Chapter 7

Heterologous Protein Production in Eukaryotic Cells

Problems of Prokaryotic Expression System

- Production of non-functional protein
  - Folding problem
    - Protein disulfide isomerase (PDI) for disulfide bond formation
  - Lacking proper posttranslational modification
    - Proteolytic cleavage
    - Glycosyltaion : 30% of mammalian proteins
      - O-linked : Ser, Thr
      - N-linked : Asn
    - Phosphorylation
    - Acetylation
    - Sulfation
    - Acylation
    - Addition of fatty acids
      - Myristoylation (myristylation) C_{14}
      - Palmitoylation (palmytylation) C_{16}
- Contamination with toxic compounds (pyrogens)

Eukaryotic Expression Vector

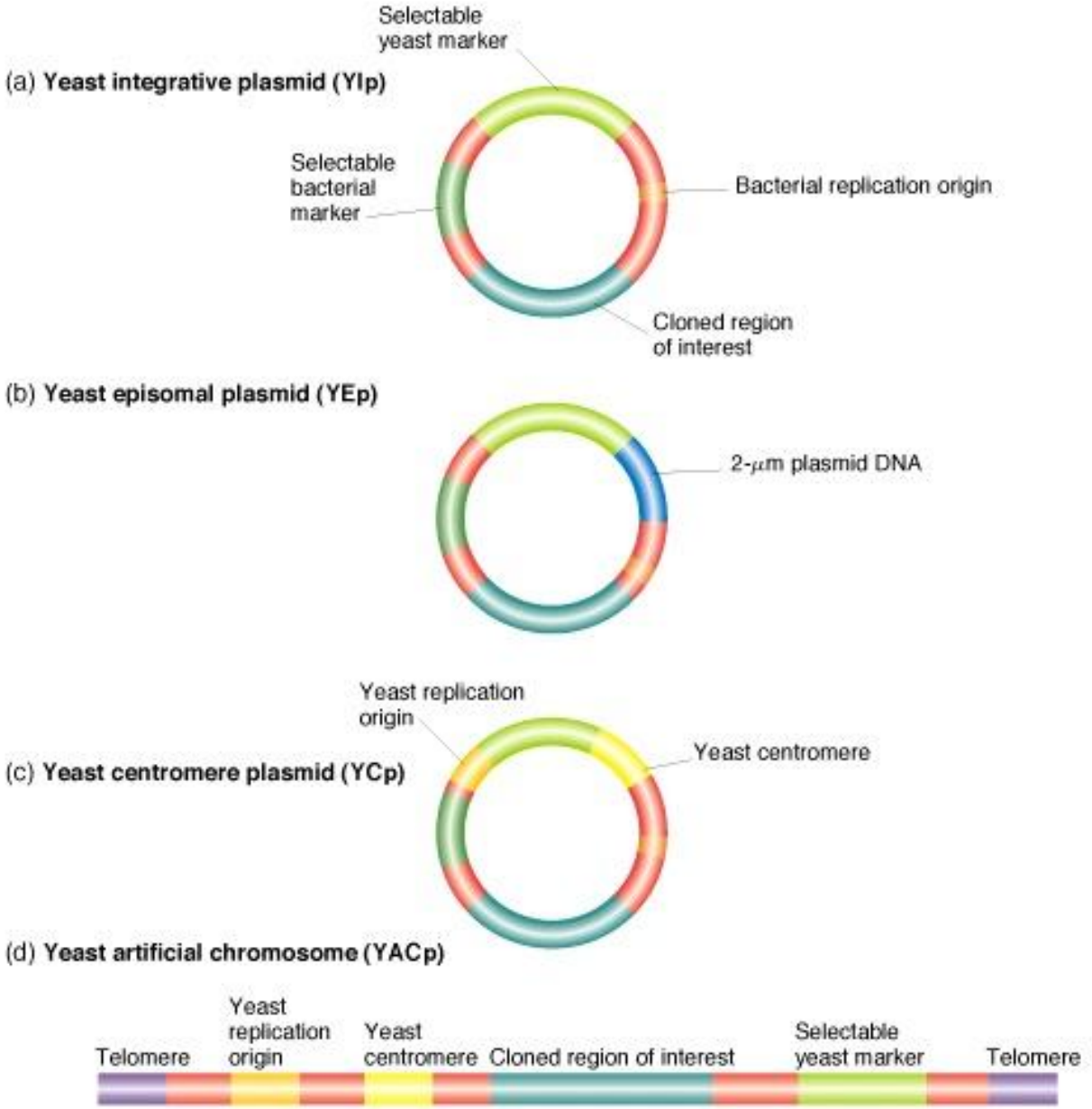
- Component of shuttle vectors
  - Eukaryotic promoter and terminator
  - Markers (both for *E. coli* and eukaryotes)
  - Replication origins for *E. coli* and eukaryotes (optional)
- Terminology of DNA introduction
  - Transformation
    - Introduction of DNA into *E. coli* or yeast
  - Transfection
    - Introduction of DNA into mammalian cells
      - Transformation of mammalian cells → becoming cancerous cells

7.1. *Saccharomyces cerevisiae* Expression Systems

Saccharomyces cerevisiae Expression Systems

- Advantage of *S. cerevisiae*
  - Well known genetics and physiology
  - Easy to grow
  - Strong promoters characterized
  - Naturally occurring 2μ plasmid
  - Posttranslational modification
  - Secretion of so few proteins
  - Generally recognized as safe (GRAS) organism
    - Suitable for production of vaccines, pharmaceuticals

S. cerevisiae Vectors

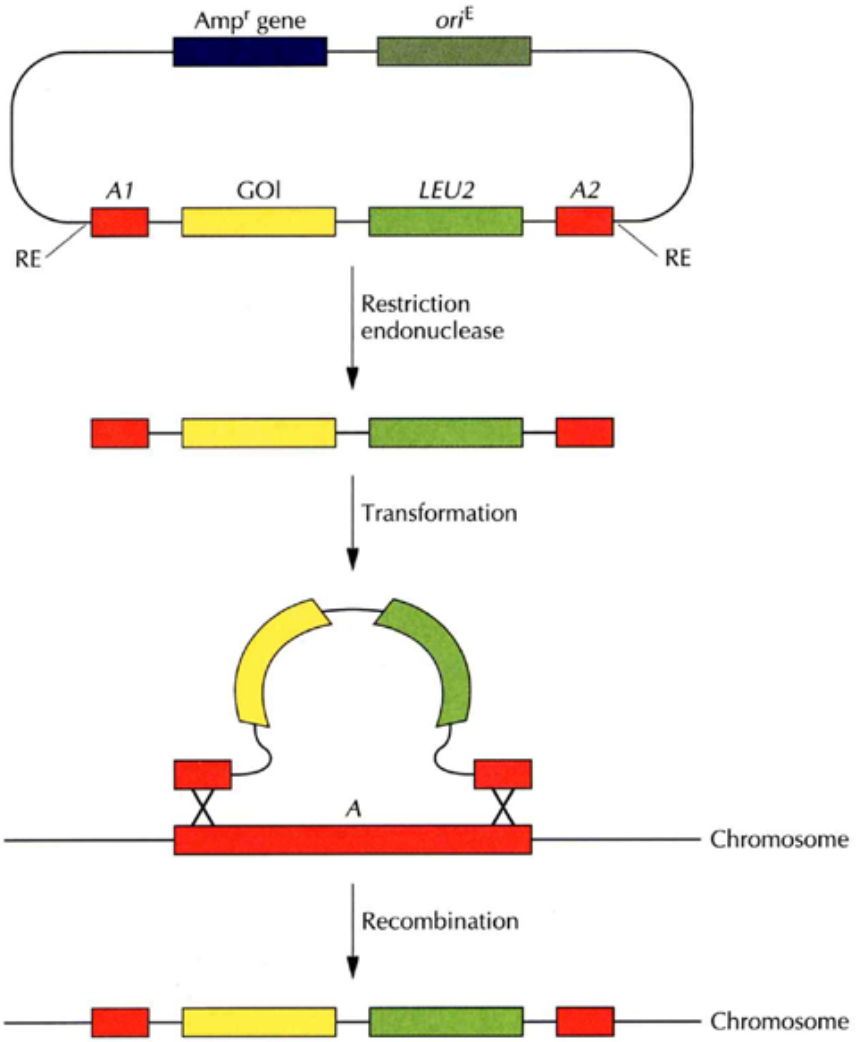


S. cerevisiae Vectors

- Selection markers
  - Genes for amino acids (His, Trp, Leu) or nucleotide (Uracil) synthesis
  - Use auxotropic strains for the selection marker
- Promoters
  - Inducible promoters
    - Gal promoter: Galactose inducible (1000X)
    - CUP1 (metallothionein) : copper inducible
  - Constitutive promoters
    - ADH1 (alcohol dehydrogenase)
    - GPD (glyceraldehyde-3-phosphate dehydrogenase)

Integration of DNA with YIp vector

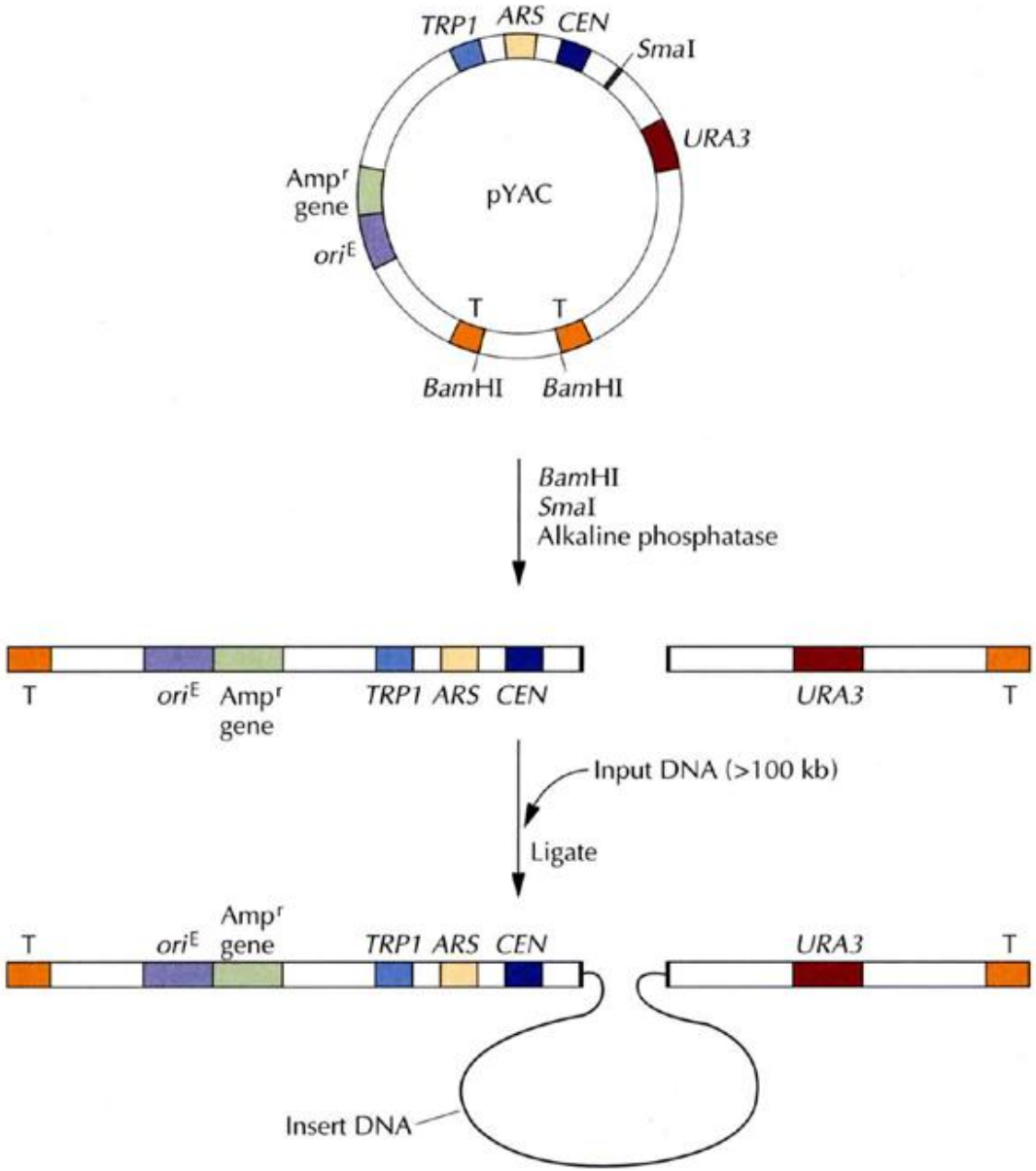
- Multiple integration into repetitive DNA sequences
  - δ sequence derived from retrotransposon



Yeast Artificial Chromosome (YAC)

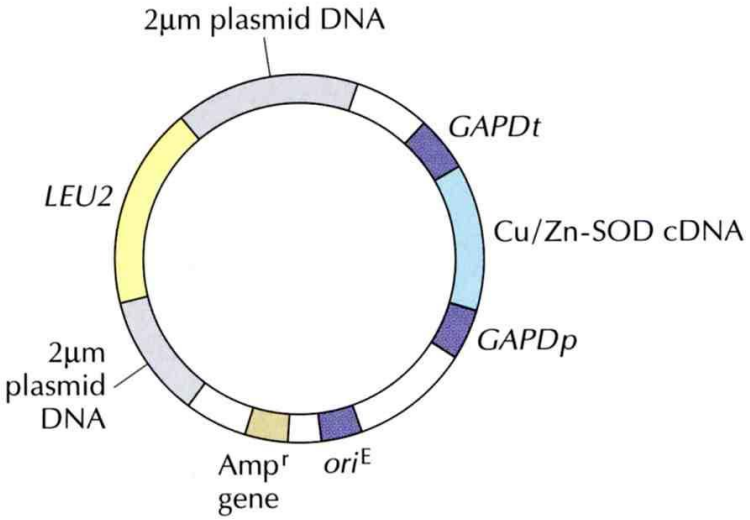
- Usage of YAC
  - To clone big DNA (100 kb)
  - Physical mapping of human genomic DNA
- Components
  - Yeast replication origin
    - Autonomous replicating sequence (ARS)
  - Yeast centromere
  - Telomeres to maintain chromosome stability

YAC


TRP1
ARS
CEN
SmaI
URA3
Amp^r gene
ori^E
pYAC
T
T
BamHI
BamHI
BamHI
SmaI
Alkaline phosphatase
T
ori^E
Amp^r gene
TRP1 ARS CEN
URA3
T
Input DNA (>100 kb)
Ligate
T
ori^E
Amp^r gene
TRP1 ARS CEN
URA3
T
Insert DNA

Intracellular Production of Heterologous Proteins in *S. cerevisiae*

- Production of Cu/Zn-SOD
  - Superoxide dismutase eliminating superoxide radical by generating H_2O_2 with H_2
  - Administration during blood reperfusion
  - Therapeutic use for inflammatory diseases
- Production of Cu/Zn-SOD in yeast
  - Proper acetylation of N-terminal ala



Secretion of Heterologous Proteins by *S. cerevisiae*

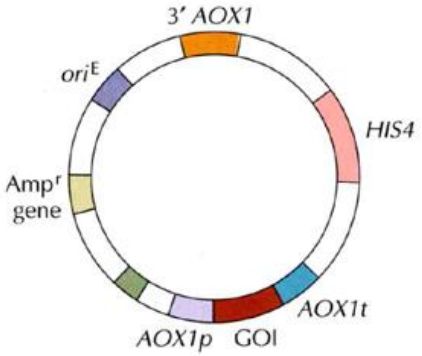
- Secretory proteins in yeast
  - All the glycosylated proteins
  - Containing leader sequence
- Addition of leader sequence for secretion
  - Leader sequence of mating type factor α
  - Lys-Arg adjacent to starting amino acid
- Strategies to enhance the secretion of recombinant proteins
  - Overexpression of PDI (protein disulfide isomerase)
    - Increase secretion of proteins with disulfide bond

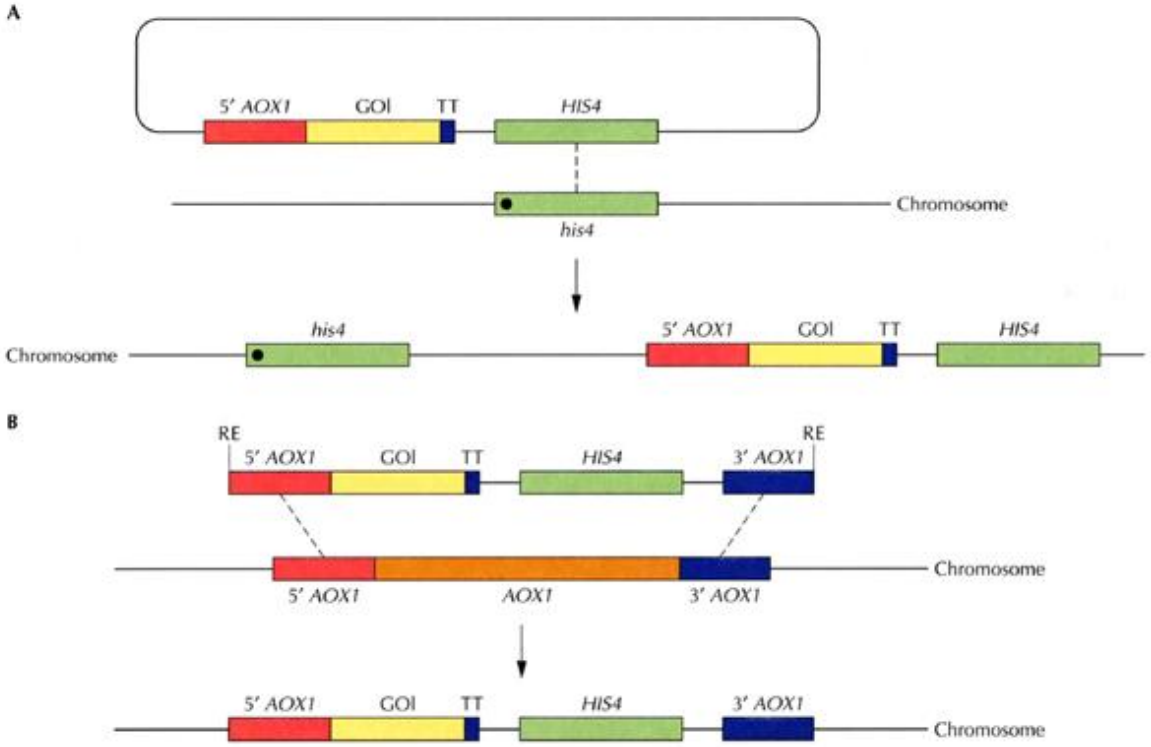
Pichia pastoris and Other Yeast Expression Systems

- Disadvantages of *S. cerevisiae*
  - Hyperglycosylation
  - Incomplete secretion
  - Production of toxic ethanol at high density growth
- *P. pastoris*
  - Methylotrophic yeast
  - Strong methano-inducible gene encoding alcohol oxidase (AOX1)
  - No ethanol production → high cell density
  - Secretion of very few proteins
- Other yeast systems
  - *Hansenula polymorpha*
    - Methylotrophic yeast
  - *Candida utilis*
  - *Aspergillus*
    - Filamentous fungus

P. pastoris Expression Vector

- Integrating vector for protein expression
  - By single or double cross over

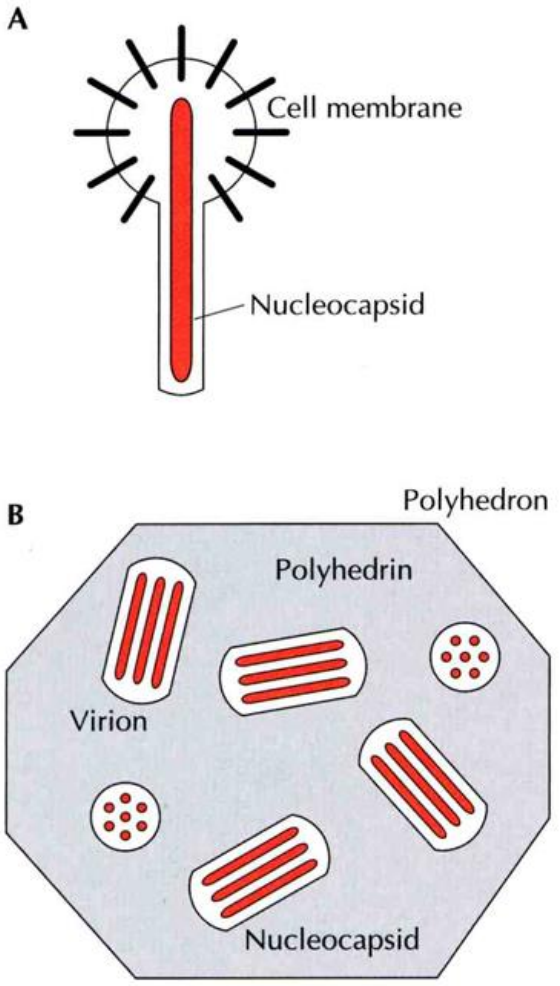




7.2. Baculovirus-Insect Cell Expression System

Baculovirus-Insect Cell Expression System

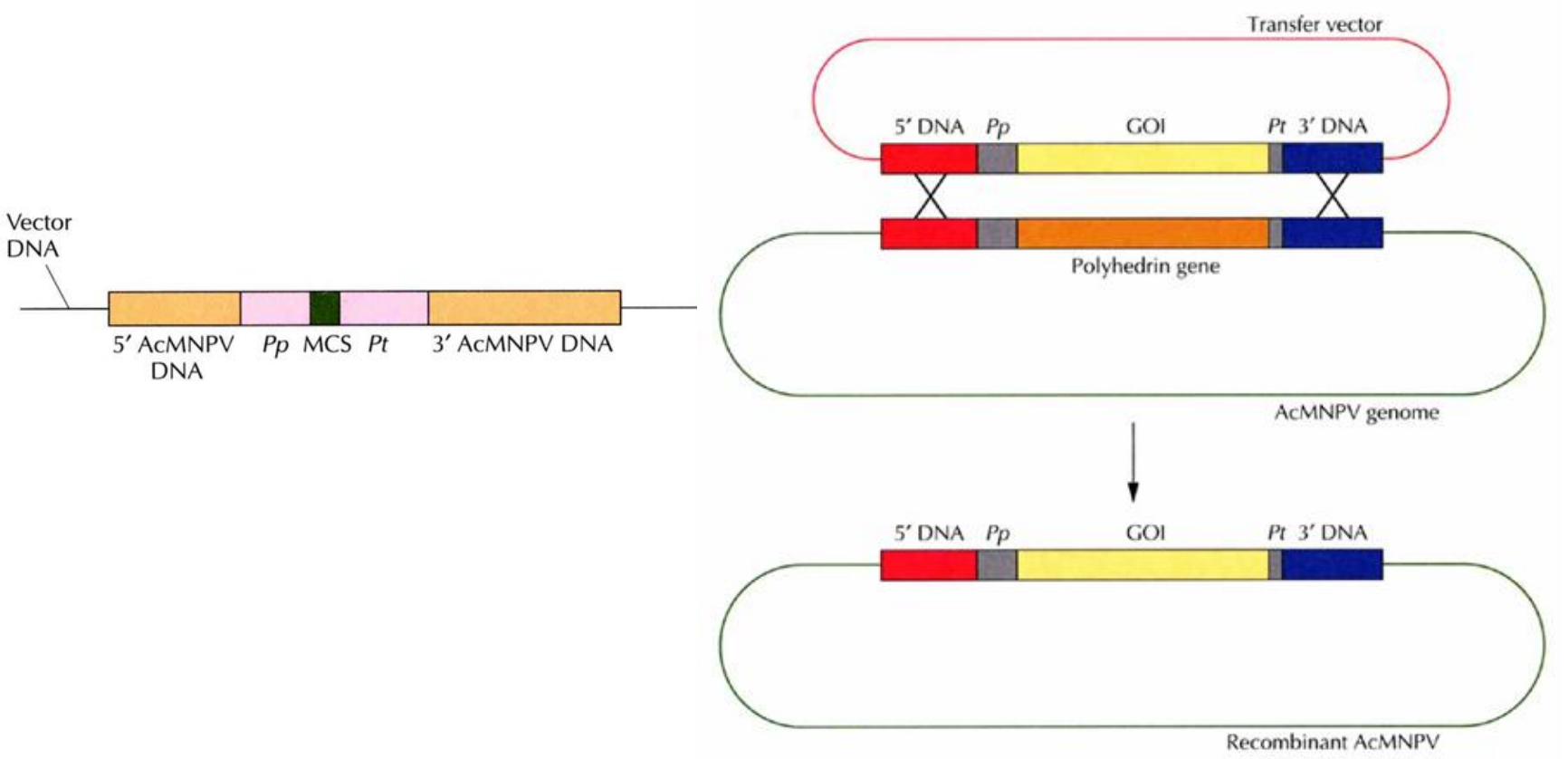
- Baculovirus
  - Infect invertebrates
  - Two forms
    - Single nucleocapsid (virus particle)
      - Budding off from an infected cell
    - Polyhedron
      - Clusters of nucleocapsids (virions) trapped in a protein matrix polyhedrin
      - Release after cell lysis



Baculovirus Expression Vector System

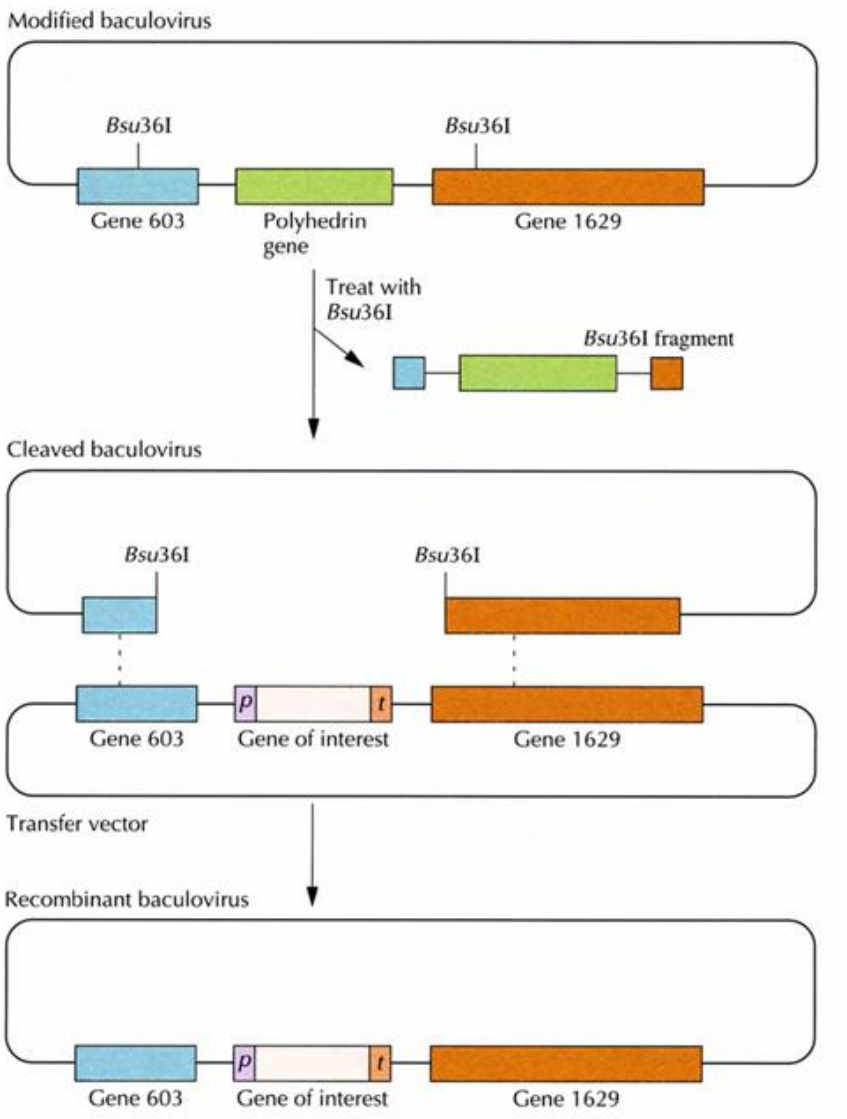
- Baculovirus system
  - AcMNPV: Autogrpha californica multiple nuclear polyhedrosis virus
  - Spodoptera frugiperda (Sf) cells for infection
- Protein expression using baculovirus system
  - Integration of GOI into AcMNPV DNA
    - Transfer vector containing GOI
      - Expression of GOI under the control of polyhedrin (polyh) gene promoter
    - Cotransfection of transfer vector and AcMNPV DNA
    - Isolation of recombinant clones
      - Occlusion-negative plaques
      - PCR
      - Introduction of lacZ to identify recombinant cones

Baculovirus Expression Vector System



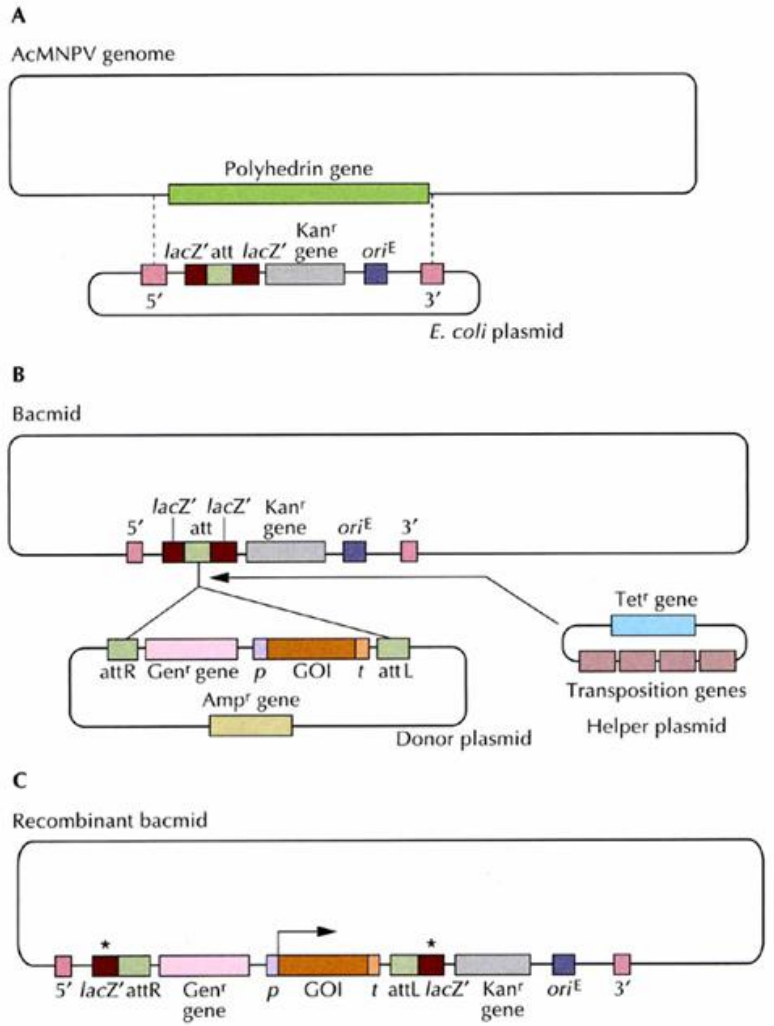
Increasing the Yield of Recombinant Baculovirus

- Linearization of AcMNPV genome before infection
  - Reduced infectivity
  - Recovery of infectivity by recombination with transfer vector
- Disruption of an essential gene



Construction of *E.coli*-Insect Cell Baculovirus Shuttle Vector

- Generaiton of recombinatn bacmid in *E. coli*
  - Using transposition
  - Identification of recombinant clones by lacZ selection
- Infection of insect cells for protein production
  - Provide a-2,6-sialyltransferase gene for proper glcosylation
  - Introduction of genes for processing enzymes



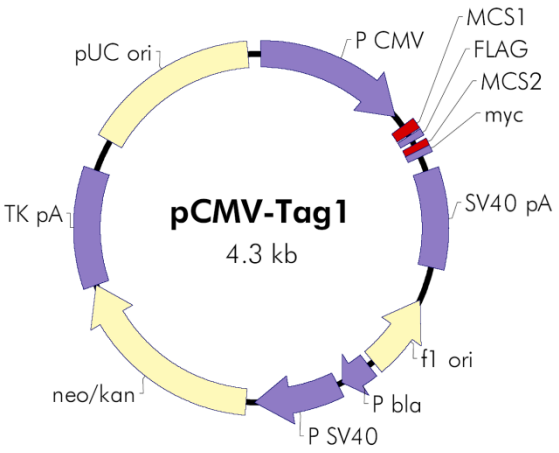
7.3. Mammalian Expression System

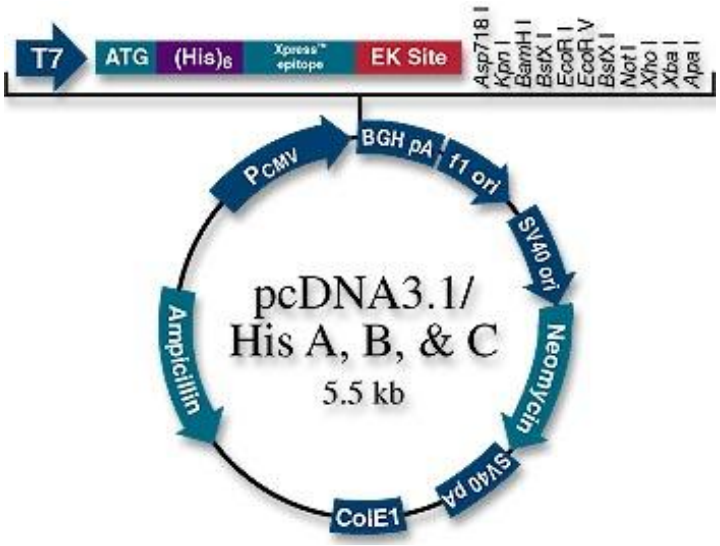
Mammalian Cell Expression System

- Cell lines
  - For short term expression
    - COS: from African green monkey kidney
    - BHK: baby hamster kidney
    - HEK-293: human embryonic kidney
  - For long term (stable) gene exrpession
    - CHO:Chinese hamster ovary

Types of Plasmid Vectors

- Non-replicating plasmid vectors
- Replicon vectors
  - SV40 (lytic virus) replication origin
    - High copy number (10^5/cell)
  - BK, BPV (latent virus) replication origin
    - Low to moderate copy number



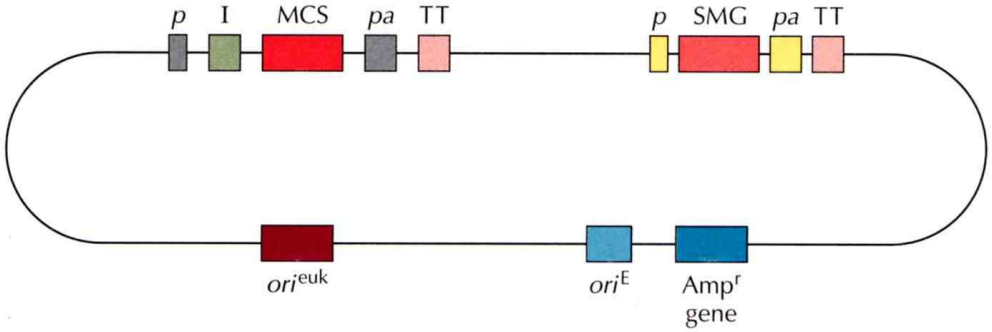


Types of Transfection

- Transient transfection
  - Maintenance of transfected DNA as extrachromosomal state until it is diluted or degraded
- Stable transformation
  - Integration of DNA into host chromosome
    - Formation of cell line
  - Maintenance as an episome

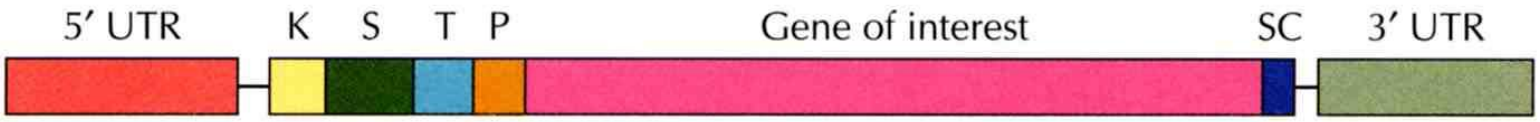
Mammalian Expression Vectors

- Eukaryotic replication origin
  - SV40 (simian virus 40)
  - Requires T antigen for replication
- Eukaryotic promoter and transcription terminator (polyadenylation signal)
  - Human virus
    - Cytomegalovirus
    - SV40
    - Herpes simplex virus
  - Mammalian genes
    - β-actin
    - Metallothionein
    - Thymidine kinase
    - Bovine growth hormone
- Intron
  - Between promoter and MCS
  - Increase expression



Translation Control Elements

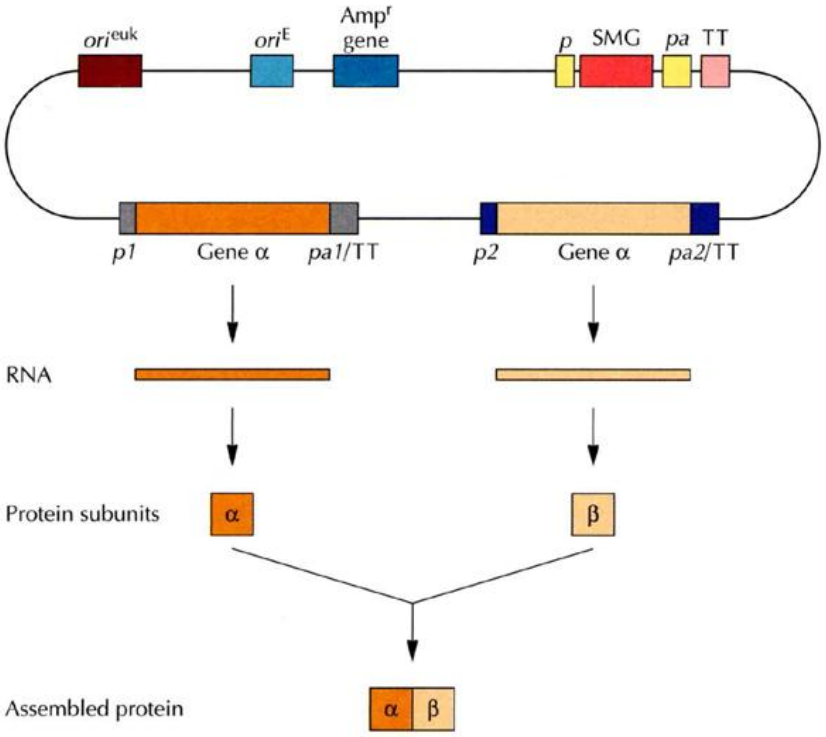
- 5’ and 3’ UTR
  - For efficient translation and mRNA stability
- Kozak sequence
  - Required for translation initiation
  - CC(A or G) CCAUGC
- Signal sequence
- Tagging sequence
- Proteolytic cleavage site



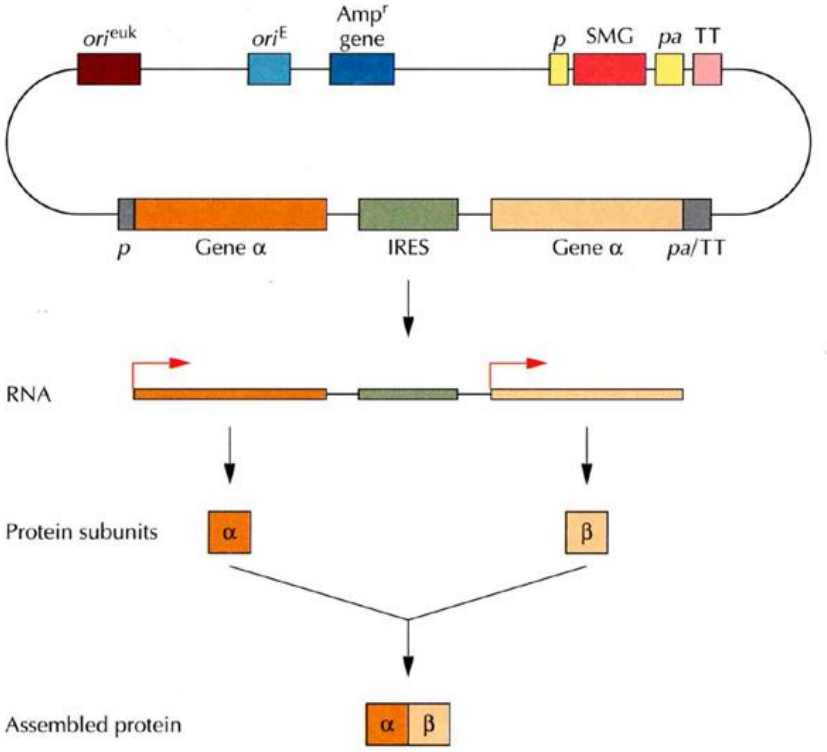
Expression of Two Genes

- Two-vector expression
  - Cotransfection of two plasmids
- One-vector expression
  - Two-gene expression vector
    - Expression of two genes by independent promoters
  - Bicistronic vector
    - Two genes are separated by an internal ribosomal entry site (IRES) from mammalian virus

Expression of Two Genes



Two-gene expression vector



Bicistronic vector

Selectable Markers

- Neo
  - Neomycin phosphotransferase
  - G-418 (geneticin) for eukaryotic cells
- Selection to increase copy number of the plasmid
  - DHFR (dihydrofolate reductase)- MTX(methotrexate) system
    - DHFR: Required for purine synthesis
    - MTX: Competitive inhibitor of DHFR
    - High MTX concentration
      - Cells producing excess DHFR survive
  - Glutamine synthetase (GS)- methionine sulfoximine (MSX) system
    - MSX inhibits GS
      - Cells producing excess GS survive