA)
    - requires the enzyme reverse transcriptase (RT)

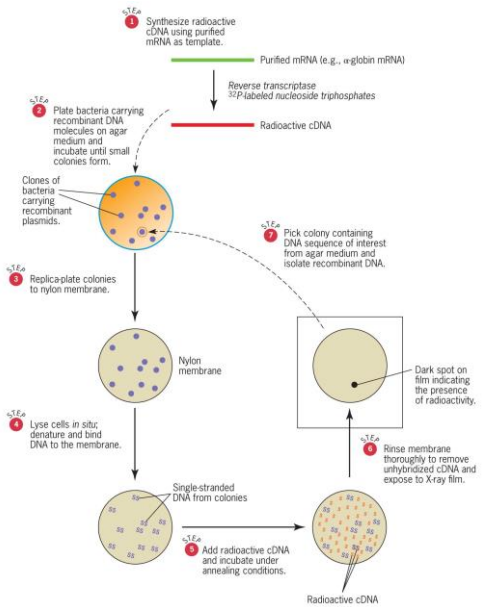
Construction of cDNA Libraries

- cDNA is synthesized from mRNA using a primer (i.e. oligo-dT since all mRNA contains poly A tail) and the RNA-dependent DNA polymerase RT (synthesizes DNA from an RNA template)
- Ribonuclease H (to remove RNA), then second strand synthesis
- DNA dependent DNA polymerase for second DNA strand
- ds cDNA fragments are inserted into cut plasmid

see diagram slide 39

Screening DNA Libraries for Genes of Interest

- (1) Genetic selection
  - Isolated DNA sequences restores the wild-type phenotype to a mutant organism
- (2) **Molecular Hybridization**
  - Radioactively labeled DNA (or RNA) is used as a hybridization probe to identify complementary DNA bound to a nylon membrane
  - Steps:
    - (i) Replica plate bacterial colonies from a genomic library onto a nylon membrane
    - (ii) Lyse cells, denature the double stranded DNA, link ss DNA onto membrane
      - Membrane containing single stranded DNA bound to regions corresponding to the location of the bacterial colonies
    - (iii) Add radioactive cDNA (prepared by RT of mRNA in presence of radioactive nucleotides) and incubate to allow hybridization of probe to membrane bound DNA
    - (iv) Wash to remove unbound probe (probe bound complementary DNA on the membrane remains attached to membrane)
    - (v) Expose membrane to X-ray film- when developed these will produce dark spots
    - (vi) Use location of spot on film to identify positive colony from the original plate
    - Diagram slide 43/44



Questions:

1. What is the generic name of enzymes that recognize palindromic sequences in dsDNA and cleave the DNA at that location? Restriction endonucleases
2. The kind of ends following restriction endonucleases digestion that results in single stranded DNA overhangs are called: cohesive (sticky) ends
3. A collection of bacteria in which each colony contains a plasmid with a unique fragment of genomic DNA is called: genomic library
4. A library made from cellular mRNA is called: cDNA library