Questions

• What is the difference between interwound and toroidal supercoiling?
  – Give an example of toroidal supercoiling that occurs inside the cell.
Supercoiling of DNA

3. DNA compaction requires special form of supercoiling
   A. Interwound: supercoiling of DNA in solution
   B. Toroidal- tight left handed turns, packing of DNA
      both forms are interconvertible
Molecular Cloning:
Construction of a recombinant DNA
Molecular Cloning: Cloning Vectors

• Plasmids
Grand-daddy of plasmids

- EcoRI
- BamHI
- PstI
- SalI

Ampicillin resistance ($amp^R$)
Tetracycline resistance ($tet^R$)

pBR322
(4,361bp)

Origin of replication (ori)

PvuII
Molecular Cloning: Cloning Vectors

- **Plasmids**
  - Origin of replication, determines the number of copies per cell
  - Marker genes: ampicillin and tetracycline resistance genes
  - Unique restriction enzyme cut sites
Creation of a polylinker or multiple cloning site

[Diagram showing the process of creating a polylinker with restriction sites for PstI, HindIII, BamHI, and Smal, and the use of DNA ligase to join the polylinker to a plasmid cloning vector cleaved with EcoRI.]
Molecular Cloning: Cloning Vectors

1. Plasmids
   - Origin of replication, determines the number of copies per cell
   - Marker genes: ampicillin and tetracycline resistance genes
   - Unique restriction enzyme cut sites
     • Polylinker of MCS
   - Small size
     • Limitation is ~15,000 bp
Questions

• How many different ways are there to construct recombinant plasmids (hint: try a Google or similar search, also check out Invitrogen)?
Invitrogen's TOPO TA Cloning System (TOPO Cloning)

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Application
Figure 2. Gateway® technology facilitates cloning of genes, into and back out of, multiple vectors via site-specific recombination. Once a gene is cloned into an Entry clone you can then move the DNA fragment into one or more destination vectors simultaneously.
DNA to be cloned

5'  
3'

dATP  terminal transferase

5'  AAAAAA -OH  3'
3'  

+  OH  TTTTTT  5'

Cloning vector

5'  3'
3'  5'

terminal transferase  dATP

5'  TTTTTT  3'
3'  AAAAAA  5'

Anneal and ligate

Recombinant DNA
Entry of DNA into cells

- Chemical Transformation
  - Treat cells with calcium chloride
  - Heat shock
- Electroporation
  - High voltage transiently makes bacterial membrane permeable
Special examples of different plasmids

- Expression vectors
What are the considerations in vector construction that need to be taken into account with over-expression of a protein?
Questions

• Why are protein expression vectors useful?
• What kind of cells are proteins generally over-expressed in and why?
• What are the considerations in vector construction that need to be taken into account with over-expression of a protein?
Special examples of different plasmids

A. Expression vectors
   – Strong promoter for transcription
   – Ribosome binding site
   – Transcription terminator
   – Some way of controlling gene – making it inducible
Fusion proteins

Tag expressed protein with another protein or a short peptide
Type of Tags

• Fluorescent proteins
  – One example is the green fluorescent protein or GFP
Transcription

- Insert
- cDNA
- GFP

[Image of a curved object with fluorescence emission]
Type of Tags

- **Fluorescent proteins**
  - Examples is the green fluorescent protein or GFP

- **Epitope tag**
  - Short peptide sequence which has an antibody that recognizes it specifically
Express tagged protein in a cell.

Make cell extract.

Precipitate tagged protein with specific antibody.

Separate precipitated proteins.

Identify new proteins in precipitate (e.g., with mass spectrometry).
Type of Tags

• Fluorescent proteins
  – Examples is the green fluorescent protein or GFP

• Epitope tag
  – Short peptide sequence which has an antibody that recognizes it specifically

• Metal chelator
  – Can bind to Ni or Co chelated and immobilized
Special examples of different plasmids

B. Shuttle vectors
   - Contains origins of replication from two different organisms
   - Can be replicated in both
   - Often used to shuttle plasmids from bacteria to yeast

C. Cosmids
   - Plasmids containing at least one cos (cohesive-end site) of lambda phage
   - Up to 44 kbps
Molecular Cloning: Cloning Vectors

2. Bacteriophages: example is lambda
   - 1/3 of genome (48.5 Kb) is non essential
   - DNA is packaged into phage particles
   - Can only fit 40 – 53 Kb of DNA
   - Have an in vitro packaging system
   - Highly efficient at transforming bacteria
   - Can clone up to 23 Kb of DNA
λDNA

Cleave by restriction enzyme and separate the fragments

Not required for lytic infection

Remaining λ DNA contains genes required for infection but is too small to package

~36 kb

Anneal and ligate

~15-kb foreign DNA fragment

Infective λ phage containing foreign DNA fragment

In vitro packaging

Chimeric DNA
3. Bacterial Artificial Chromosomes (BAC)
   - 100 to 300 Kb in size
   - Have selectable markers
   - Stable origin of replication
   - Size of inserts is ~100 KB
   - Uses electroporation
Cloning sites (include lacZ)

F plasmid
par genes

BAC vector
CmR
ori

restriction endonuclease

Large foreign DNA fragment with appropriate sticky ends

DNA ligase

Recombinant BAC

electroporation

selection of chloramphenicol-resistant cells

Agar containing chloramphenicol and substrate for β-galactosidase

Colonies with recombinant BACs are white.
Molecular Cloning: Cloning Vectors

4. Yeast Artificial Chromosomes
BamHI digestion creates linear chromosome with telomeric ends

EcoRI digestion creates two arms

Left arm has selectable marker X

Right arm has selectable marker Y

Fragments of genomic DNA generated by light digestion with EcoRI

Ligate

YAC

Enzymatic digestion of cell wall

Transform

Select for X and Y

Yeast cell

Yeast spheroplast

Yeast with YAC clone
Molecular Cloning: Cloning Vectors

4. Yeast Artificial Chromosomes (YAC)
   - ARS or origin of replication
   - Selectable markers
   - CEN or centromere sequence for proper segregation
   - Telomere sequences
   - Suitable for very large DNAs
A DNA library

• Collection of DNA clones
• Source for gene discovery
• Largest example is genomic library
• Other subsets would be such things as:
  – cDNA libraries
• Can scan these libraries by DNA hybridization
Colonies grown on master plate

Velvet pressed to master plate and transferred to nitrocellulose filter

Treat with alkali and dry

DNA bound to filter

Anneal labeled probe, wash and dry

Radioactive probe hybridizes with its complementary DNA

Autoradiograph and compare with master plate

Autoradiograph film

Colonies detected by probe

Blackening identifies colonies containing the desired DNA
• Why are cDNA libraries still so important in the era of genomics?
• What information can be derived from cDNA that are not obtained generally by DNA microarrays?
Full-length cDNA clones and the sequence information derived thereof are the entry to:

- **Proteomics**: Only full-length cDNAs enable the synthesis, use, and analysis of proteins.
- **Gene Regulation**: Only full-length cDNAs allow for promoter identification, and as such give you access to the regulatory information that drives gene expression.
- **RNAi**: The more information you have the greater your chances to prepare a specific RNAi probe.
- **Splicing**: Only full-length cDNA information can tell you about the true exon composition of transcripts.
- **Genomics**: Full-length cDNAs are needed for gene discovery and correct mapping of genomic locations.
- **SNP Analysis**: Only a complete and correct annotation of a gene enables functional characterization of SNPs.
Next time on to protein methods

Change in the syllabus – will not be doing protein structure in this section
Questions

• Why would you need to purify protein?
• What properties of proteins can be used to separate and purify them from each other?
• What is column chromatography and how does it apply to protein purification?
• What causes proteins to denature?