MMG 301 Lec. 28

Genetic Engineering Basics

Questions for Today:

1. How does one obtain a DNA fragment containing the desired gene
   - using restriction enzymes?
   - using the Polymerase Chain Reaction (PCR)?
2. How does one join the DNA fragment with a cloning vector?
3. After introducing an expression system into a host (Lec. 26), how does one isolate the desired clone?
4. What are desired properties in a cloning vectors?
5. What types of applications are possible?

Use of Restriction Enzymes for preparing a collection of DNA fragments

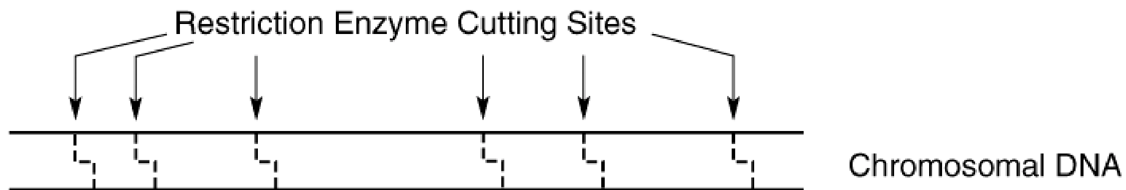
[The following description of restriction enzymes (restriction endonucleases) provides greater detail than described in Lec.23]

Initial Goal: Make a "*Library*", a large population of heterogeneous fragments joined into a single type of cloning vector.

Over 500 restriction endonucleases are now commercially available. These include 4-cutters, 6-cutters, 8-cutters, and others. (Original purpose of these enzymes? Restriction of foreign DNA while methylated self DNA is left undamaged)

Sequence of single-strand overhangs must be complementary to that of vector; but any blunt end can be ligated to any other blunt end.

ORNL-DWG 92M-66!



enzyme	organism	Recognition site	overhang
*Bam*HI	*Bacillus amyloliquefaciens* H	5'..G▼GATCC..3' 3'..CCATG▲G..5'	GATCC..3' G..5'
*Eco*RI	*Escherichia coli RY13*	5'..G▼AATTC..3' 3'..CTTAA▲G..5'	AATTC..3' G..5'
*Nco*I	*Nocardia corallina*	5'..C▼CATGG..3' 3'..GGATC▲C..5'	CATGG..3' G..5'
*Sma*I	*Serratia marcescens*	5'..CCC▼GGG..3' 3'..GGG▲CCC..5'	GGG..3' CCC..5'
*Mbo*I	*Moraxella bovis*	5'..N▼GATCN..3' 3'..NCTAG▲N..5'	GATCN..3' N..5'

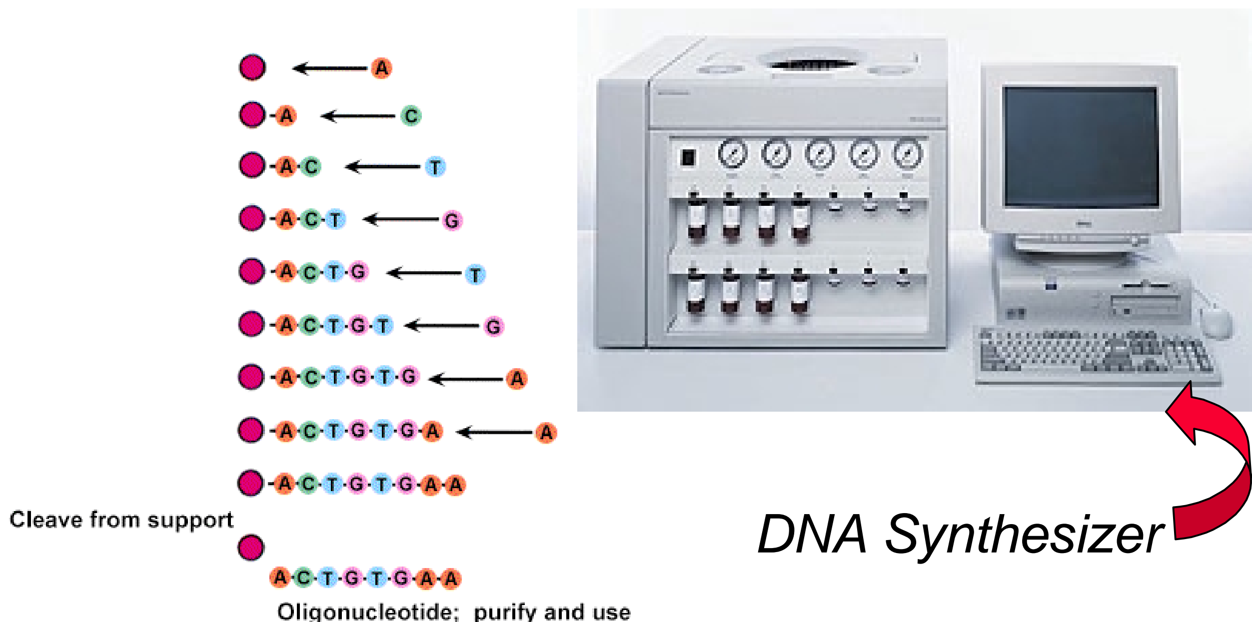
(N = any base) **Result = collection of DNA fragments, not just the single desired fragment!**

Need a selection method to find desired clone

Use of the Polymerase Chain Reaction (PCR) to make *a specific* DNA fragment

(This approach is used if the sequence of the gene of interest is already known)

Requires an <u>oligonucleotide</u> – a short piece of synthetic single-stranded DNA (= a primer)
Primers are typically 25-35 bp long
Primer hybridizes with a complementary DNA strand and is used for polymerization of a new strand



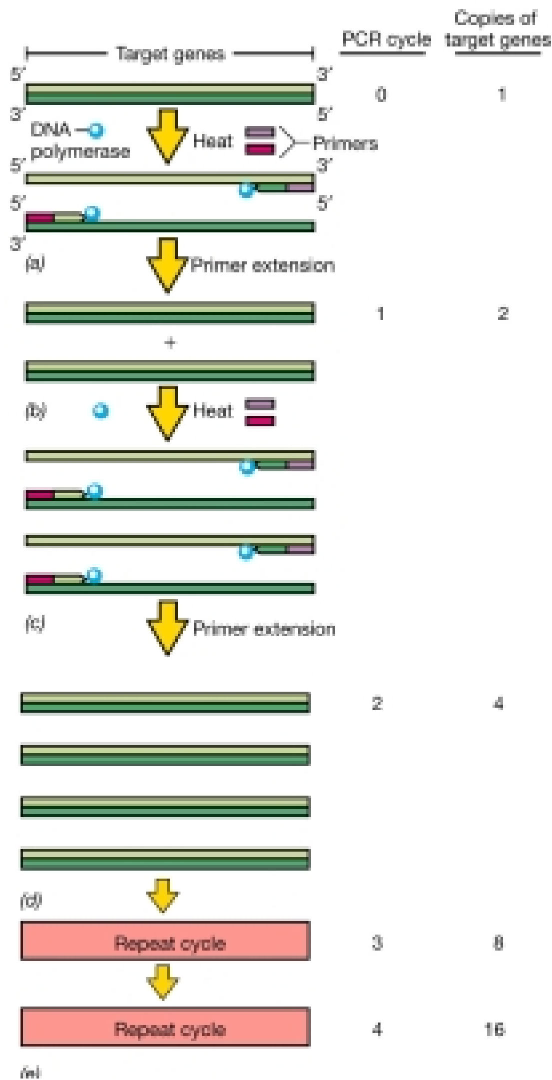
Other components needed:

- high temp DNA polymerase (*Taq, Pfu*, next page)
- template DNA (examples: bacterial chromosomal DNA; mammalian chromosomal DNA)
- deoxynucleotide triphosphates (dATP, dGTP, dTTP, dCTP)

Thermally stable DNA polymerases

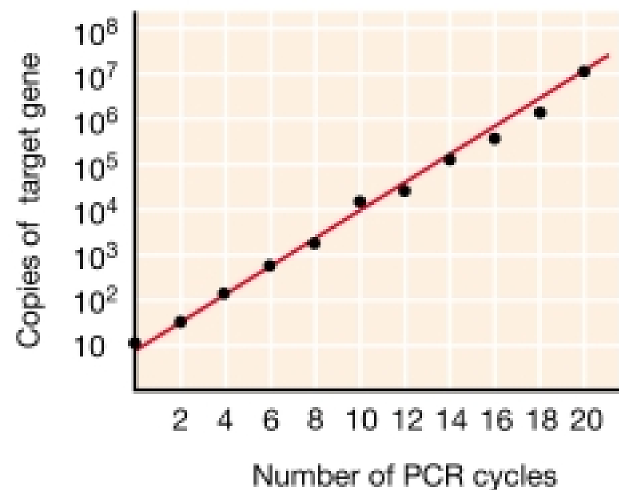
•*Taq* – *Thermus aquaticus*, a thermophilic bacterium; enzyme is stable at 95°C and works optimally at 72°C

•*Pfu* – *Pyrococcus furiosus* (Euryarchaeota; remember that?)



Properties of DNA that are used for PCR: Strands separate (denature) at high temp and anneal (reform proper base pairing) upon cooling

Semilog plot



Reactions are carried out in the presence of a large molar excess of primers.

heated lid

temp block

display panel

programming pad

Mix all ingredients in a tube, pop into *thermocycler*, and run a standard program

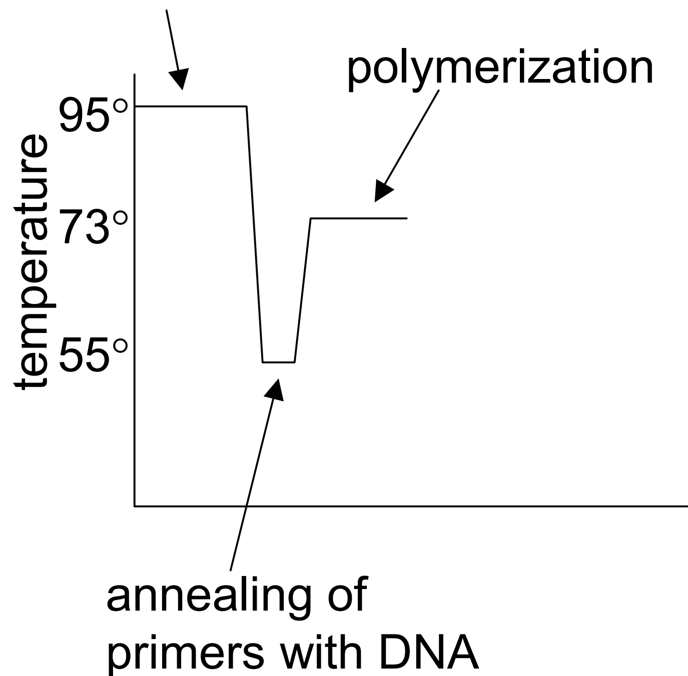
Typical PCR program:

Program:

1. 1 min 95°
2. 1 min 95°
3. 1 min 55°
4. 2 min 73°
5. Repeat

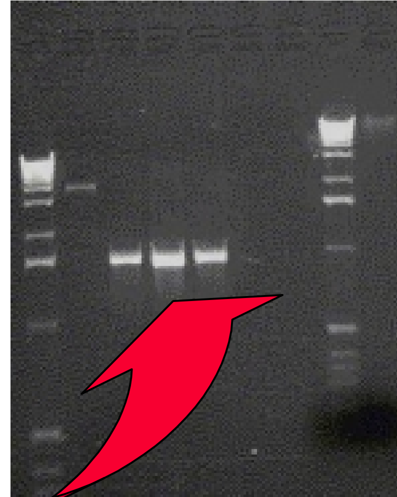
   (~ 29 times)

6. 5 min 73°
7. 20 hrs 4°

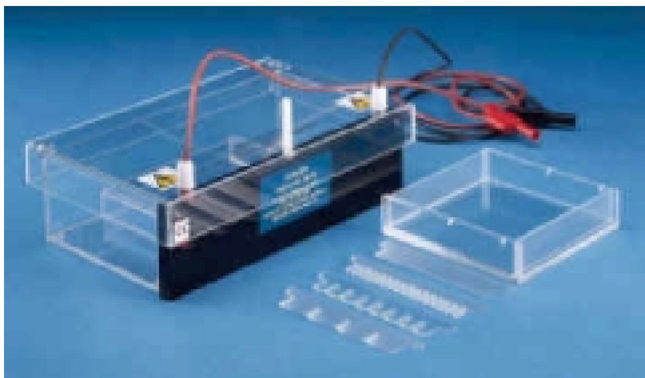


Can visualize the production of a specific fragment by gel electrophoresis

- Usually use Agarose (derived from seaweed)
- Can also use polyacrylamide gels

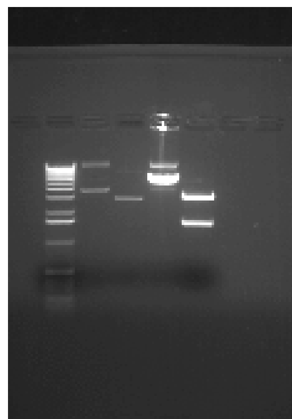


PCR product – DNA fragment from *Pseudomonas* genome



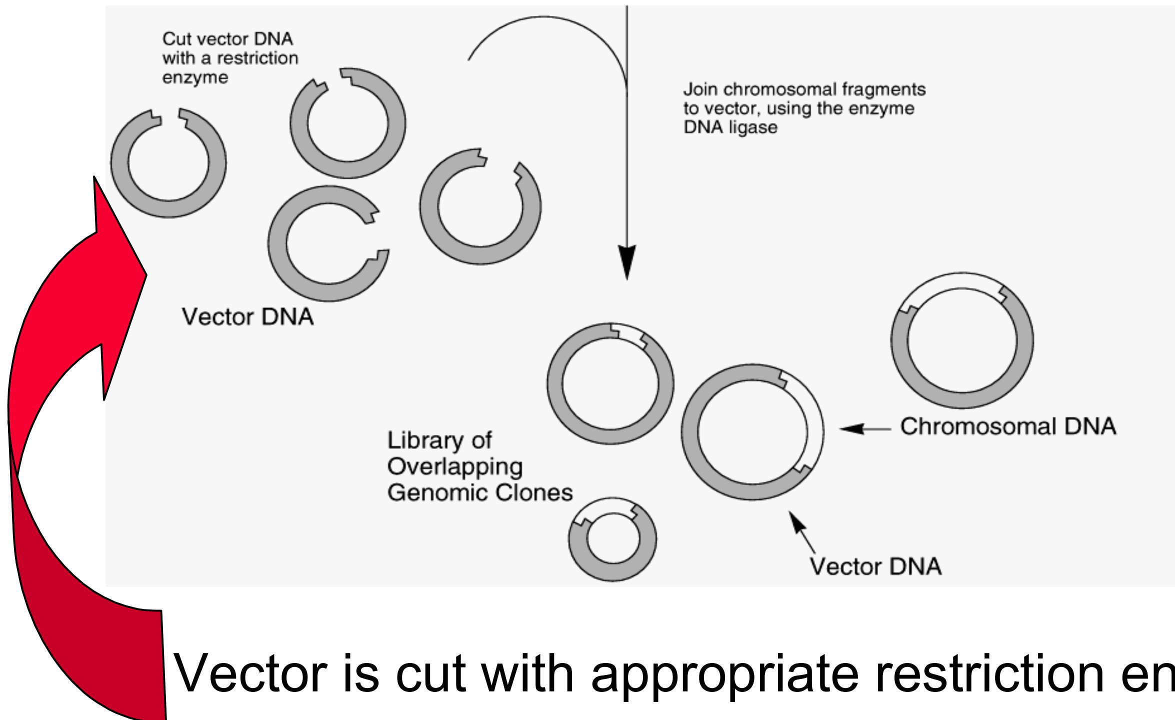
Electrophoresis apparatus

This same approach works to visualize fragments derived from endonuclease digests (e.g., plasmids stained with ethidium bromide)

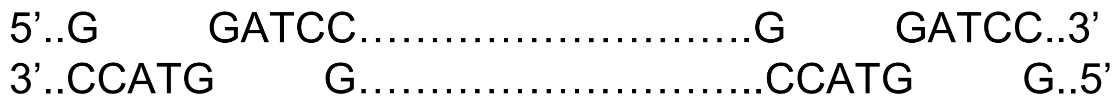


Joining DNA fragments into Cloning Vectors with DNA ligase

DNA from PCR or random restriction fragments

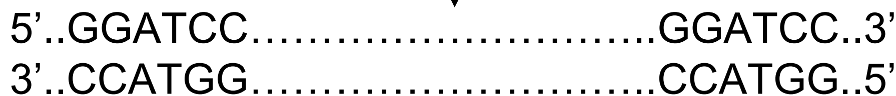


Vector is cut with appropriate restriction enzyme



DNA ligase + ATP

Use ligase from phage T4



-- For a PCR fragment, this gives a single product, so no selection is needed.

-- In the case of a restriction digest, one gets a large family of products with different inserts in the vector (as illustrated), so need to select.

Selection of desired clone from a library

After transferring the vector + DNA into a cell (e.g., by electroporation, transformation, transduction, conjugation) one has a *library*. Now we want to identify the clone(s) containing the gene of interest. Approaches include:

a. Select for a new enzyme activity – examples: ability of the *E. coli* to grow on a new substrate that requires the desired gene.

b. Gene complementation – transform library into an *E. coli* with a mutation in the chromosomal gene of interest. Grow on selective growth medium. If a foreign DNA fragment codes for an enzyme that complements the *E. coli* mutated gene, then cells with that plasmid will grow.

c. Antibody detection – must have antibodies made against the protein of interest. Screen *E. coli* colonies using labeled antibodies specific to the desired protein.

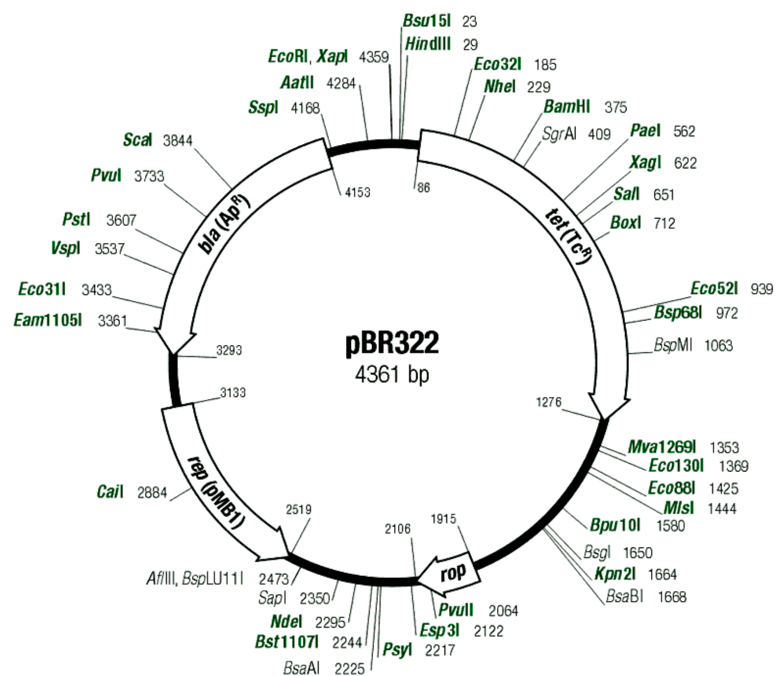
d. DNA or RNA probe – use short, labeled oligonucleotide as a hybridization probe to screen colonies.

Cloning vectors: plasmids, bacteriophage, phagemids, cosmids, etc.

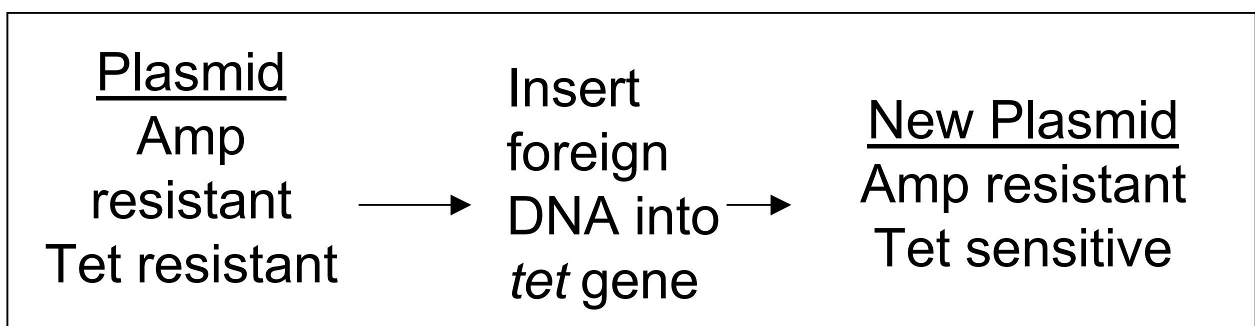
Plasmids:

pBR322 One of the first plasmids utilized

- •Ampicillin resistance: (β-lactamase) used to select for cells that contain plasmid (others are killed)
- •Tetracycline resistance
- •Origin of replication
- •Can select for insertion of fragments using antibiotic genes



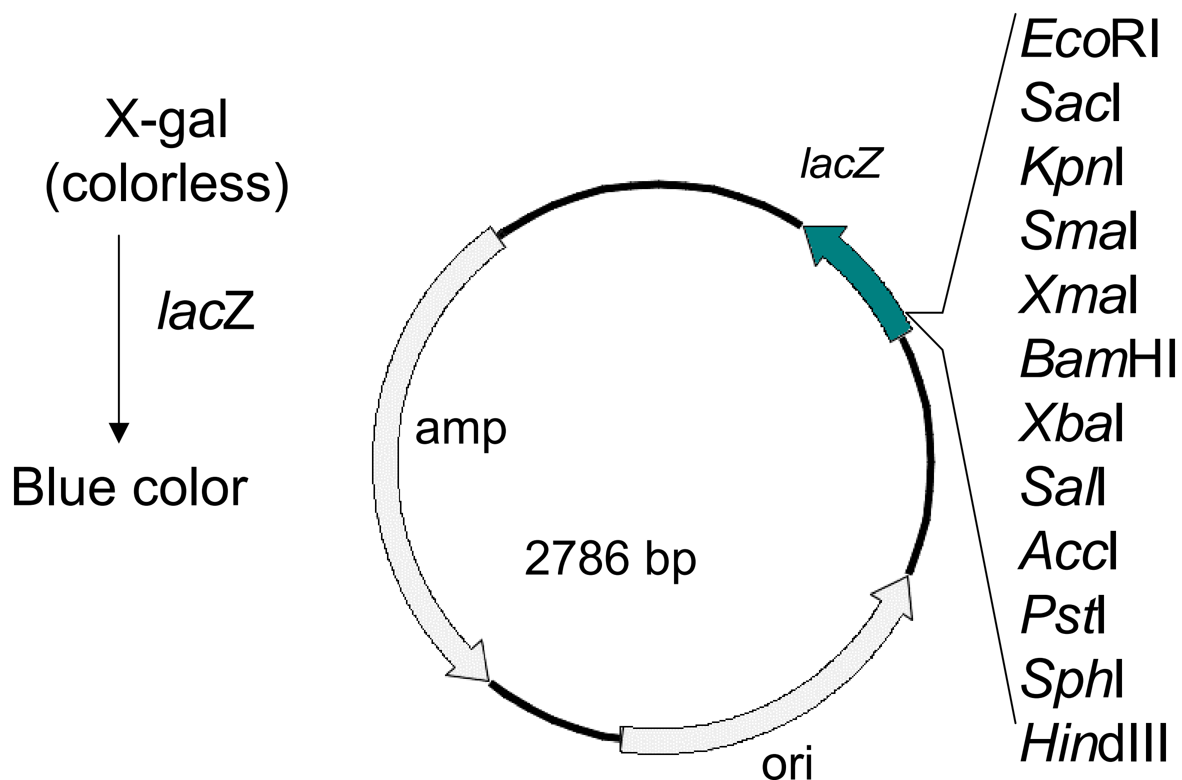
Insertional inactivation:



pUC18

- Multiple cloning site – a “polylinker” containing many restriction enzyme sites

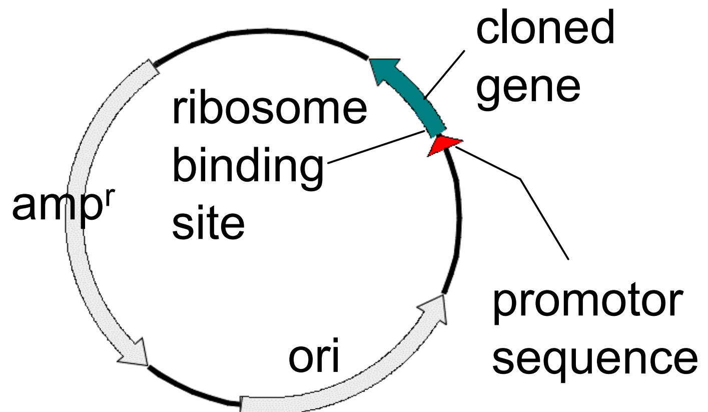
- insertional inactivation of the *lac*Z enzyme (β-galactosidase) leads to inability to hydrolyze the substrate X-gal: cells produce blue colonies for no insert and white colonies if there is an insert



Many other plasmids have been designed to have specific functions: variable “copy number”, different “incompatibility groups” (needed for 2 plasmids in the same cell), regulation of expression, etc.

Regulated or high-level expression in plasmid vectors

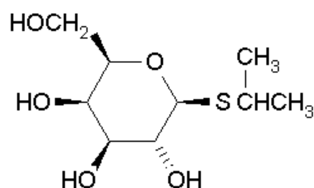
Can choose an optimal promoter, ribosome binding site, new polymerase, etc.



Common promotors:

- *lac*- from the *lac* operon
- *tac*- a hybrid of the *trp* and *lac* promotors
- lambda P_L (induced by increased temperature)
- phage T7 promotor (used in pET vectors for *E. coli* host that has T7 RNA polymerase. These vectors give very high level expression so that >20% of cellular protein is derived from the inserted gene!)

Turn on *lac* promotor using IPTG. The *lac* promotor turns on T7 RNA polymerase gene. T7 RNA polymerase transcribes the cloned gene.



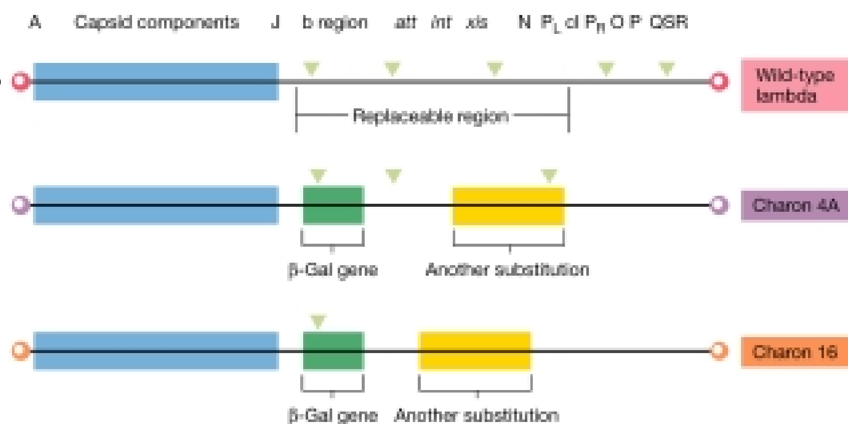
IPTG: gratuitous inducer of the *lac* operon

Bacteriophage vectors

•Modified bacteriophage M13 contains a polylinker region and *lacZ*; e.g., *M13mp18* (different number designation for various polylinkers)

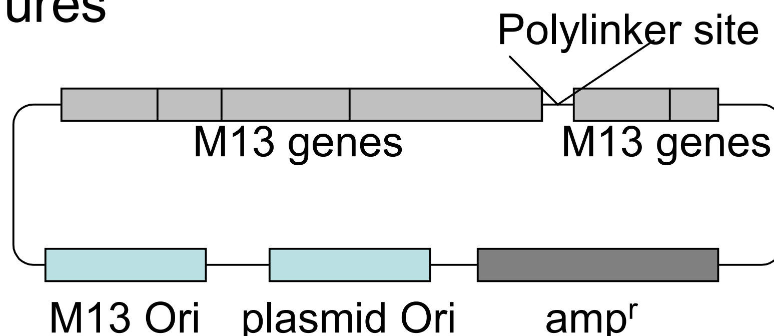
- •Double-stranded replicative form DNA is isolated and used for cloning
- •Foreign DNA fragments are cloned into the polylinker (see Lec.23)

•*Lambda* versions



Phagemids (plasmid + phage): a plasmid with both an M13 and plasmid origins of replication

- •Grow and manipulate as a plasmid, but in *E. coli* with helper phage, plasmid can be packaged into M13 phage capsids
- •Single-stranded DNA can then be isolated from the phage and used for DNA sequencing or other procedures



<u>cosmid</u> – a plasmid containing the *cos* site of lambda

Able to package DNA into lambda particles

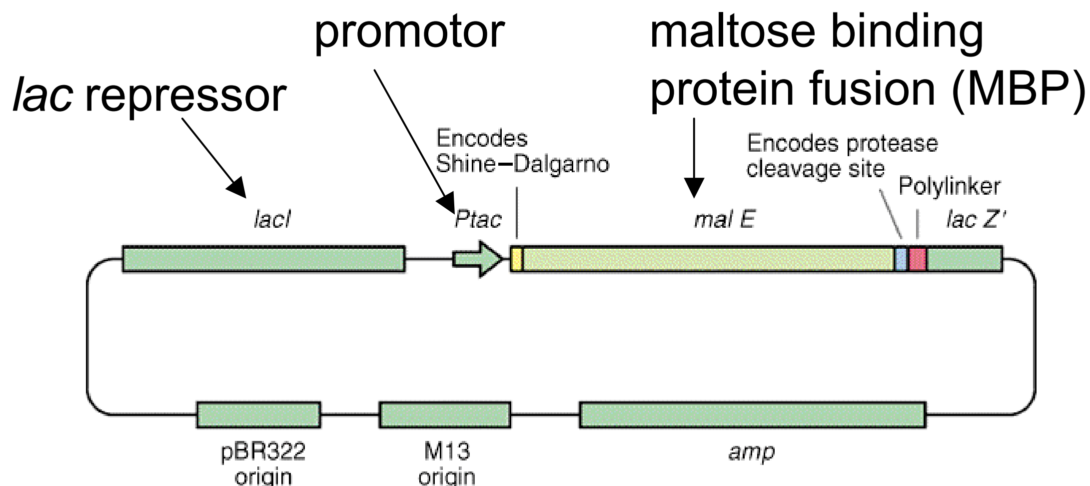
Can clone large segments of DNA (up to 30-40 kbp)

<u>Other cloning vectors</u>

- •artificial chromosomes –
  - •yeast (YAC): propagate like a separate chromosome; large DNA inserts
  - •bacterial (BAC): based on *E. coli* F plasmid; inserts up to 300 kbp
- • shuttle vector - has origin of replication for eukaryotic organism (yeast, insect cells) AND an origin of replication for *E. coli*

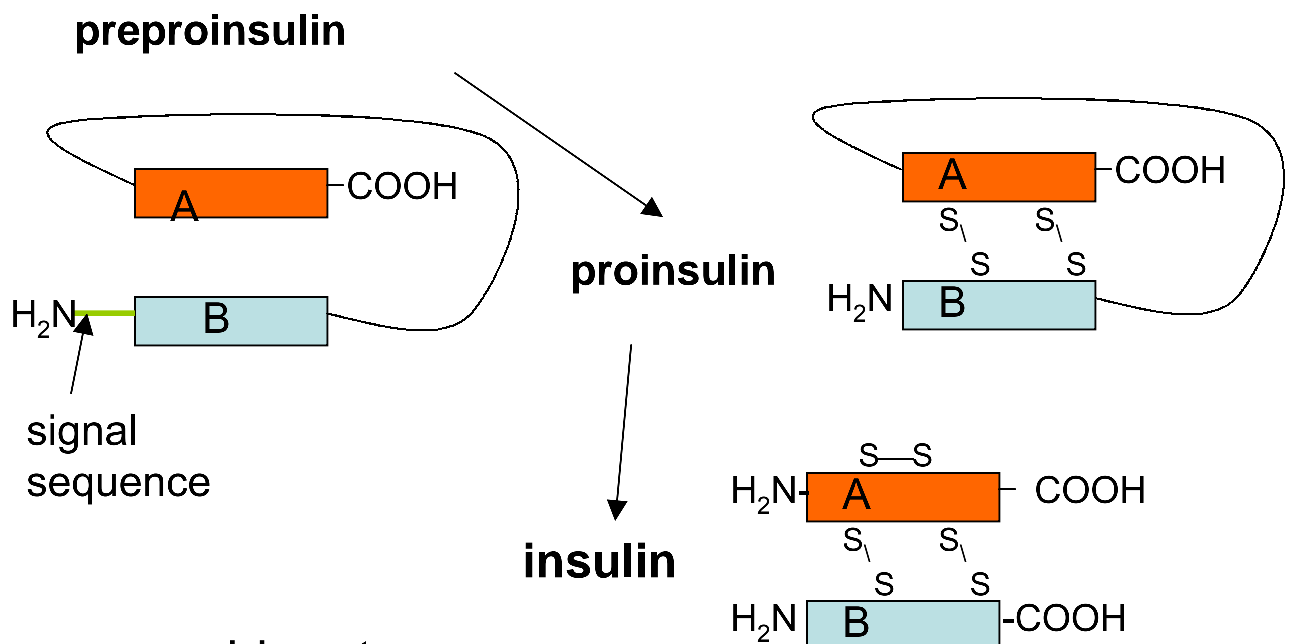
<u>Fusion vectors</u>: plasmids that create an <u>in frame</u> fusion of the gene of interest with another gene.

•The resulting fusion protein may have increased solubility or be more easily purified (e.g., single step purification using maltose binding protein or 6His-tag)



Examples of Applications:

1. Genes encoding human proteins can be cloned to provide enzymes, hormones, or other components to individuals who make these at insufficient levels. Example = Insulin: In mammals, insulin made as a precursor protein which is then processed in the cell to become the hormone



Two recombinant approaches are used:

-- In bacteria, produce proinsulin as a fusion protein (has better stability in *E. coli*) followed by chemical cleavage

-- produce A and B chains separately on plasmids followed by chemical joining

2. <u>Vaccines</u>

• a protein from a pathogenic organism can be produced in microbes, purified, and used to elicit an immune response. Example: Hepatitus B vaccine using a viral surface protein

• recombinant DNA is cloned into vaccinia virus, injected into humans, and used to elicit an immune response to the antigen.

▪ plasmid DNA containing the gene for an antigenic protein is taken up in cells and used to direct production of a protein, resulting in immunity for the organism.

3. <u>Functional Genomics</u>: characterize roles of genes

example: what are roles for various human proteins? Clone the genes as fusions to DNA encoding a His-tag, express in bacteria, affinity purify in high-throughput approach (132 genes).

(Proc. Natl. Acad. Sci. 99:2654, 2002)