BIOC4004 - Industrial Biochemistry
Lecture 06 - Wed Jan 21, 04

Topics for the Day:

- Seminar topics
- Protein Expression and purification
  - Expression vectors
  - Fusion proteins
  - Uses of fusion proteins
  - Eukaryotic expression

notes:

ftp://bioc4004@132.246.88.66/

user: bioc4004, password: bioc4004

Oral Presentation Topics:

- improvement of industrial enzymes through directed protein engineering
- combinatorial mutagenesis and protein engineering
- pathway engineering and overexpression to produce useful compounds
- Harnessing microorganisms for Bioremediation
- Development of novel antibiotics
- Genomes of exotic species; gene prospecting
- Genomics/Proteomics approaches to drug design
- Novel diagnostic techniques
- Pathway engineering in Agrifood
- Antibody vaccines

Submission	Member 1	Member 2	Member 3	Member 4	Topic 1
1	Thad B.	Sandra V.	???	???	Agrifoods
2	Keldeagh L.	Chris J.	Pratik L.	Zain B.	Pathways
3	Sharon H.	Jen W.	Bethany D.	Kerry R.	Vacc or Ab eng.
4	Johanna L.	Jenny N.	Kathy C.	Mona L.	Diagnostics
5	Frances C.	Jason F.	Greg S.	Justin S.	Vacc or Ab eng.
6	Maria B.	Martha P.	Monique B.	???	Prot. Engineering
7	Jonathan G.	Robyn E.	Jason O'B.	Steve A.	Bioremediation

Need group:

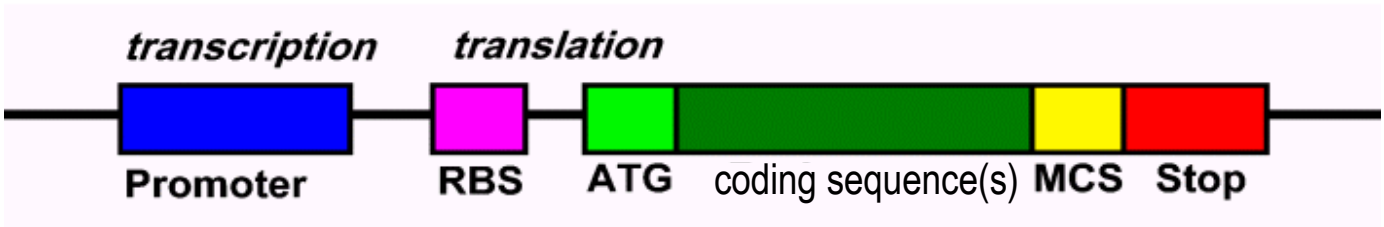
Alex Duong	
Ali Shafea	
Daniel Kolczynski	
Hardeep Nahal	
Thaila Riden	

Jen Eng

- **PLEASE READ THE MARKING SCHEME !!!!!**
- **if you need the computer projector, get a form from the AV dept.**
- **Need to submit a small summary and 1 question for class discussion**

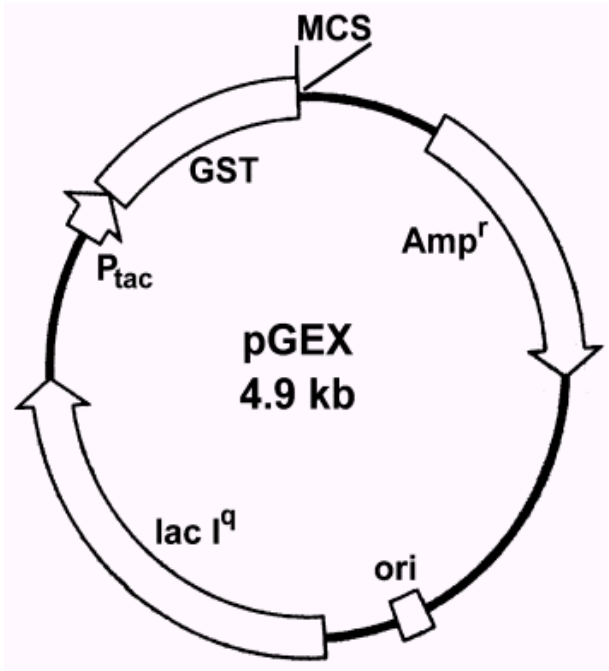
Producing Recombinant Proteins: expression vectors

- Why use expression vectors ?
  - "over-production" of a selected protein
  - expression of an engineered protein
  - library screening
    - activity screening
    - antibody screening
    - screening by "other interactions"
      - another protein
      - a compound



What's necessary ?

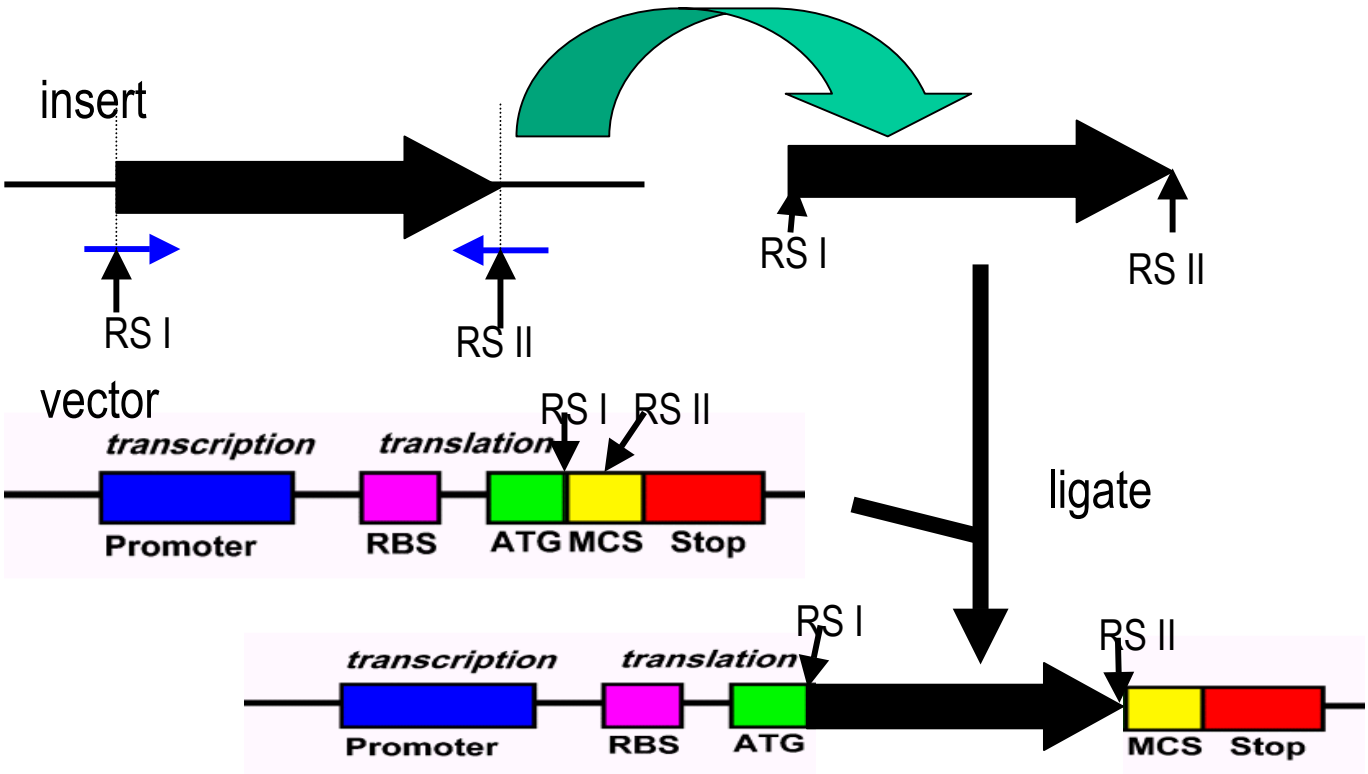
- good transcription level
  - strong promoter
- good transcriptional regulation
  - regulatory sequences
  - inducible promoter ?
- all of the signals to ensure good translation
  - Ribosomal Binding Site
  - in-frame cloning of the coding sequence
  - translation termination



- <u>additionally:</u>
  - some allow cloning of a fusion protein
    - purification tags
    - detection
  - expression system should be protease deficient

Cloning into an Expression Vector

- Cut-out insert from current vector
  - or PCR amplify incorporating restriction sites into primers



Insert |EcoRI|
NNN GAA TTC TTG ACT AGG NNN ...
... Glu Phe Leu Thr Arg Xaa ...

Vector

|EcoRI |
P_{tac} ... ATG AAT TCN NNN
Met Asn Ser Xaa

⇨

|EcoRI |
P_{tac} ... ATG AAT TCT TGA CTA GGN
Met Asn Ser ***

|EcoRI|
P_{tac} ... ATG GAA TTC NNN
Met Glu Phe Xaa

⇨

|EcoRI|
P_{tac} ... ATG GAA TTC TTG ACT AGG NNN ...
Met Glu Phe Leu Thr Arg Xaa ...

|EcoRI |
P_{tac} ... ATG GGA ATT CNN
Met Gly Ile Xaa

⇨

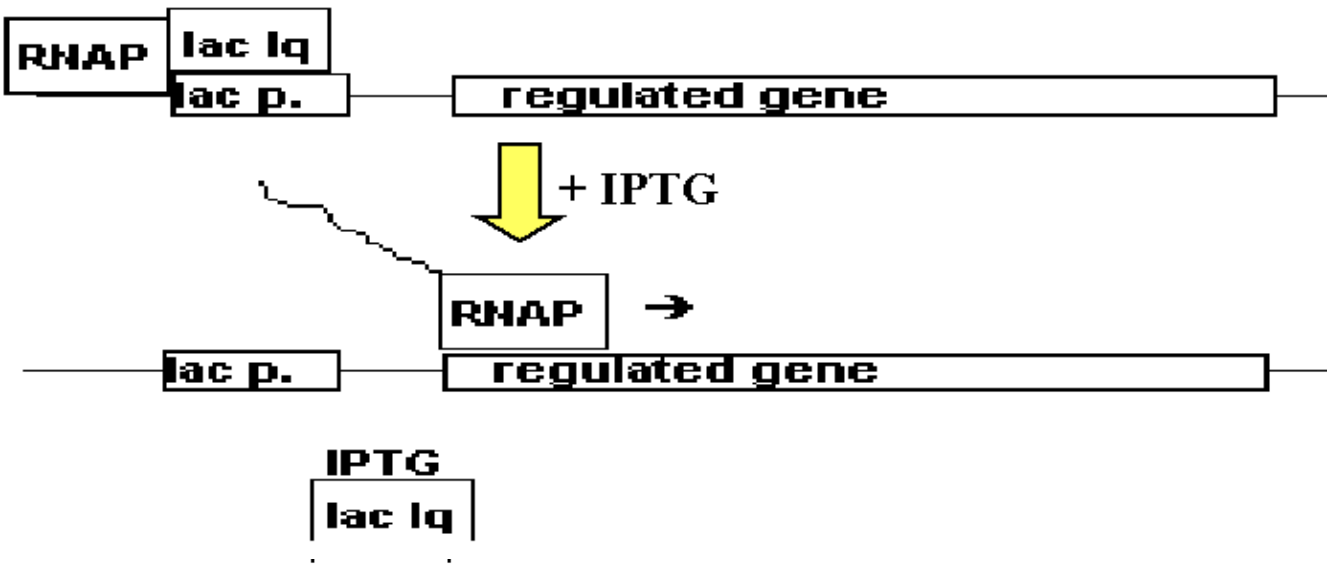
|EcoRI |
P_{tac} ... ATG GGA ATT CTT GAC TAG GNN
Met Gly Ile Leu Asp ***

- correct orientation(5'-N to 3'-C orientation)
- in-frame to avoid premature termination

Expression Vectors: inducible promoters

- a lot of them are based on the *lac* operon promoter
- inactive because of repressor binding
- inducer binds repressor, which falls off promoter
    - activation
    - inducer: galactose or galactose analogues (IPTG)

Promoter	Repressor	Induction
lac	$lacI^q$	IPTG
tac	$lacI^q$	IPTG

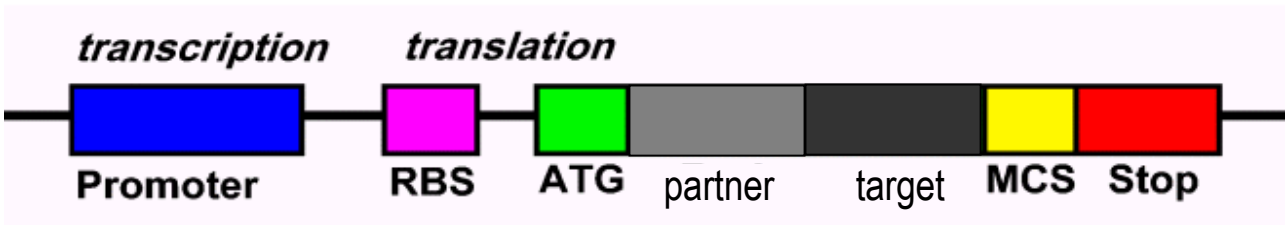


- regulated expression is good because…
    - sometimes *E. coli* doesn't like your pet protein
        - protease activity
        - "inclusion bodies"
        - cytotoxic
- you can delay expression until the culture is well underway!!!!

The Fusion Protein Concept

Reasons why you may want to express your protein as a fusion protein ?

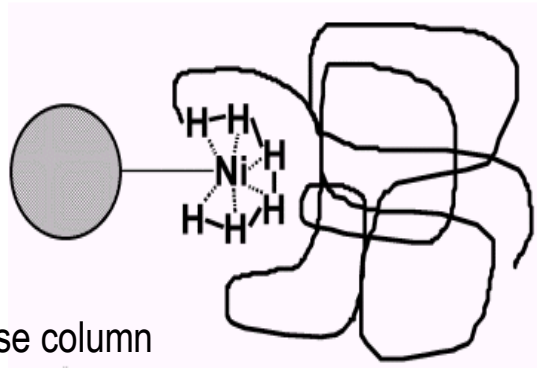
- Tag for affinity purification or column immobilization
  - Glutathione-S-Transferase (GST)
  - Thioredoxin
  - Maltose binding protein
  - poly-His
- Tag for detection
  - antibody epitopes (c-myc)
  - green-fluorescent protein (GFP)
  - “two-hybrid assay”
  - lacZ
- Export signals



Fusion Partner	Affinity Ligand
Glutathione-S-Transferase	glutathione
Thioredoxin	phenylarsine oxide
Maltose Binding Protein	amylose
Six Histidine Residues (His_6)	nickel

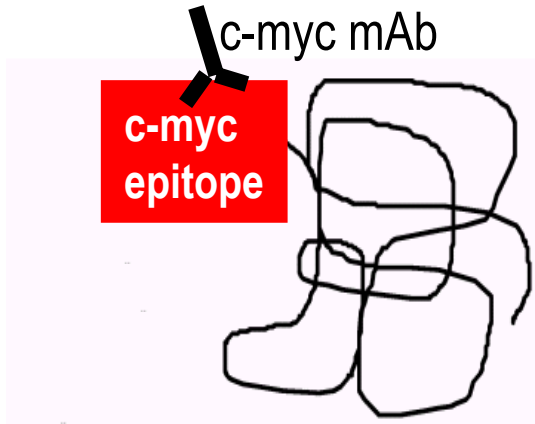
The Fusion Protein Concept at work!!!!

Metal Chelate Chromatography



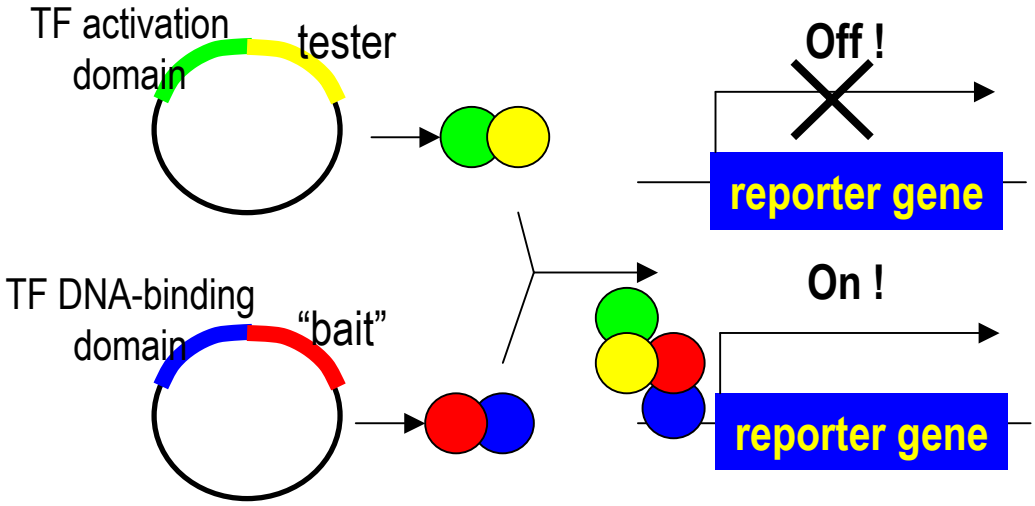
- popular method for analytical protein purification
- fusion protein with 6 X His tag on either end
  - negligible effect on target protein structure
- His-tag chelates metal ions
- purify protein by running through Nickel-sepharose column

Epitope Tagging



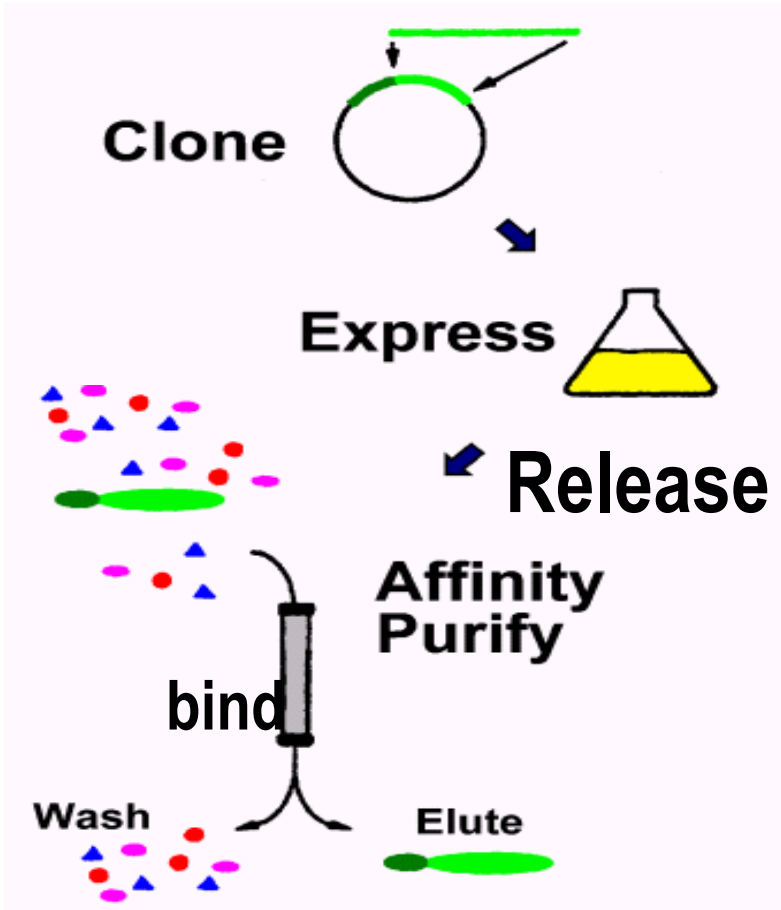
- addition of small antigenic tag
- 6-12 residues in length
- epitope for a given monoclonal antibody (mAb)
- can be used to purify protein, mostly for detection
  - westerns blotting
  - immunohistochemistry

Yeast Two-hybrid Assay



- used to seek protein-protein interactions
- test a library of tester proteins vs a "bait" fusion protein
- interaction brings DNA-binding and activation domains together: activation
- look to see which tester proteins activate transcription

Expression and purification of a fusion protein



Potential Problems

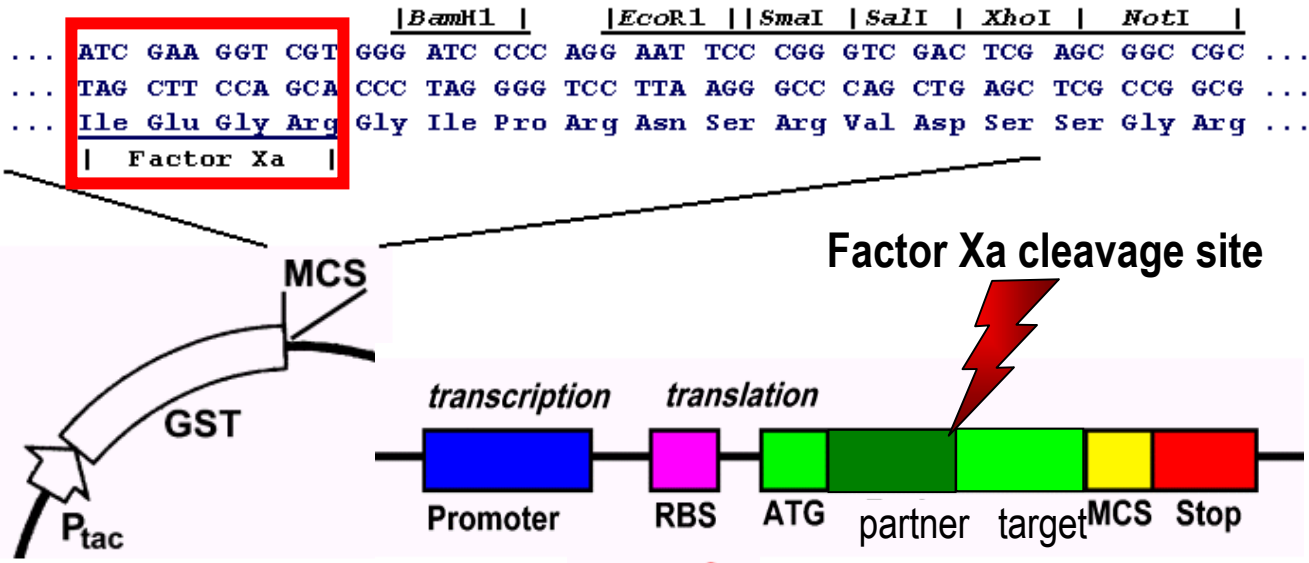
- cytotoxicity
- proteolysis
- inclusion bodies

} Solved by inducible expression system !!!

- Adverse effects of fusion partner on structure
    - denature (Urea) and re-fold (may not work!!!)
    - may not be able to use fusion
        - purify by more conventional separation methods
            - differential precipitation & centrifugation
            - chromatography

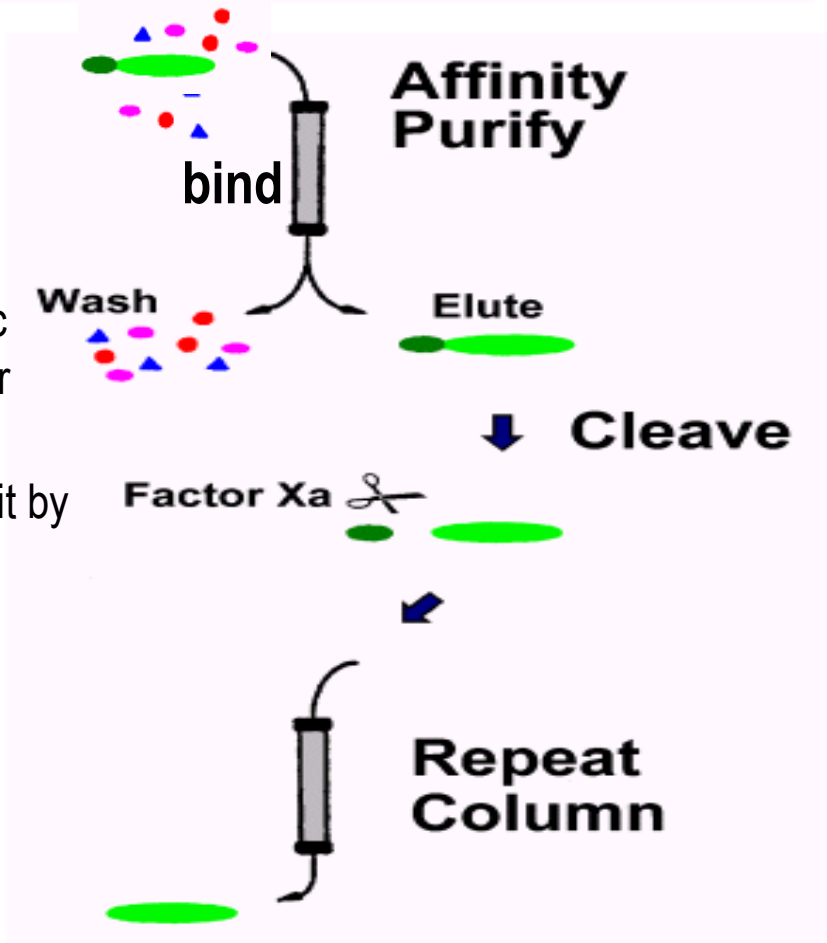
It was good while it lasted…getting rid of a fusion partner

Factor Xa cleavage site

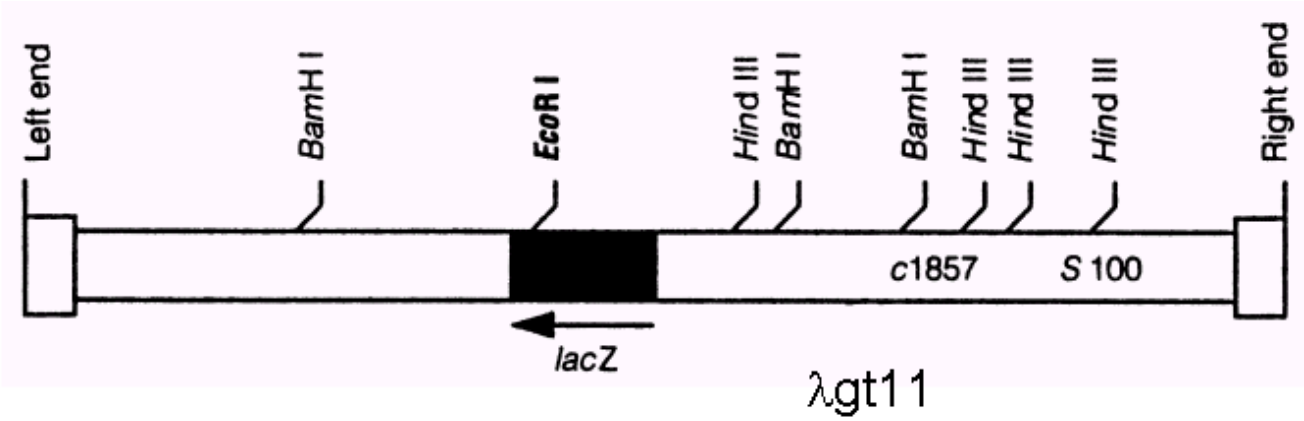


Some vectors carry an engineered site for proteolytic cleavage of the fusion partner

The fusion protein can be split by Factor Xa cleavage !!!!

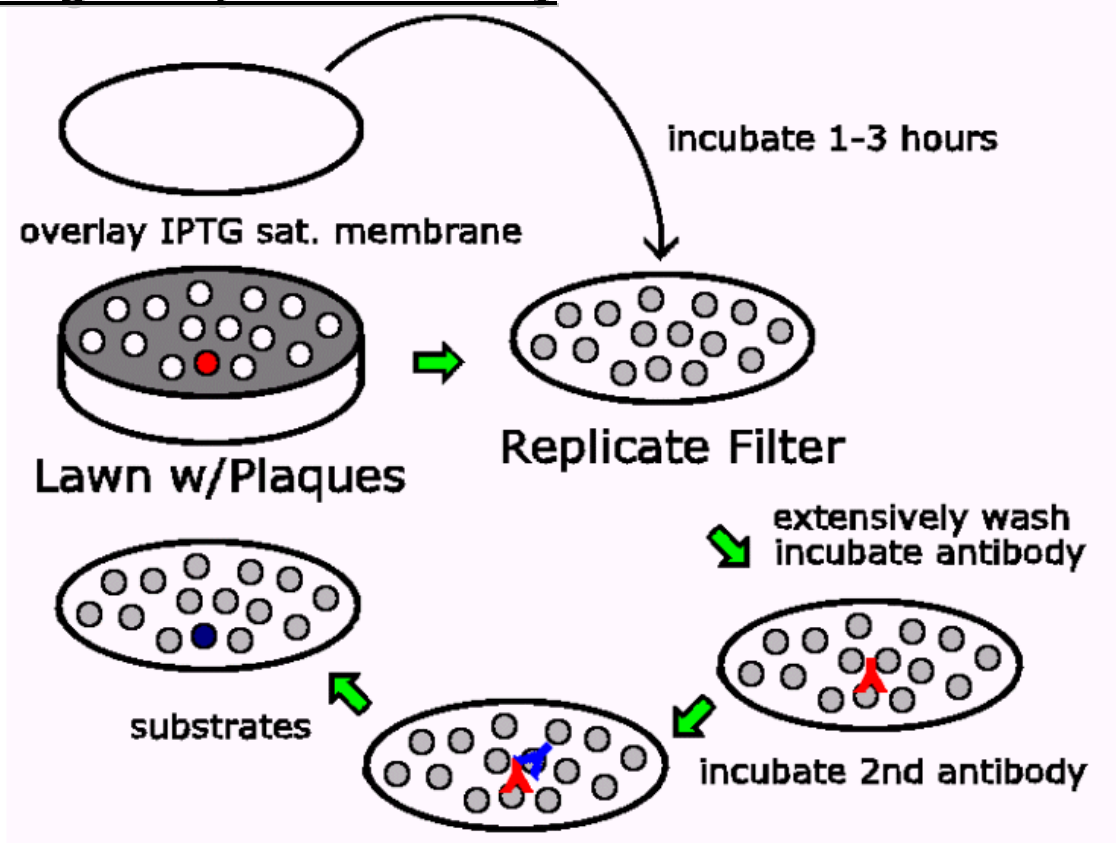


Expression Libraries



- prepare library in expression vector
  - cDNA better than gDNA
  - 6X possible reading frames
- screen with protein based probe
  - antibody/western blot
  - ligand binding or other activity

Screening an Expression Library



E. coli's great but….it's still a prokaryote

Problems with expression of eukaryotic proteins in prokaryotic expression systems:

- **genetic stability of the coding sequence**
- **protein stability once expressed**
- **potential problem with correct folding**
- **post translational modification machinery is very different**

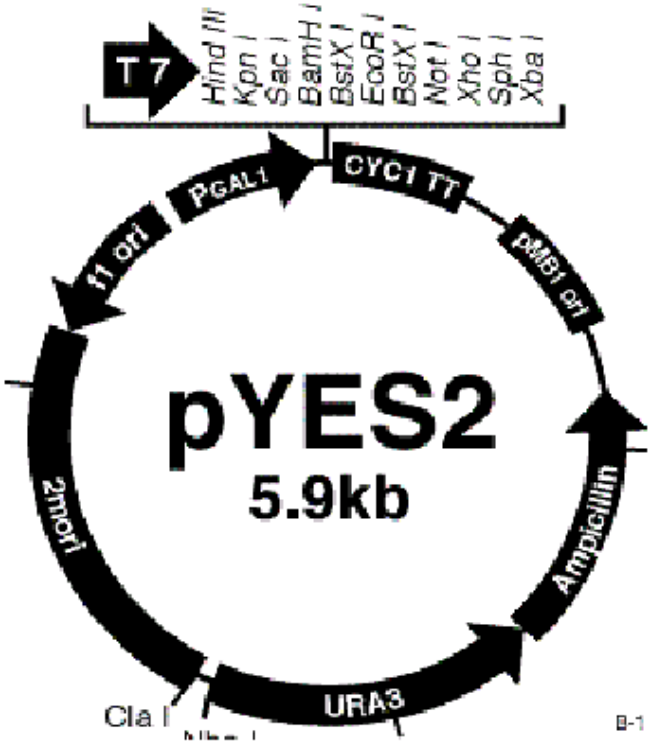
Eukaryotic Expression Systems

- in theory, plasmids can be introduced into any host
- yeast are easy to maintain in lab
  - *Saccharomyces cerevisiae*
  - *Pichia pastoris*
- viruses
  - several mammalian
  - baculovirus (insect)

vaccinia (lytic)
adenovirus (lytic)
papilloma (episomal)
retrovirus (integrated)

Shuttle Vectors

- ***E. coli*** **replication origin and selectable marker**
- **eukaryotic replication origin, selectable marker, promoters/enhancers, polyA signals**



PGal1	galactose-inducible promoter (yeast)
cyc1 TT	transcription terminator
pMB1 ori	*E. coli* origin of replication (pUC)
Amp^r	*E. coli* selectable marker
URA3	yeast selectable marker (ura3 host)
2μ ori	yeast origin of replication
f1 ori	ssDNA origin of replication
T7	phage promoter (in vitro transcription)

- for preparative purposes: exploit the ease of use of E. coli
  - make lots of plasmid quickly
- can be transformed into eukaryotic cells
  - yeast: LiCl transformation
  - mammalian: calcium phosphate
  - protoplast fusion
  - liposomes
  - electroporation
  - transformation
  - microprojectile
  - microinjection
- has all the sequences necessary for expression and translation

Protein Purification

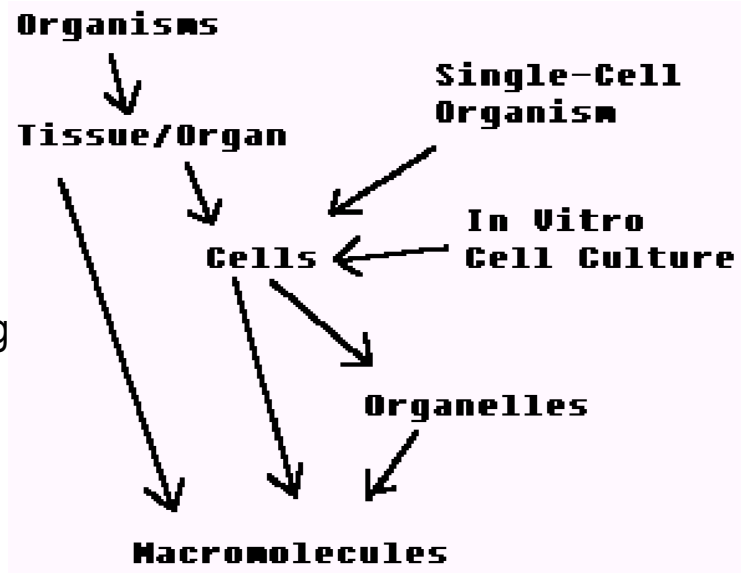
In order to work on a protein, you must isolate it and purify it

Why purify ?

- Need protein for functional or structural studies
- Need protein for industrial or pharmaceutical application
- Development of antibody for the protein
- Identification of protein sequence

How to Purify ?

- Develop method according to
    - Scale (analytical, medical, industrial)
    - Level of purity required (crude, highly purified, pyrogen-free)
    - Application (functional, structural, antibody, sequencing…)
        - native conformation vs non-native conformation
    - Source material (original, recombinant)



- For analytical purposes:
    - need lower levels of protein:
        - enzymology: 0.1-100 mg
        - crystallography: 0.1-1.0 g

- For industrial purposes:
    - therapeutic: 10g-100 kg
    - industrial: kg to tonnes

- Facilitate things by starting with “enriched” source
    - ie. recombinant source necessary for industrial uses!! (less expensive)

Protein Purification

- Need to extract protein from source material in soluble form
- Disrupt the source material (unless protein secreted outside cell)
- Separate protein of interest from others based on
    - physical characteristics
    - chemical characteristics

Cellular Disruption Methods

TECHNIQUE	COMMENTS
homogenation	Cells are placed in homogenizer or blender and disrupted by shear forces or grinding. Many different types of homogenizers are available depending on the application. Most consist of a pestle with a defined clearance and can be hand or motor driven. Homogenation can be combined with osmotic methods. Heating can be a problem.
presses	Cells are placed under high pressure in a stainless steel chamber. The chamber is opened while under pressure and the cells exit through a small orifice at a controlled rate. The rapid exposure to atmospheric pressure causes the cells to lyse. Optimal pressure for cell disruption needs to be determined empirically for each type of cell and experimental condition.
N_2-cavitation	Cells are placed under high pressure in a N_2 atmosphere. The pressure is suddenly released, causing the N_2 dissolved within cells to boil off and rupture the cells. Both the pressure and rate of pressure release can be controlled.
osmotic, hypotonic	Cells are placed in an isomolar solution of a permeable solute or a hypotonic solution. Water will enter cell which results in swelling until the membrane ruptures. The technique is gentle, but may not lyse cells with walls or rigid cytoskeletons(eg., bacteria, plants, etc.).
sonication	Ultrasonic waves are used to disrupt cells. Heating is a problem and the process tends to vesiculate membranes and subcellular compartments.
freeze-thaw	Ice-crystals formed during freezing will rupture cell membranes. It will generally take multiple cycles of freeze-thaw to disrupt all of the cells.
detergents	Detergents will disrupt the lipid bilayer allowing the contents to be released and will solubilize membrane proteins. Many detergents denature proteins and they are often difficult to remove.
chaotropic agents	Chaotropic agents solubilize proteins by disrupting the structure of water. Protein denaturation is a problem.
enzymatic	Bacteria, yeasts and plant cells are often treated with enzymes to remove the cell wall. Enzymatic treatment is often combined with other disruption methods.