Biotechnologie Moléculaire

3ème année

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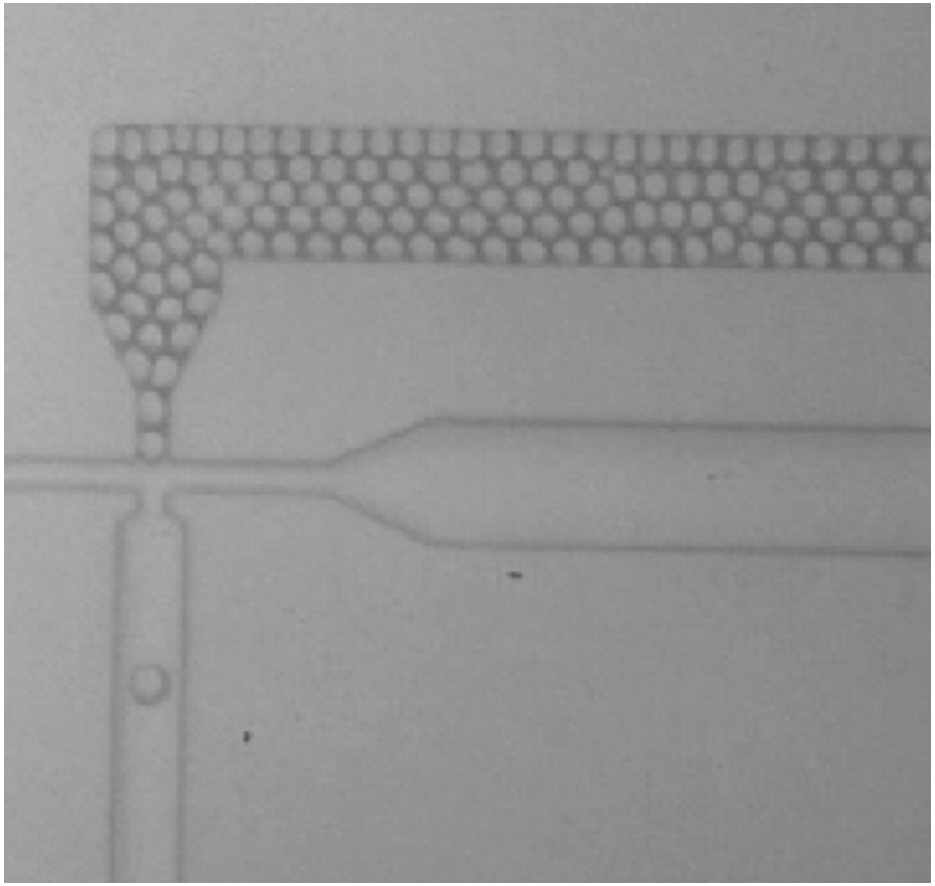

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All Powerpoint presentations and supporting material (animations/ movies) available on the intranet:

https://cours.espci.fr/site.php?id=258

Use your normal intranet username and password

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3ème année

URL for films from Cold Spring Harbor Dolan DNA learning Center:

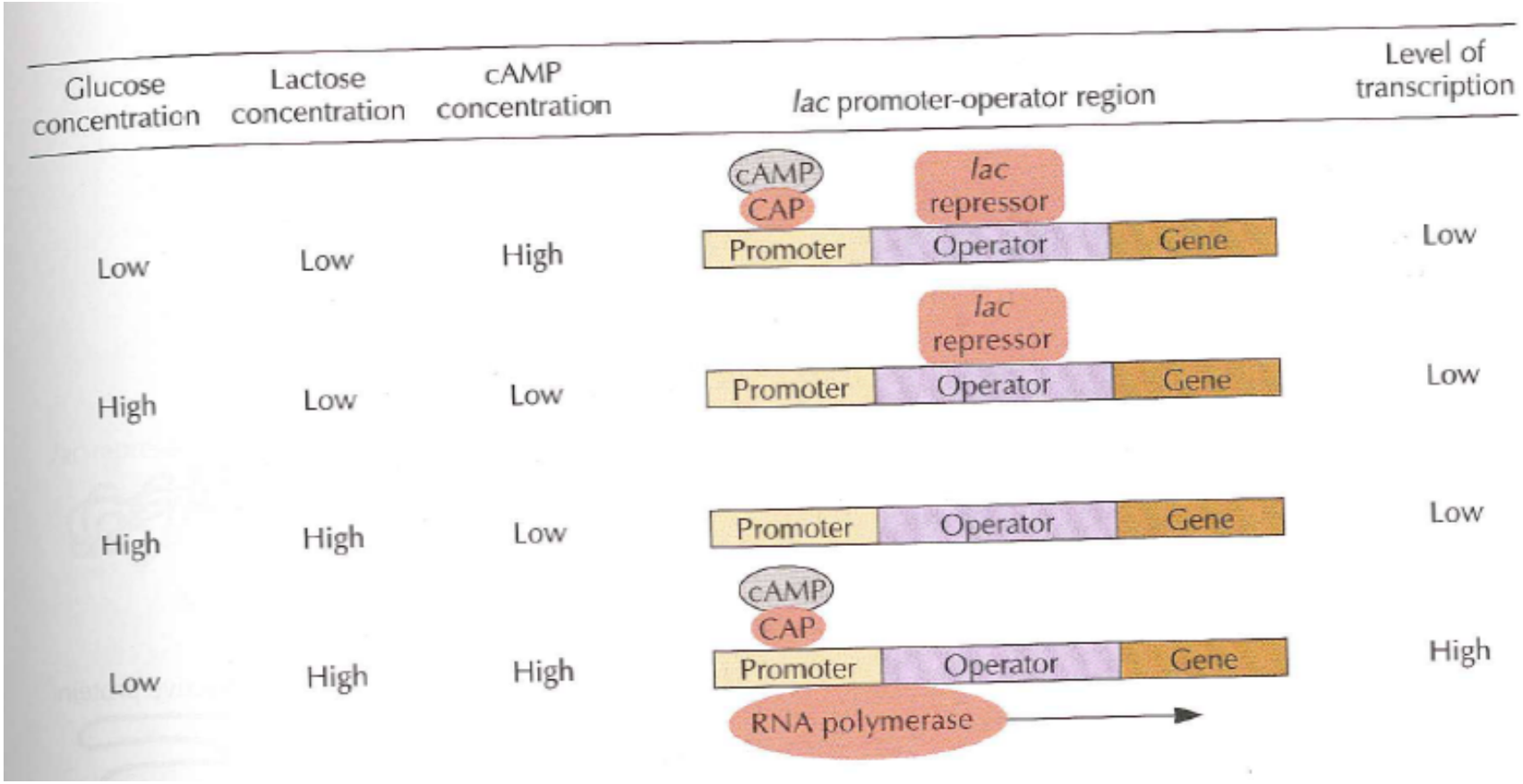
http://www.dnalc.org/resources/animations/

You can download the films for PC or for Mac

Manipulation of Gene Expression in Prokaryotes

Gene Expression from Strong and Regulatable Promoters

The *E. coli lac* promoter

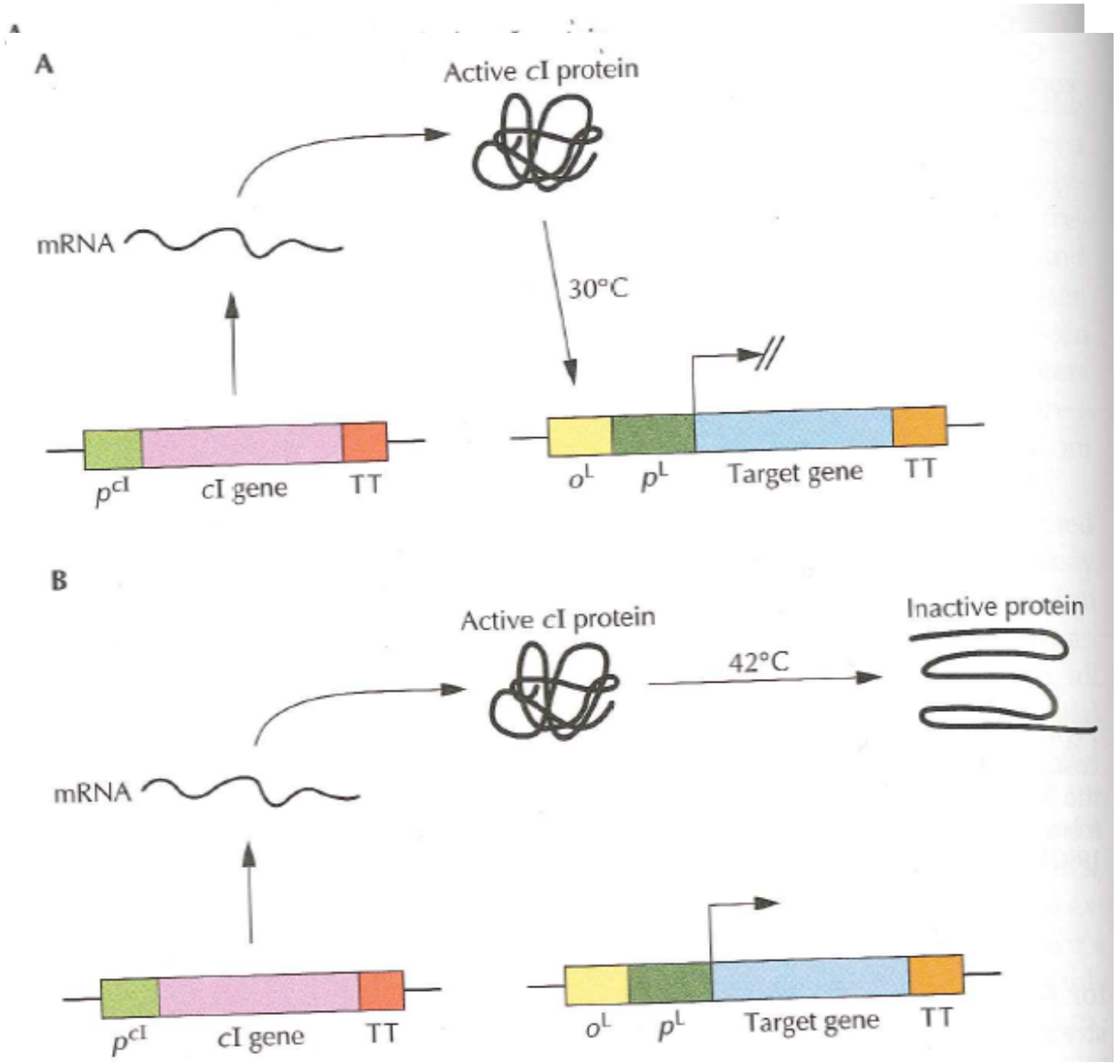


Effect of the concentrations of glucose, lactose and cAMP on the level of transcription (cAMP concentration is high when the glucose level is low)

IPTG, a non-metabolisable analogue of lactose, can also be used for induction

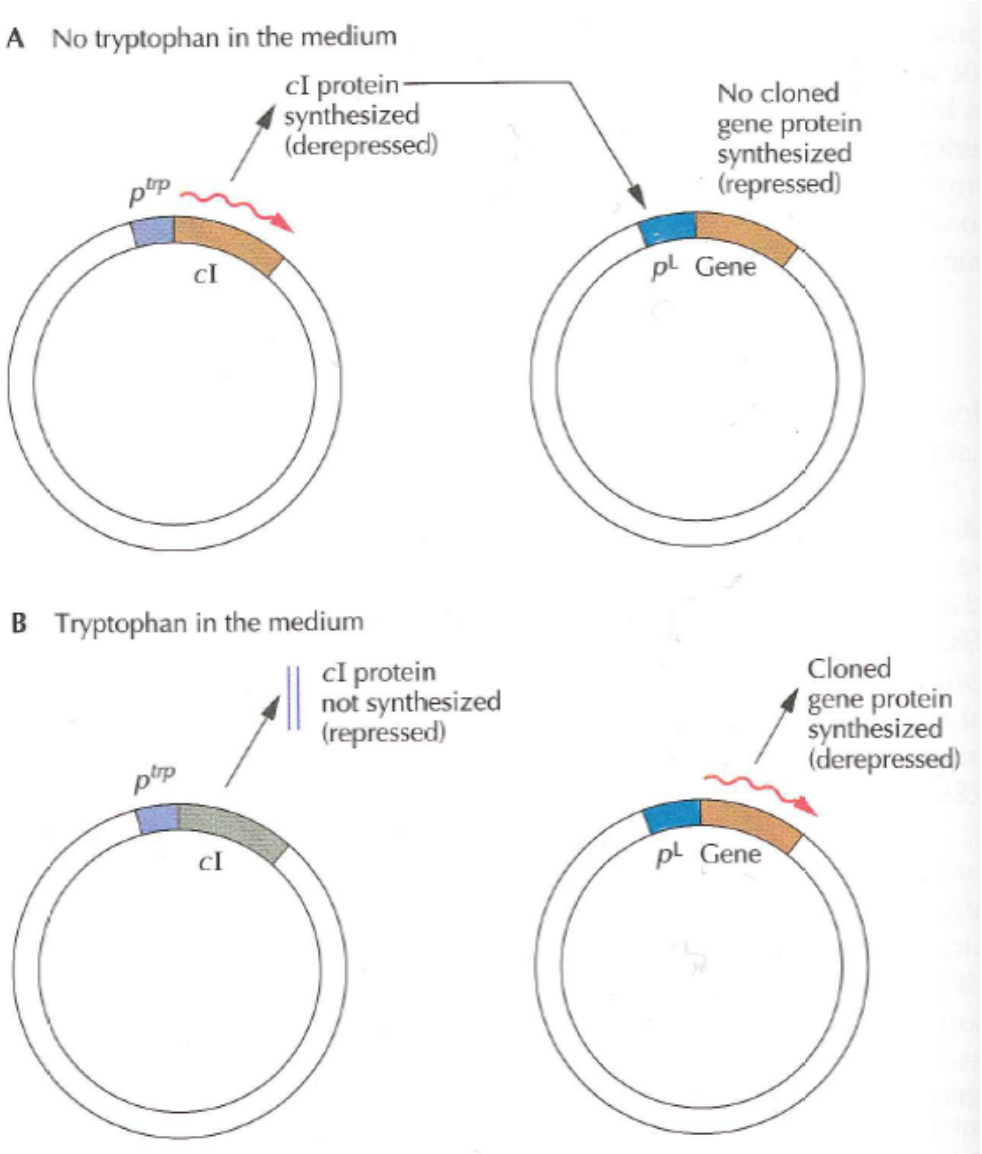
Gene Expression from Strong and Regulatable Promoters

Regulation of gene expression by the phage λ p^L promoter



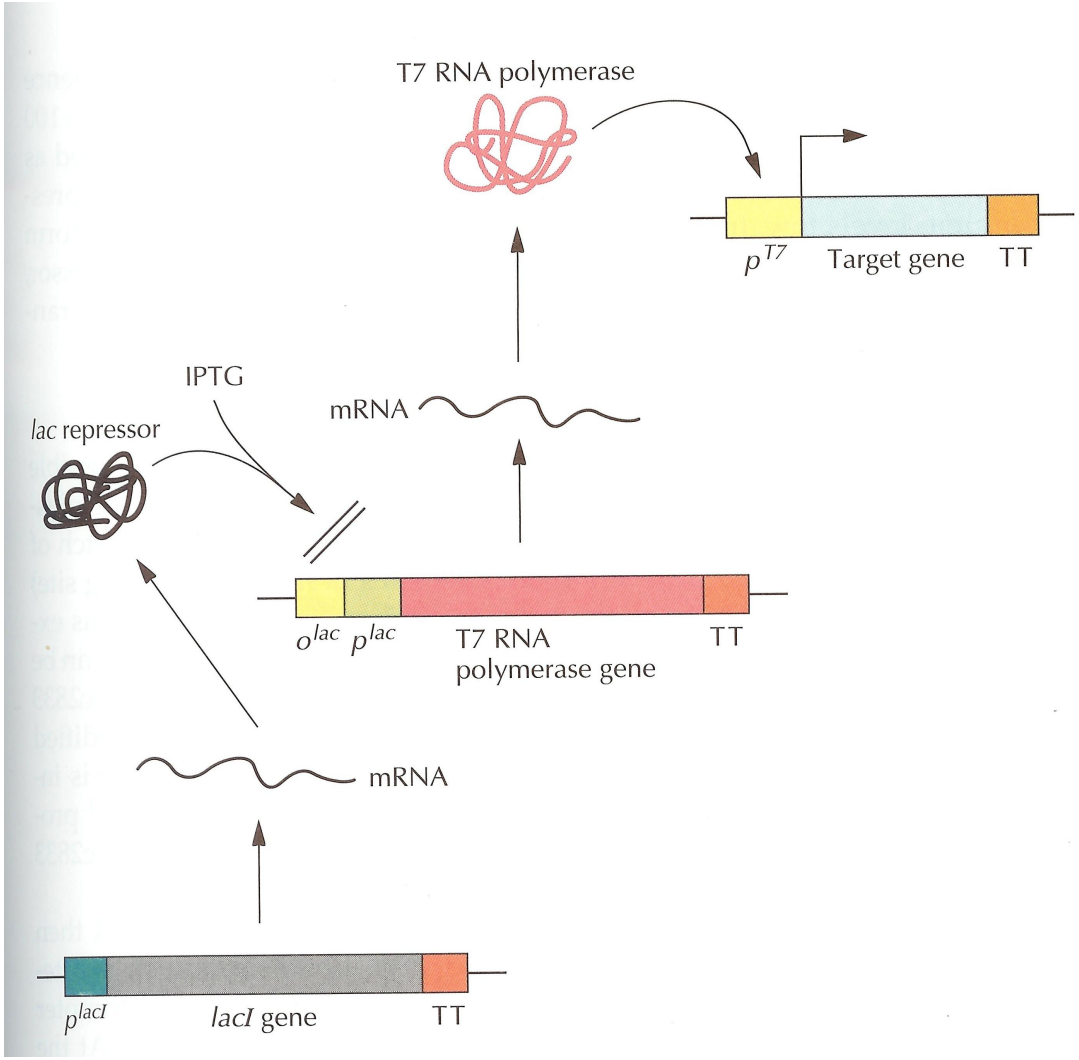
Gene Expression from Strong and Regulatable Promoters

Dual plasmid systems for controlling the phage λ p^L promoter



Gene Expression from Strong and Regulatable Promoters

Regulation of gene expression by the bacteriophage T7 promoter



Gene Expression from Strong and Regulatable Promoters

Expression in microorganisms other than *E. coli*

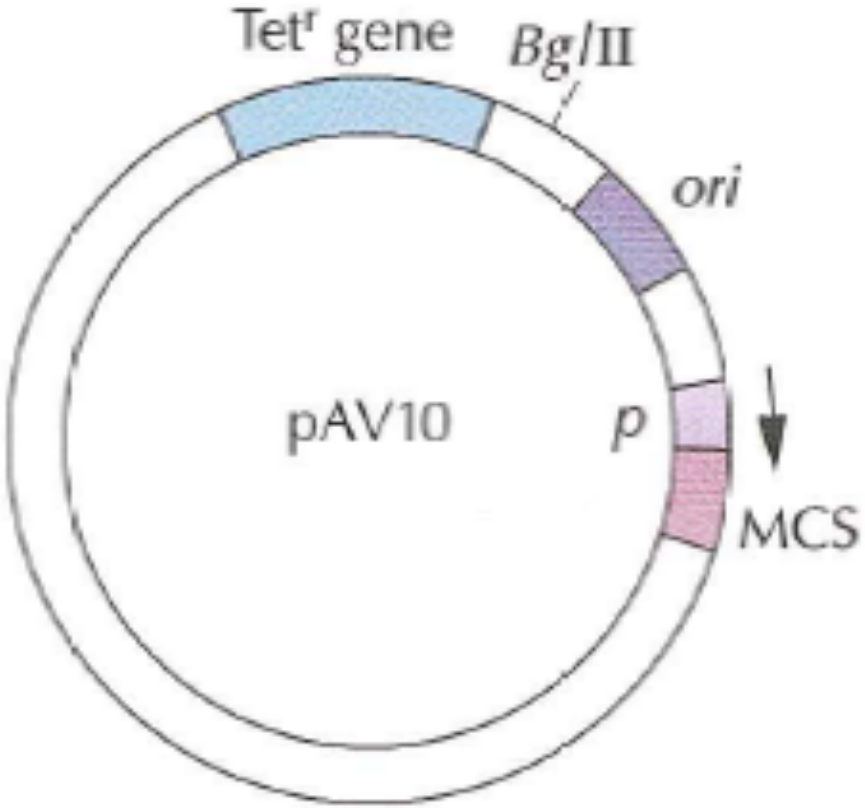
Promoter	β-Galactosidase activity (U) in:			
	Escherichia coli	*Rhizobium meliloti*	*Rhizobium leguminosarum*	*Pseudomonas putida*
None	16	110	130	150
Nm	1,400	21,800	13,900	16,300
lac	2,000	9,050	6,250	9,800
tac	11,300	2,850	1,150	2,950
S1	40	3,300	1,200	3,350

Adapted from Labes et al., *Gene* 89:37–46, 1990.

β-galactosidase expressed by various gram-negative bacteria

Gene Expression from Strong and Regulatable Promoters

"Universal" gram-negative bacterial expression vectors



Uses two overlapping promoters (*p*) from transposon 5 (Tn5).
Allows efficient expression in *E. coli* and 6 other species

Fusion Proteins

DNA constructs can be made which express a foreign protein fused to another protein (on a single polypeptide)

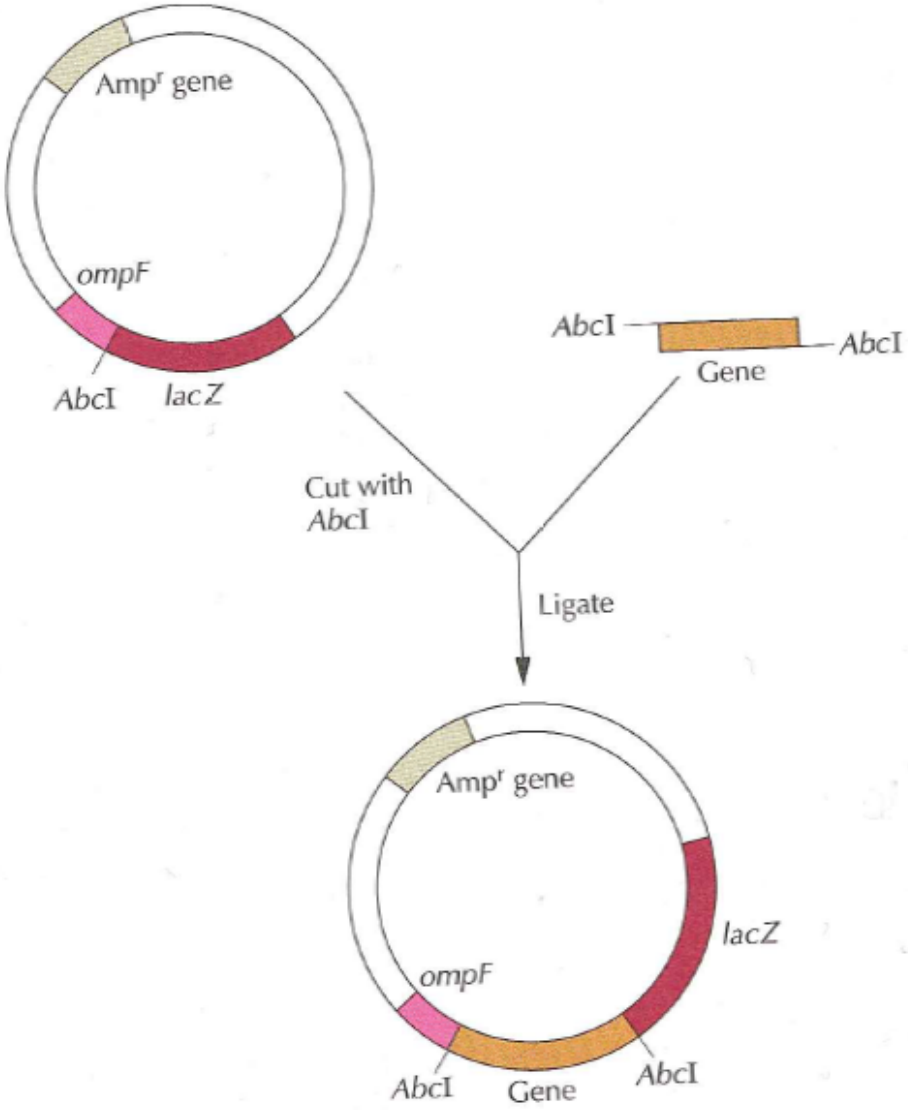
This can be useful to:

- Improve the expression level of the foreign protein

- Facilitate purification of the foreign protein

Fusion Proteins

An example of a fusion protein cloning vector



After transcription and translation a tribrid protein is produced comprising:

1.The N-terminal region of the outer membrane protein (*ompF)*
2.The cloned gene (gene)
3.A truncated β-galactosidase gene (*lac*Z)

Fusion Proteins

To facilitate the purification of foreign proteins in *E. coli*

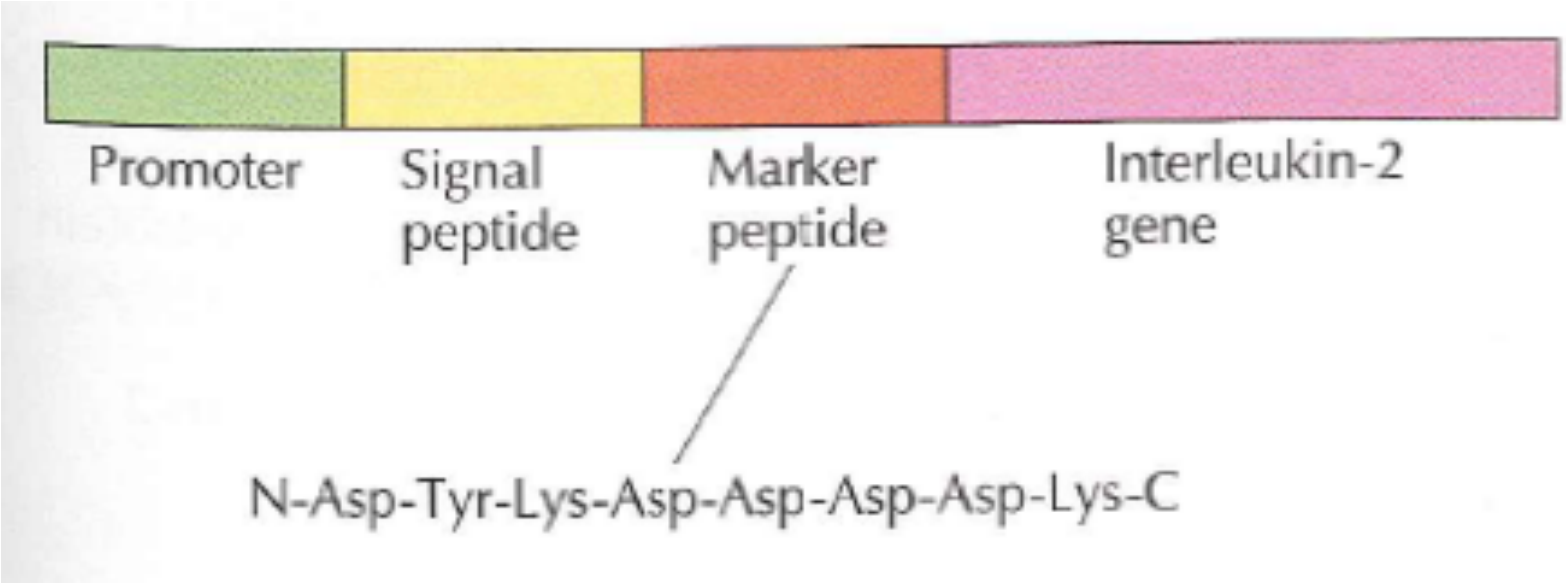
Fusion partner	Size	Ligand	Elution condition
ZZ	14 kDa	IgG	Low pH
His tail	6–10 aa	Ni^{2+}	Imidazole
Strep-tag	10 aa	Streptavidin	Iminobiotin
PinPoint	13 kDa	Streptavidin	Biotin
MBP	40 kDa	Amylose	Maltose
β-Lactamase	27 kDa	Phenyl-boronate	Borate
GST	25 kDa	Glutathione	Reducing agent
Flag	8 aa	Specific MAb	Low calcium

Adapted from Nygren et al., *Trends Biotechnol.* **12**:184–188, 1994.

Abbreviations: aa, amino acids; kDa, kilodaltons; ZZ, a fragment of *Staphyloccus aureus* protein A; His, histidine; Strep-tag, a peptide with affinity for streptavidin; PinPoint, a protein fragment which is biotinylated in vivo in *E. coli*; MBP, maltose binding protein; GST, glutathione *S*-transferase; Flag, a peptide recognized by enterokinase; MAb, monoclonal antibody.

Fusion Proteins

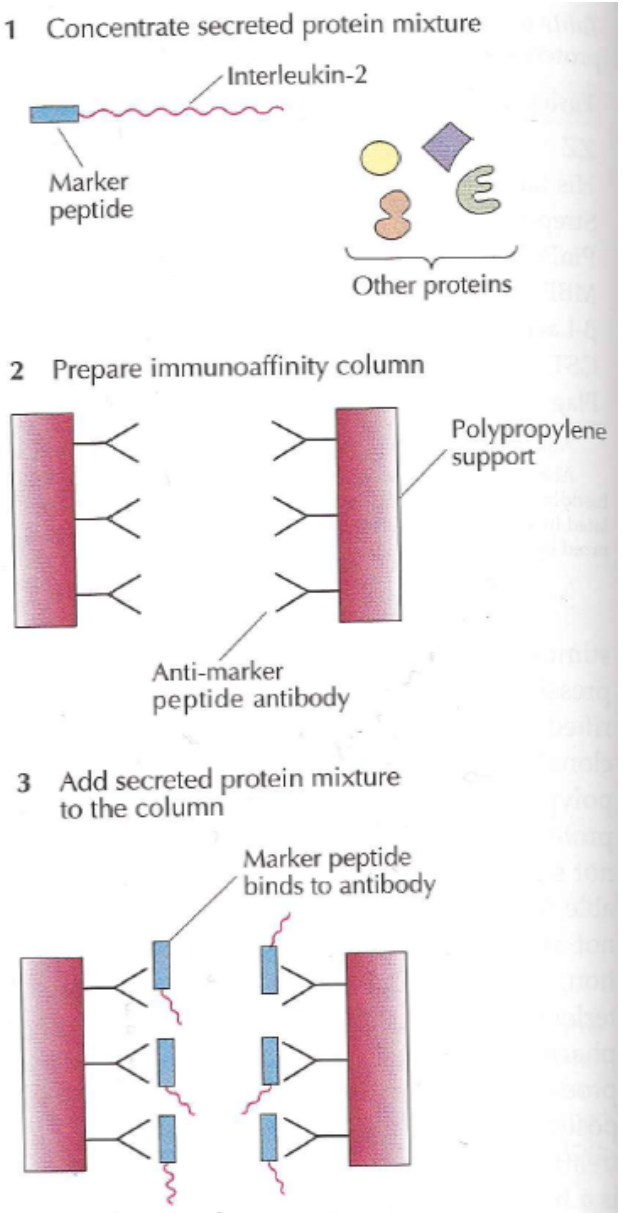
To facilitate the purification of foreign proteins in *E. coli*



The genetic construct used to produce a fusion protein consisting of a marker peptide and interleukin-2

Fusion Proteins

To facilitate the purification of foreign proteins in *E. coli*



Immunoaffinity purification of a fusion protein

Increasing Expression Level

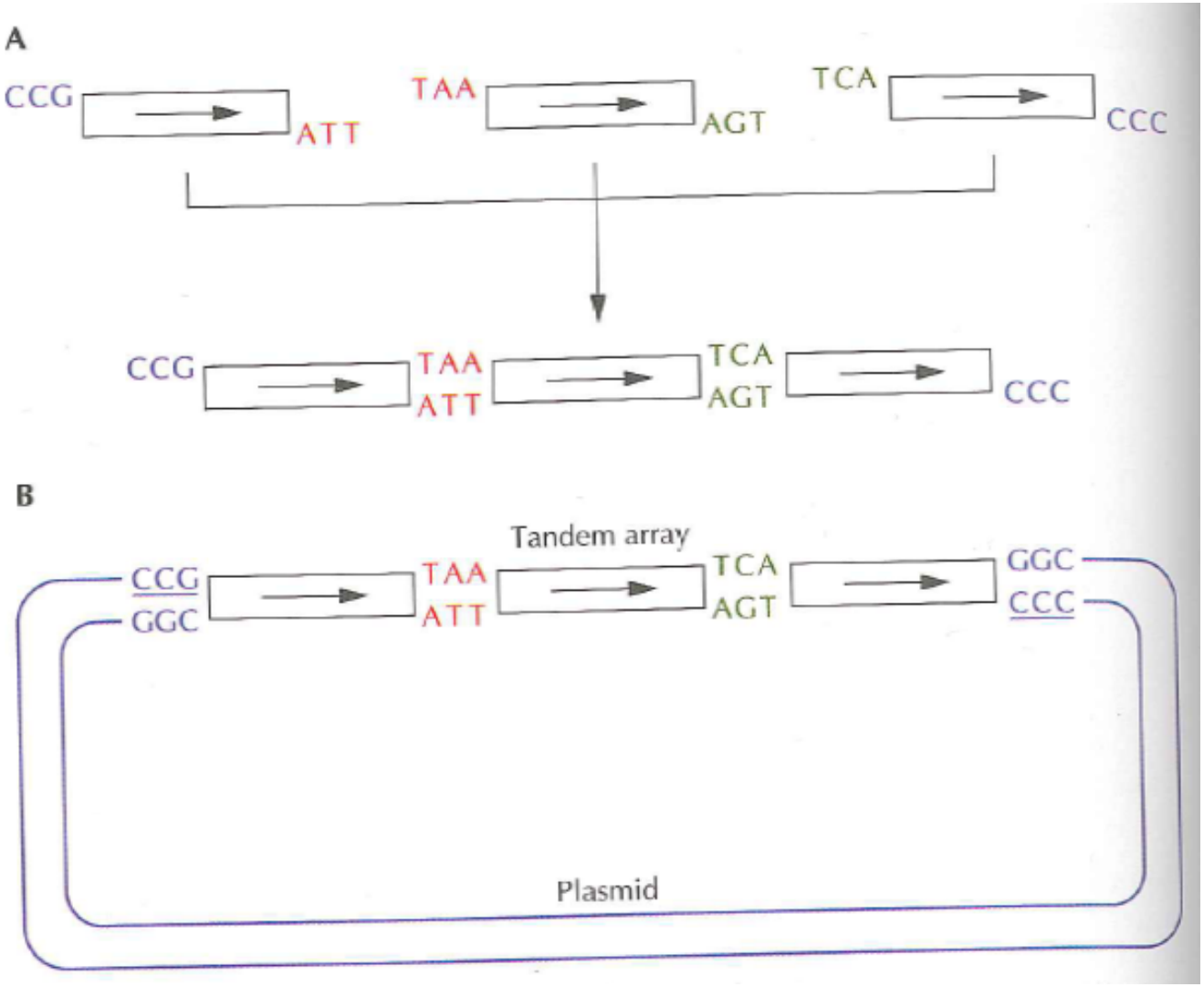
The number of plasmids per cell (the copy number) can be increased

However the higher the copy number, the more of the cell's resources are diverted to the production of plasmid encoded proteins and the metabolic activities of the host strain are constrained.

An alternative is to express unidirectional tandem gene arrays cloned in a single plasmid.

Increasing Expression Level

Unidirectional tandem gene arrays



The individual gene fragments are typically generated by PCR using primers that append Type IIS restriction enzyme sites which can be cleaved to create the sticky ends (as in Golden Gate cloning)

Unfortunately tandem gene arrays are often not genetically stable due to homologous recombination causing deletions

Increasing Expression Level

Optimising protein expression

Using a strong promoter to optimise transcription of mRNA may not be sufficient to maximise the yield of the cloned product.

It may also be necessary to optimise:

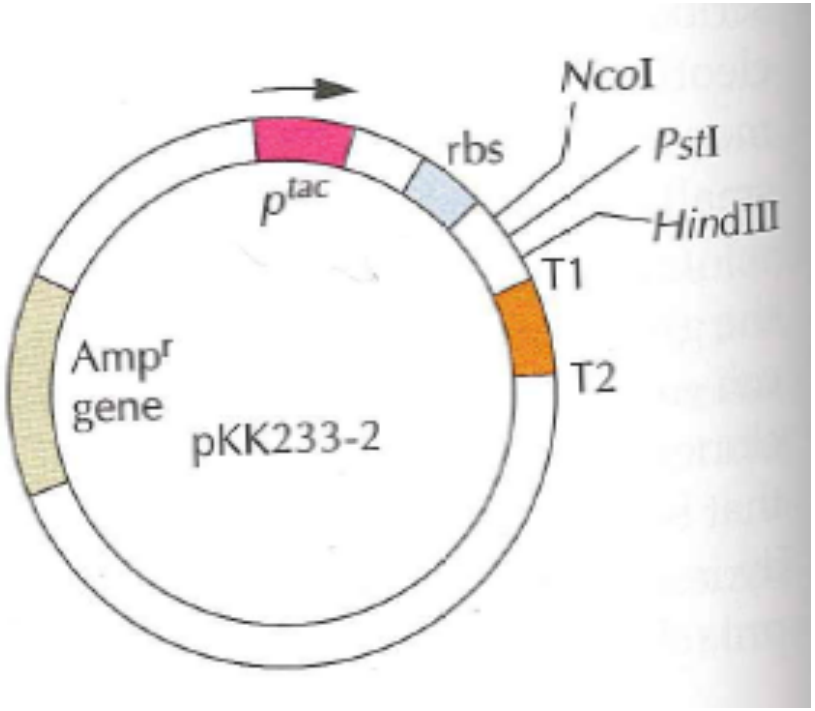
1. Translation of the protein
2. Stability of the protein

Increasing Expression Level

Optimising protein expression

For example, the expression vector pKK233-2 contains:

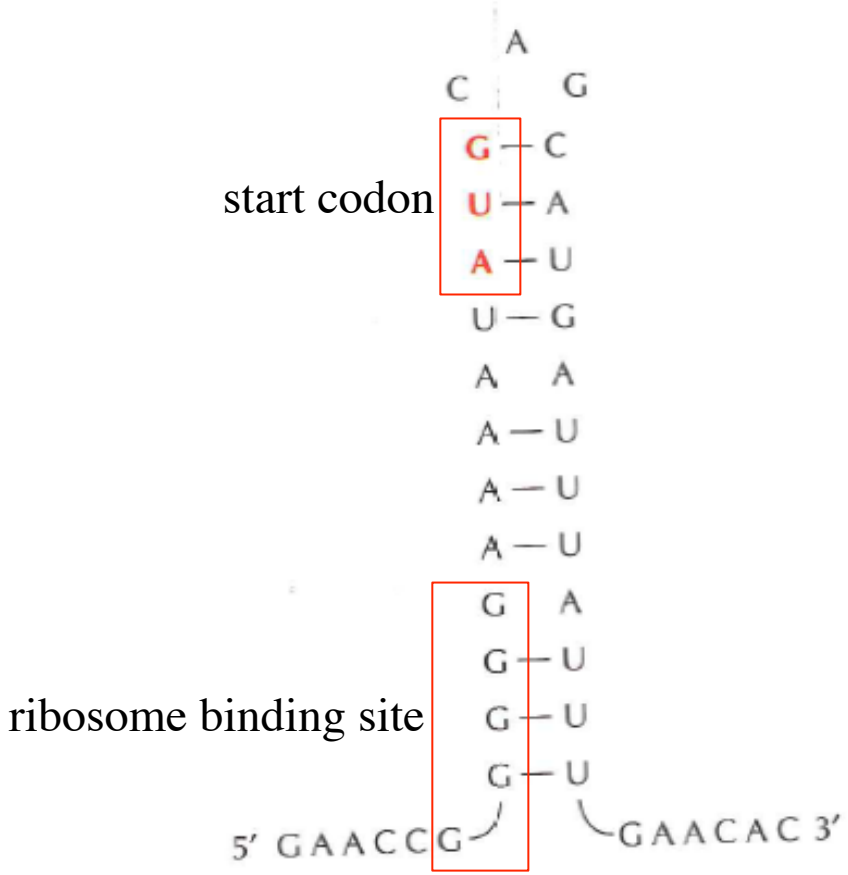
- An ampicillin resistance gene as a selectable marker
- The strong *tac* promoter
- The efficient *lac*Z ribosome-binding site (rbs)
- An ATG start codon optimally located 8 nucleotides downtream from the ribosome-binding site
- The transcription terminators T1 and T2 from bacteriophage λ to increase mRNA stability



Increasing Expression Level

Optimising protein expression

It is also important to ensure that the region comprising the region of mRNA comprising the ribosome binding site and the start codon (AUG) are not sequestered in a double-stranded region, otherwise translation will not be efficient.



Increasing Expression Level

Optimising protein expression

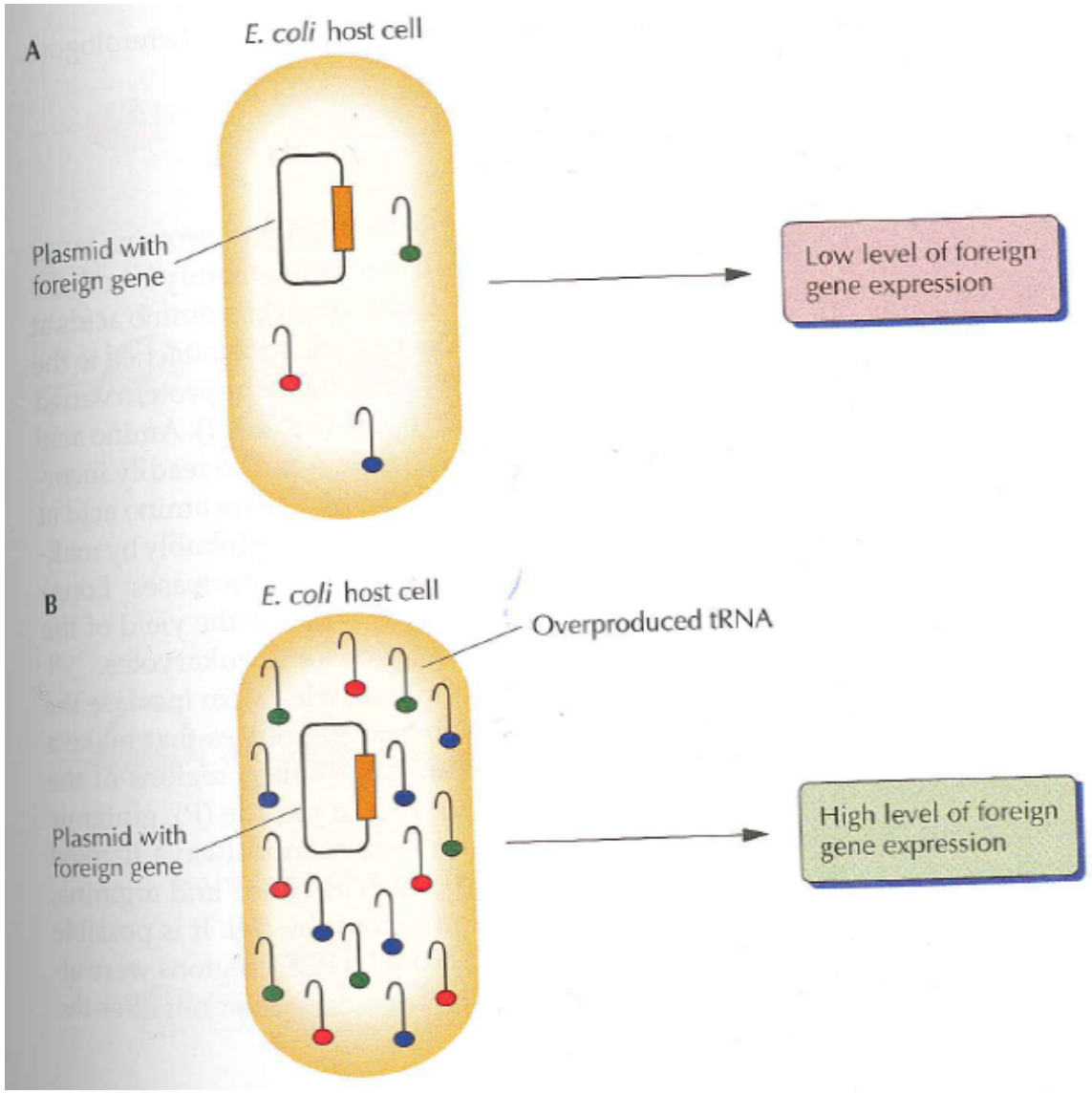
Foreign cloned genes often contain codons which are rarely used by *E. coli* (such as AGG, AGA, AUA, CUA and CGA). The host cell therefore my not produce enough transfer RNA to translate these codons reducing protein yield.

This problem can be overcome in several ways:

1. If the cloned gene is eukaryotic it can be cloned and expressed in a eukaryotic expression system
2. By creating a synthetic gene which only use codons which are commonly used in *E. coli* (codon optimisation)
3. Using *E. coli* engineered to overexpress rare tRNAs

Increasing Expression Level

Using *E. coli* cells engineered to overexpress rare tRNAs



Increasing Expression Level

Increasing protein stability

Amino acid added	Half-life
Met, Ser, Ala	>20 h
Thr, Val, Gly	>20 h
Ile, Glu	>30 min
Tyr, Gln	~10 min
Pro	~7 min
Phe, Leu, Asp, Lys	~3 min
Arg	~2 min

Adapted from Bachmair et al., *Science* **234**:179–186, 1986.

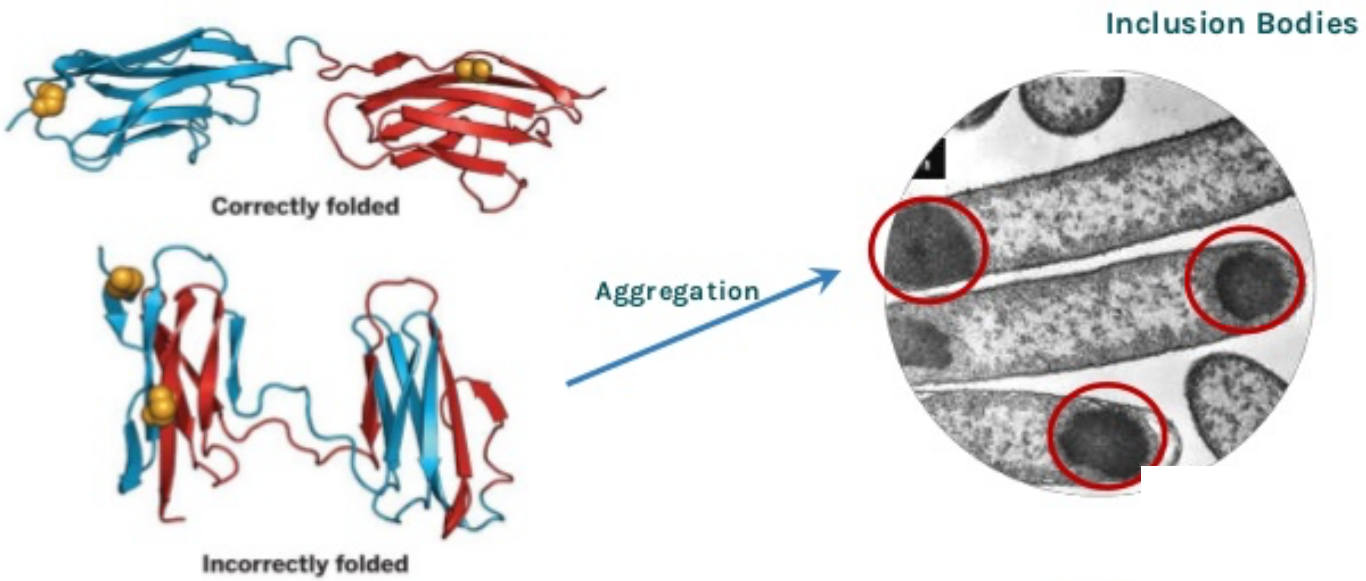
Stability of β-galactosidase with different amino acids at its N-terminus

Changing the amino acid at the N-terminus of a protein can often significantly change it's half-life in *E. coli*

So called PEST sequences rich in proline (P), glutamate (E), serine (S) and threonine (T) in the protein can make the protein more susceptible to proteolysis and stability of the protein can be improved by genetic manipulation of the PEST sequences.

Increasing Expression Level
Protein Folding

Many proteins overexpressed in *E. coli* accumulate in insoluble, intracellular, biologically inactive inclusion bodies



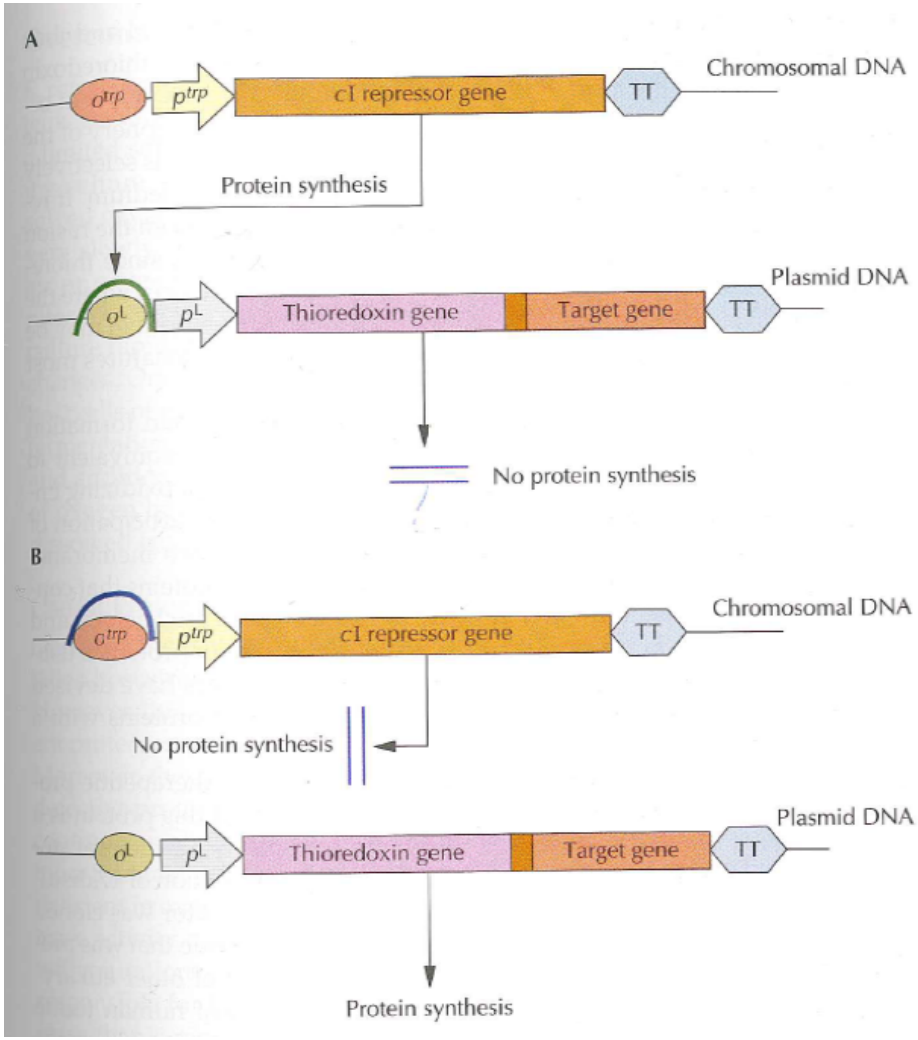
Active protein can often be recovered from inclusion bodies after using protein solubilisation and refolding procedures.

However, in many cases it is preferable to produce soluble, active protein from the beginning.

There are a number of ways to achieve this.

Increasing Expression Level

Producing folded protein by making a fusion protein



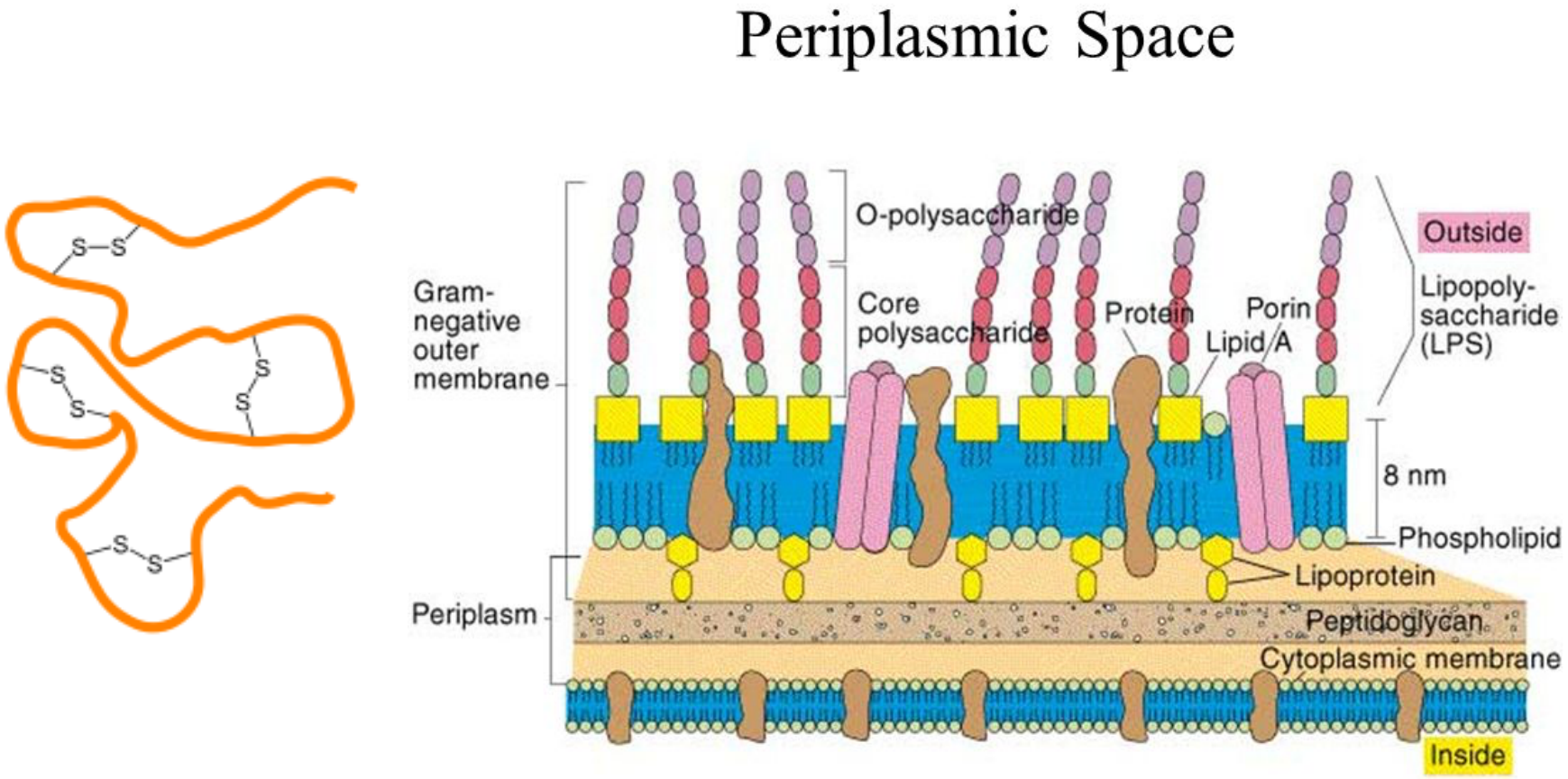
No tryptophan: No protein synthesis

With tryptophan: protein synthesis

For example, proteins fused to the 11.7 kDa protein Thioredoxin can remain soluble even when up to 40% of the cellular protein consists of the fusion protein.

Increasing Expression Level

Producing folded disulfide bonded proteins

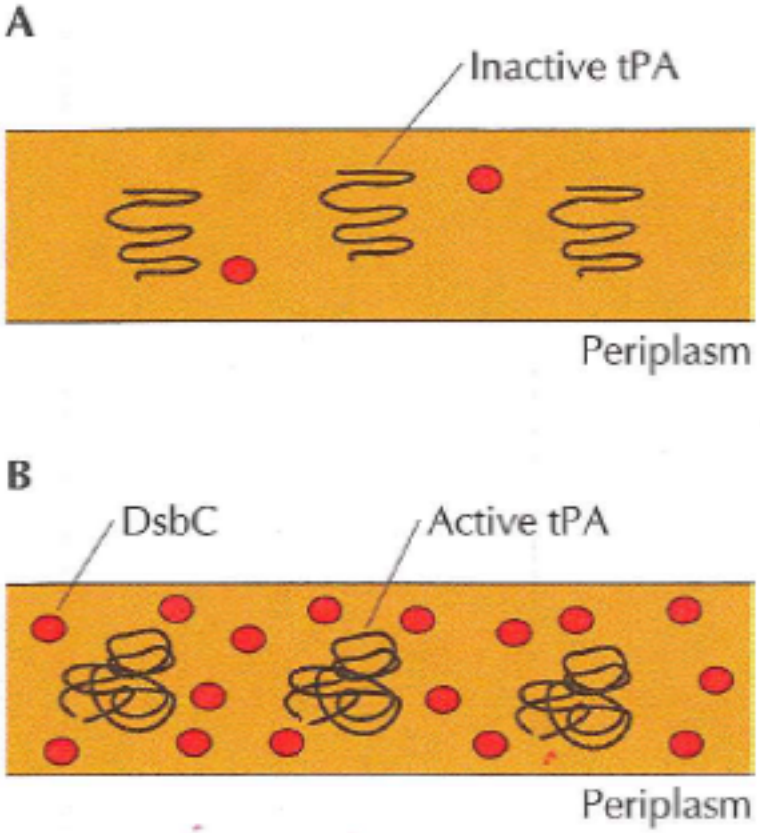


Disulfide bonds do not form in the reducing environment of cytoplasm.

They do, however, form in the oxidising environment of the *E. coli* periplasm or the eukaryotic endoplasmic reticulum.

Increasing Expression Level

Producing folded disulfide bonded proteins



Tissue plasminogen activator (tPA), an important therapeutic "clot-buster" drug, contains 17 disulfide bonds.

Expression of active tPA in *E. coli* required that it be directed to the periplasm by fusion of a leader peptide and that disulfide bond formation be catalysed by overproduction of the *E. coli* enzyme DsbC.

Disulfide bonds do not form in the reducing environment of cytoplasm.

They do, however, form in the oxidising environment of the *E. coli* periplasm or the eukaryotic endoplasmic reticulum.

Heterologous Protein Production in Eukaryotic Cells

Eukaryotic Proteins in Prokaryotes

Where is the problem?

Prokaryotic expression systems sometimes fail to produce functional versions of eukaryotic proteins

This is mostly due to:

1. Improper folding

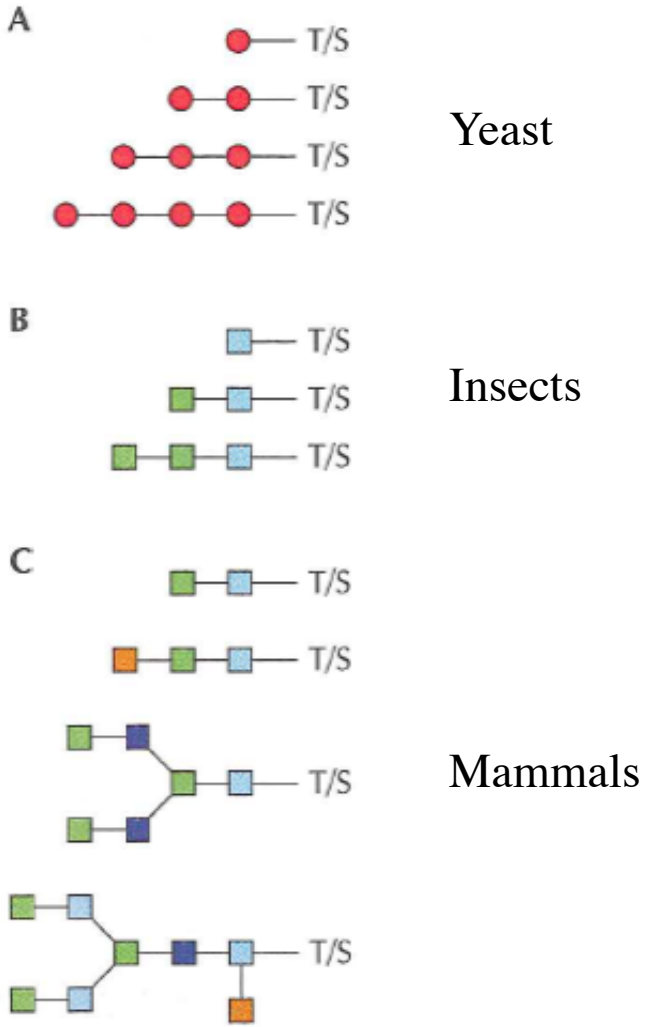
2. Inability to modify certain amino acid side chains (post-translational modification), e.g.

    • To form disulphide bonds: in eukarotes this is catalysed by the enzyme protein disulphide isomerase (PDI). Aberrant disulfide bond formation changes the configuration of the protein and can reduce stability and abolish activity.

    • To add sugars (glycosylation) – about 30% of all mammalian proteins are glycosylated

Post-Translation Modification in Eukaryotes

Glycosylation



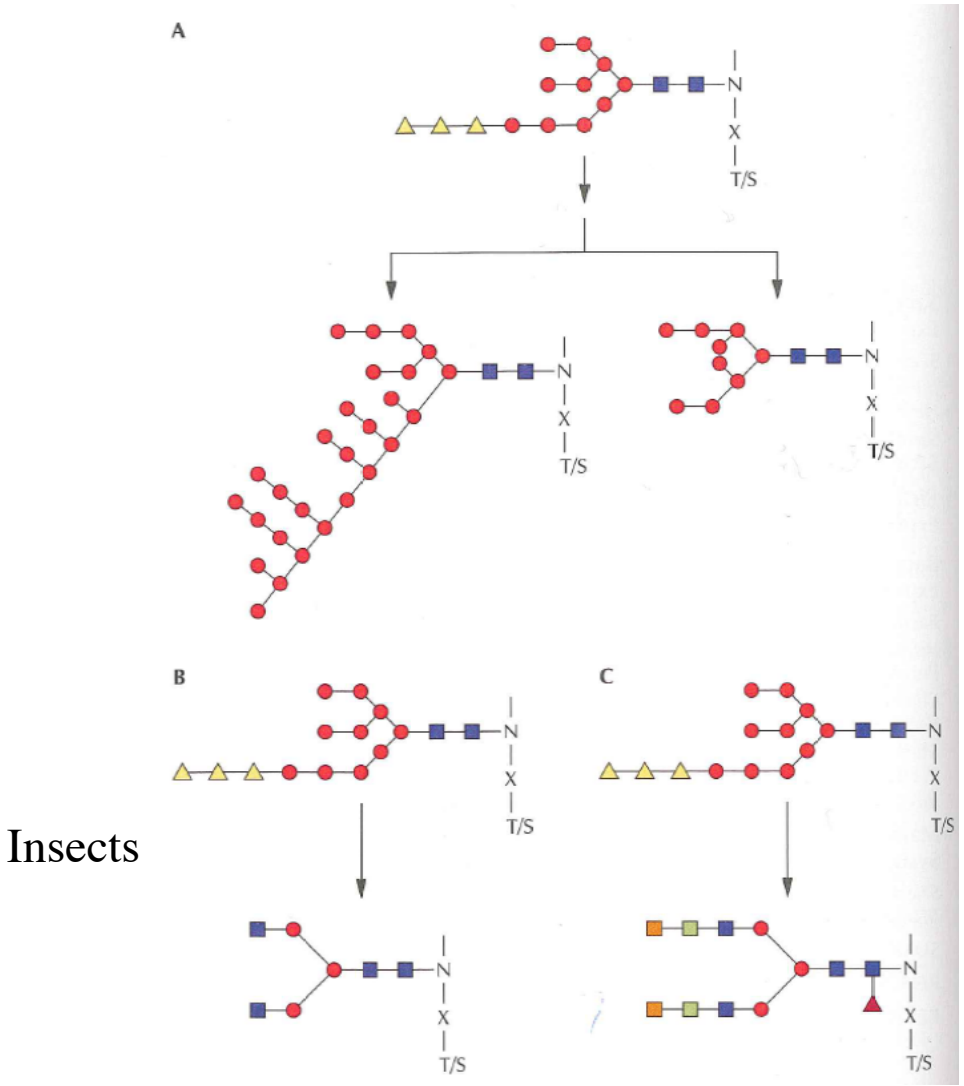
S, serine; T, threonine; red circles, mannose; dark blue squares, *N*-acetylglucosamine; light blue squares, *N*-acetylgalactosamine; green squares, galactose; orange squares, sialic acid.

Examples of O-linked glycosylation (of Serine or Threonine) in different organisms

Post-Translation Modification in Eukaryotes

Glycosylation

Yellow triangles = glucose
Green squares = galactose
Orange squares = sialic acid
Maroon triangle = fucose

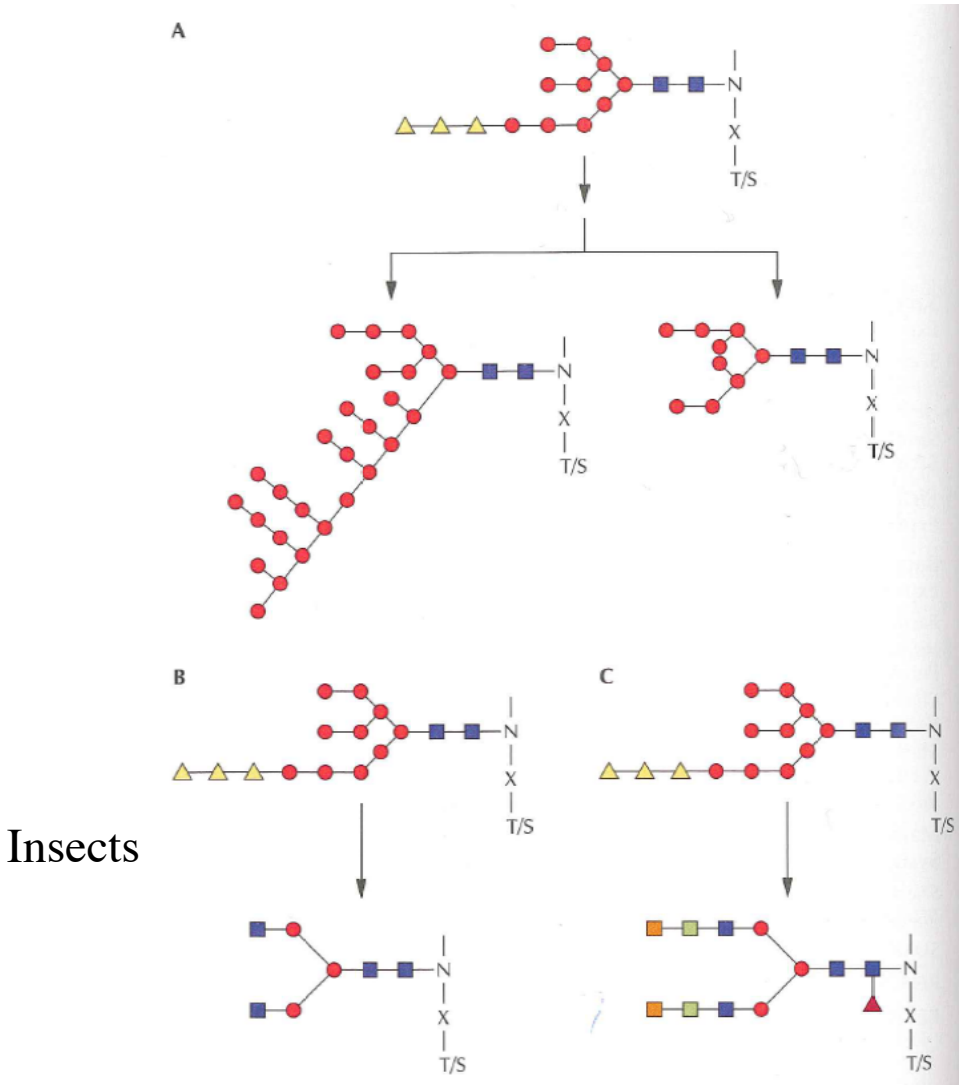


Examples of N-linked glycosylation (of Asparagine) in different organisms

Post-Translation Modification in Eukaryotes

Glycosylation

Yellow triangles = glucose
Green squares = galactose
Orange squares = sialic acid
Maroon triangle = fucose



Examples of N-linked glycosylation (of Asparagine) in different organisms

Eukaryotic Expression Vectors

General features

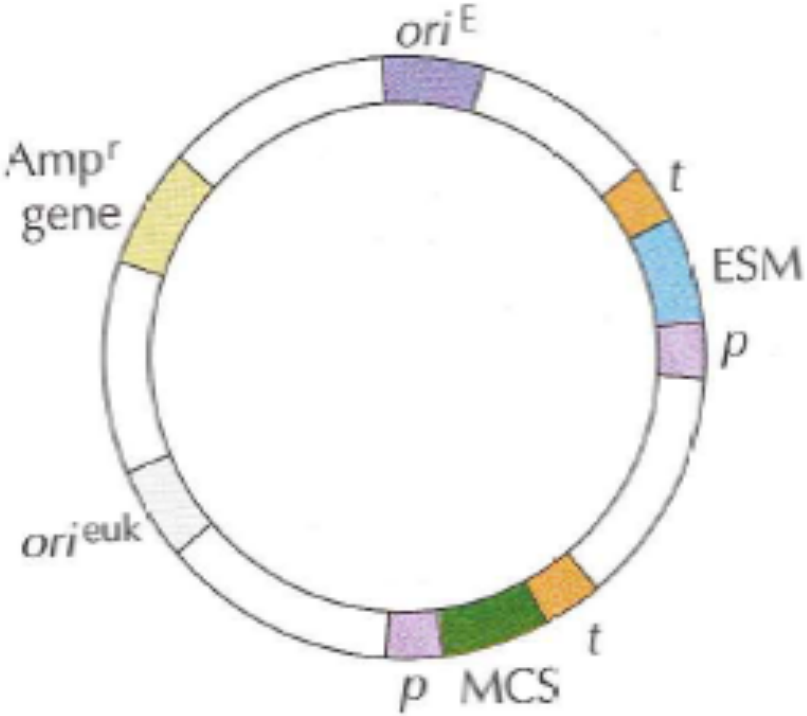


Figure 7.3 Generalized eukaryotic expression vector. The major features of a eukaryotic expression vector are a eukaryotic transcription unit with a promoter (*p*), a multiple cloning site (MCS) for a gene of interest, and a DNA segment with termination and polyadenylation signals (*t*); a eukaryotic selectable marker (ESM) gene system; an origin of replication that functions in the eukaryotic cell (ori^{euk}); an origin of replication that functions in *E. coli* (ori^{E}); and an *E. coli* selectable marker gene (Amp^{r}).

A generalized eukaryotic expression vector.

In principle, eukaryotic expression vectors do not differ from prokaryotic expression vectors

However, they contains an *E. coli* (ori^{E}) and a eukaryotic (ori^{euk}) origin of replication and *E. coli* and eukaryotic selectable markers

Sacharomyces cerevisiae Expression Systems

Advantages

Yeast is a useful expression system because:

1. It is single celled, well characterized genetically and physiologically, and grown readily in small cultures and large-scale bioreactors.

2. Several strong promoters are known, and yeast plasmids can be constructed based on the naturally occurring yeast 2 µm plasmid.

3. Yeast can carry out many post-translational modifications.

4. Yeast secretes few proteins naturally, so extracellular proteins engineered for secretion in yeast are already pretty pure, aiding purification.

5. As it is used for baking and brewing it is classed as “generally recognized as safe” by the U.S. Food and Drug Administration.

Sacharomyces cerevisiae Expression Systems

Recombinant proteins produced in *S. cerevisiae*

VACCINES
- Hepatitis B virus surface antigen
- Malaria circumsporozoite protein
- HIV-1 envelope protein

DIAGNOSTICS
- Hepatitis C virus protein
- HIV-1 antigens

HUMAN THERAPEUTIC AGENTS
- Epidermal growth factor
- Insulin
- Insulin-like growth factor
- Platelet-derived growth factor
- Proinsulin
- Fibroblast growth factor
- Granulocyte-macrophage colony-stimulating factor
- α_1 antitrypsin
- Blood coagulation factor XIIIa
- Hirudin
- Human growth factor
- Human serum albumin

Sacharomyces cerevisiae Expression Systems

Classes of *S. cerevisiae* expression vectors

There are three main classes of *S. cerevisiae* expression vectors:

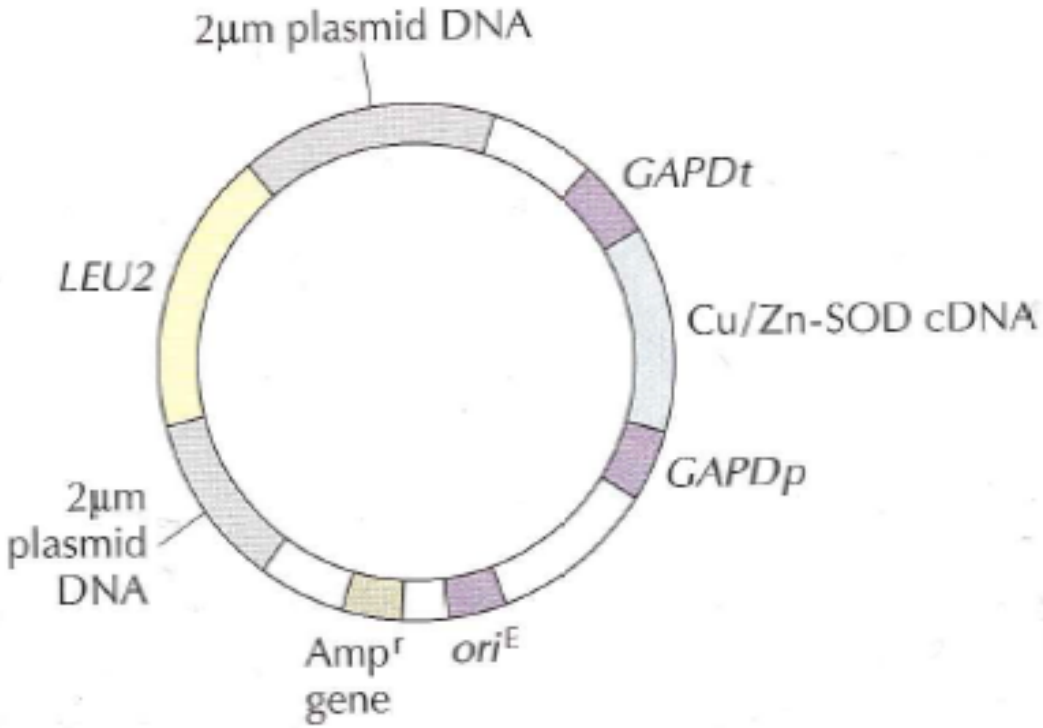
1. Yeast episomal plasmids (YEps).

2. Yeast integrating plasmids (YIps).

3. Yeast artificial chromosomes (YACs).

Sacharomyces cerevisiae Expression Systems

Yeast episomal plasmids (YEps)



This example contains:

1. A yeast gene for leucine biosynthesis (*LEU2*) – a yeast selectable marker.

2. A 2 µm plasmid origin of replication – to allow replication of the plasmid in yeast.

3. The ampicillin resistance gene (Amp^r) – a bacterial selectable marker

4. An *E. coli* origin of replication (ori^E) – to allow replication of the plasmid in bacteria.

5. The cDNA for the human enzyme Cu/Zn superoxide dismutase (Cu/Zn-SOD) – the foreign protein to be expressed.

6. The promoter (GAPDp) and termination-polyadenylation sequences (GAPDt) of the *S. cerevisiae* gyceraldehyde phosphate dehydrogenase gene.

YEps work well, but are often unstable under large-scale (≥10 litres) growth conditions.

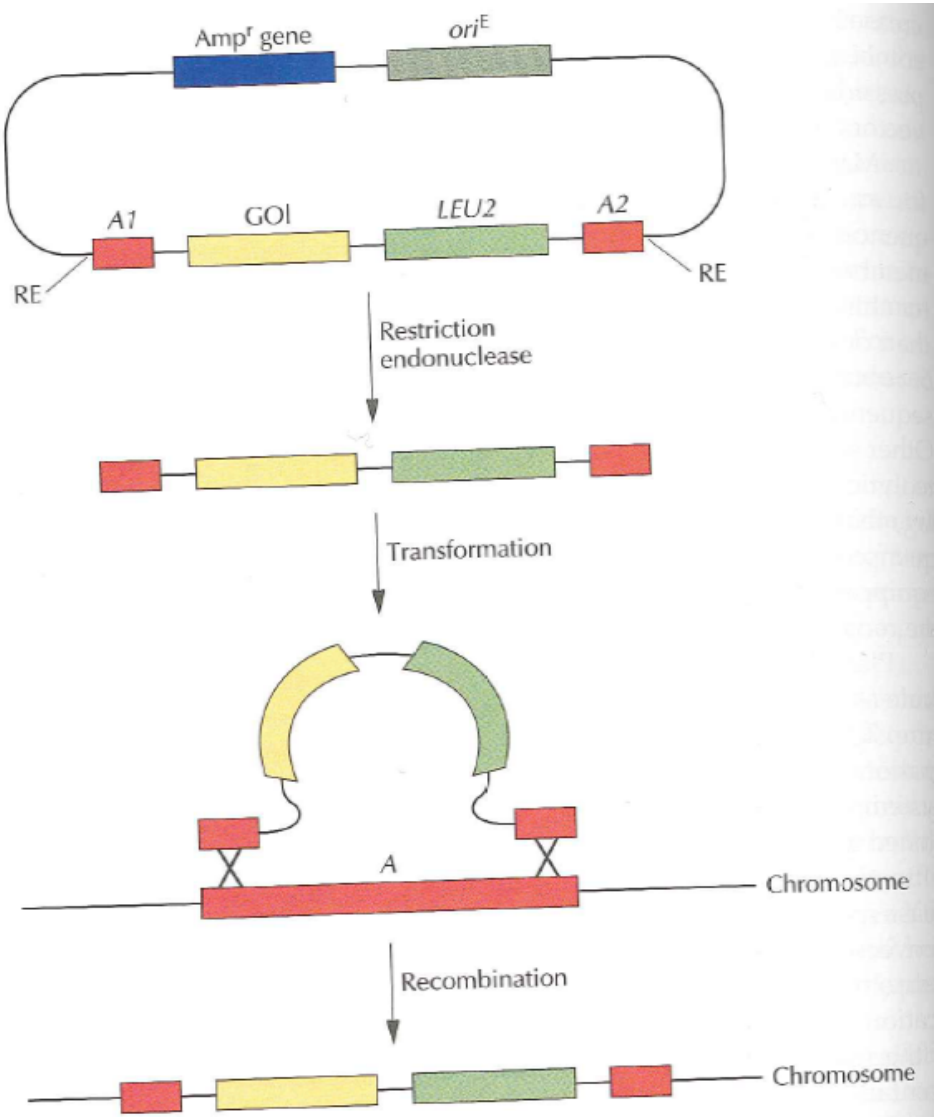
Sacharomyces cerevisiae Expression Systems

Promoters for *S. cerevisiae* expression vectors

Promoter	Expression conditions	Status
Acid phosphatase (*PH05*)	Phosphate-deficient medium	Inducible
Alcohol dehydrogenase I (*ADHI*)	2–5% Glucose	Constitutive
Alcohol dehydrogenase II (*ADHII*)	0.1–0.2% Glucose	Inducible
Cytochrome c_1 (*CYC1*)	Glucose	Repressible
Gal-1-P Glc-1-P uridyltransferase	Galactose	Inducible
Galactokinase (*GAL1*)	Galactose	Inducible
Glyceraldehyde-3-phosphate dehydrogenase (*GAPD, GAPDH*)	2–5% Glucose	Constitutive
Metallothionein (*CUP1*)	0.03–0.1 mM copper	Inducible
Phosphoglycerate kinase (*PGK*)	2–5% Glucose	Constitutive
Triose phosphate isomerase (*TPI*)	2–5% Glucose	Constitutive
UDP galactose epimerase (*GAL10*)	Galactose	Inducible

Sacharomyces cerevisiae Expression Systems

Yeast integrating plasmids (YIps)



This vector contains:

1. A yeast gene for leucine biosynthesis (*LEU2*) – a yeast selectable marker.

2. The Gene of Interest (GOI) with transcription and translation control elements

3. The ampicillin resistance gene (Amp^r) – a bacterial selectable marker.

4. An *E. coli* origin of replication (ori^E) – to allow replication of the plasmid in bacteria.

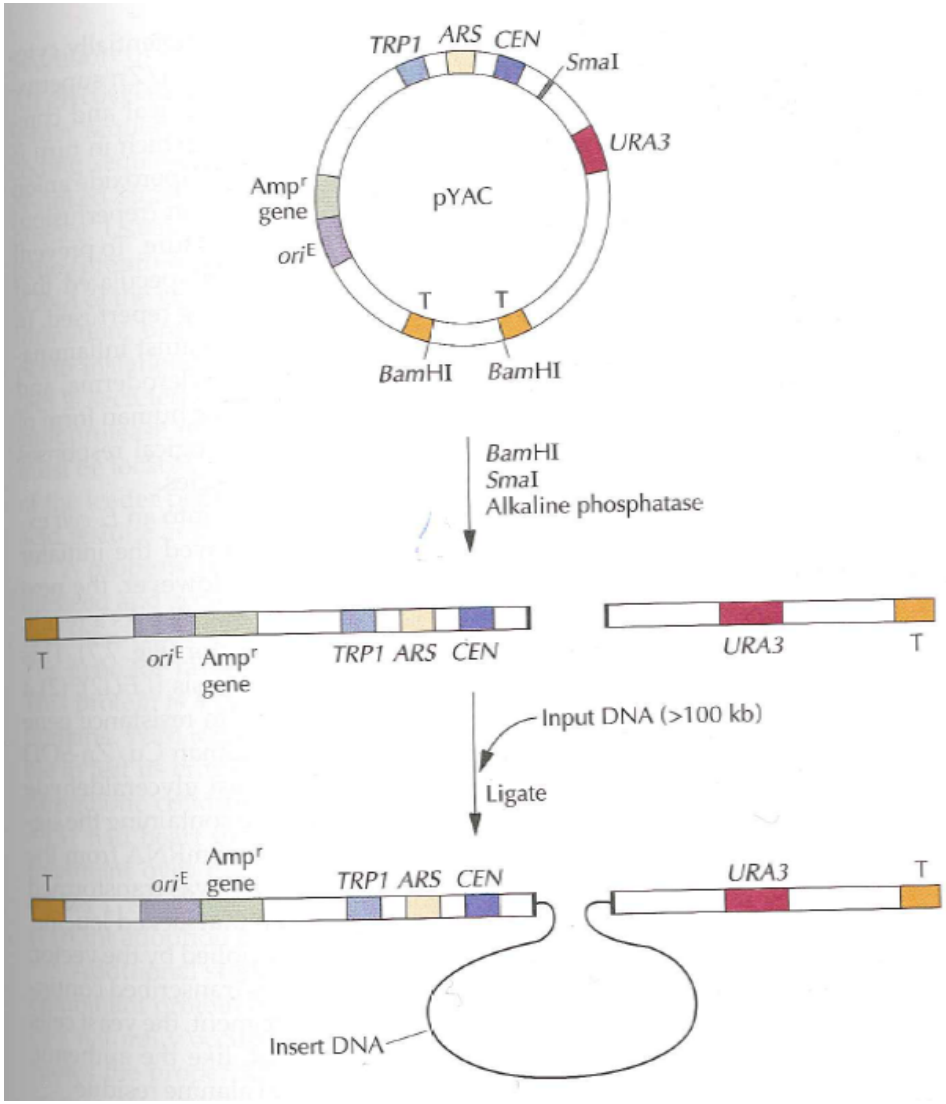
5. Two segments from the ends of a non-essential yeast gene (A1 and A2).

Note, there is no yeast origin of replication. The GOI and LEU2 are only replicated if they are integrated into the yeast chromosome by homologous recombination.

The system is genetically stable, but protein yield is low as there is only a single copy.

Sacharomyces cerevisiae Expression Systems

Yeast artificial chromosomes (YACs)



The pYAC plasmid contains:

1. The ampicillin resistance gene (Amp^r) – a bacterial selectable marker.

2. An *E. coli* origin of replication (ori^E) – to allow replication of the plasmid in bacteria.

3. A yeast autonomous replicating sequence (ARS) – allows replication of the YAC in yeast.

4. A centromere (CEN) – to enable the YAC to segregate like a chromosome.

5. A gene from the uracil biosynthesis pathway (URA3) – a selectable marker in yeast.

6. A gene from the tryptophan biosynthesis pathway (TRP1) – a selectable marker in yeast.

7. Two yeast chromosome telomere sequences (T) – to allow the YAC to be maintained stably.

Large DNAs (>100 kb) can be stably maintained as a separate chromosome in double mutant *ura*3 and *trp*1 cells.

Pichia pastoris and Other Yeast Expression Systems

S. cerevisiae is not always ideal:

- Yields of protein may be poor.
- Proteins may be hyperglycosylated (>100 mannose residues in N-linked side chains.
- Proteins designed for secretion may be maintained in the periplasmic space.
- Ethanol is produced at high cell densities, which is toxic to the cells, reducing protein yield.

Pichia pastoris (a methylotropic yeast) has a number of advantages:

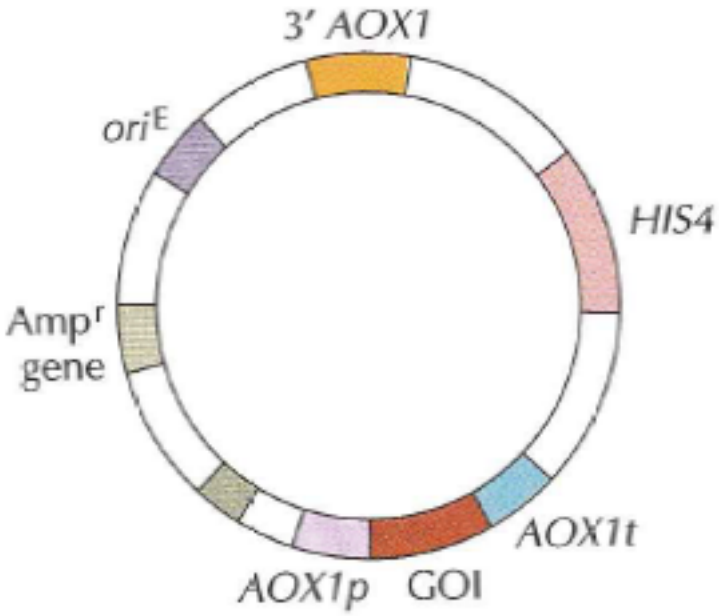
- It has a highly efficient, tightly regulated methanol inducible promoter (the *AOX1* promoter) – in the presence of methanol the expressed gene may make up to 30% of cellular protein.
- *P. pastoris* do not make ethanol and can therefore achieve high cell densities with high protein yield.
- *P. pastoris* normally does not secrete many proteins, simplifying the purification of secreted recombinant proteins.

Other yeasts, including *Hansenula polymorpha*, *Shizosaccharomyces pombe* and *Candida utilis* have also been used to make recombinant human proteins.

Several species of the filamentous fungus *Aspergillus* have been used to produce enzymes for the food, beverage, pulp and paper industries.

Pichia Pastoris Expression Systems

An integrating *P. pastoris* expression vector



The plasmid contains:

1. The ampicillin resistance gene (Amp^{r}) – a bacterial selectable marker.

2. An *E. coli* origin of replication (ori^{E}) – to allow replication of the plasmid in bacteria.

3. The Gene of Interest (GOI).

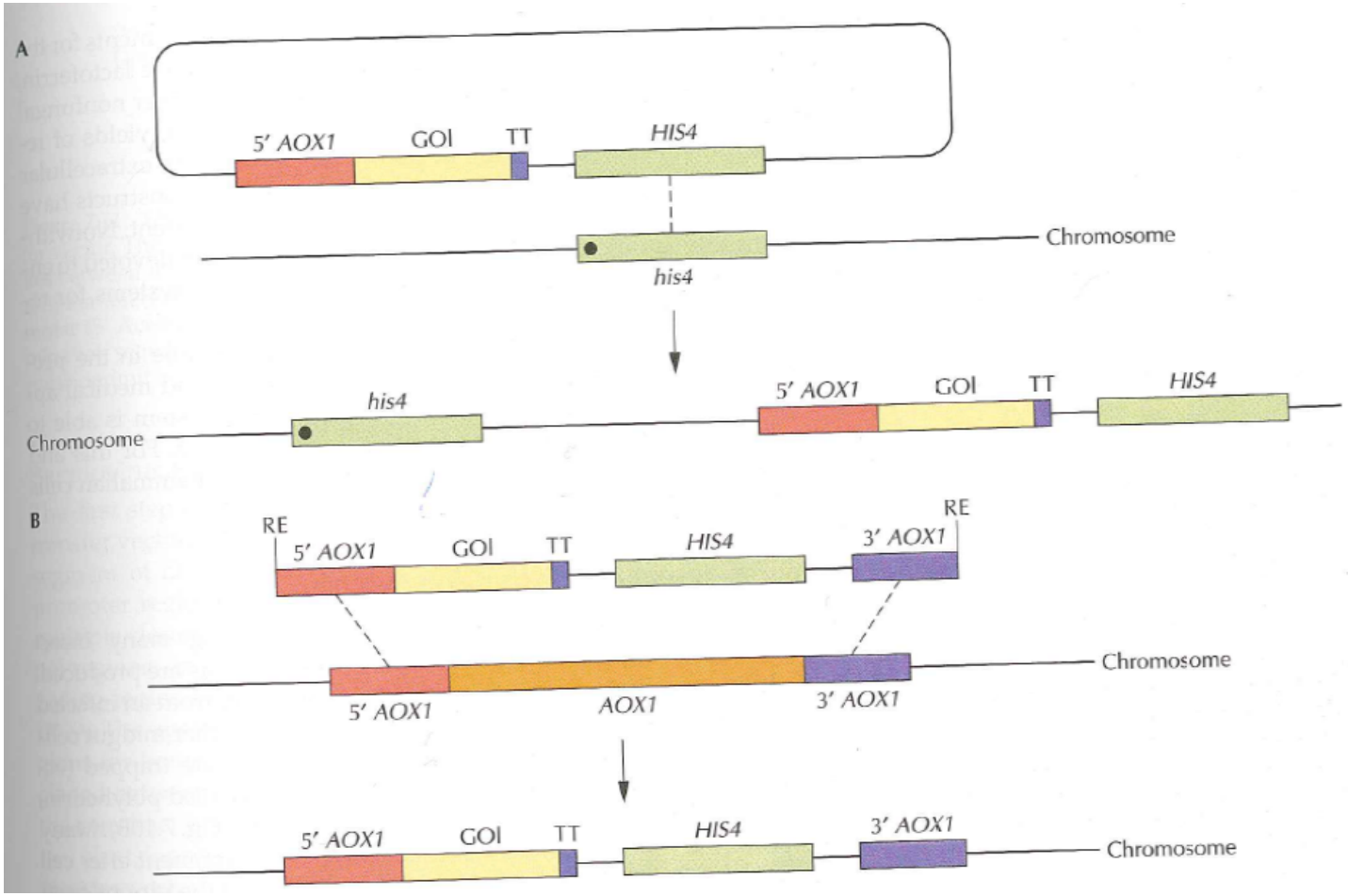
4. The promoter (AOX1p) and termination-polyadenylation sequences (AOX1t) of the *P. pastoris* alcohol oxidase 1 gene.

5. A gene from the histidine biosynthesis pathway (HIS4) – a selectable marker in *P. pastoris*.

6. The 3'-end of the AOX1 gene of *P. pastoris* – to enable recombination into the chromosomal AOX1 gene.

Pichia Pastoris Expression Systems

An integrating *P. pastoris* expression vector



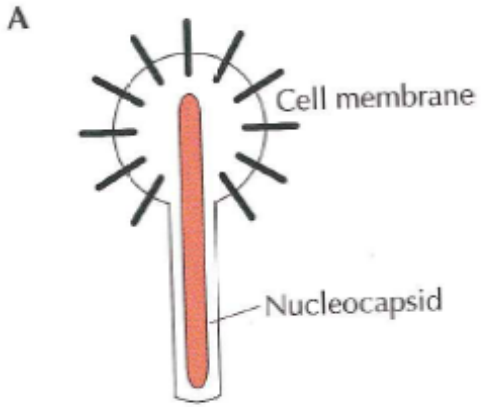
Single recombination with *HIS4*

Double recombination with *AOX1*

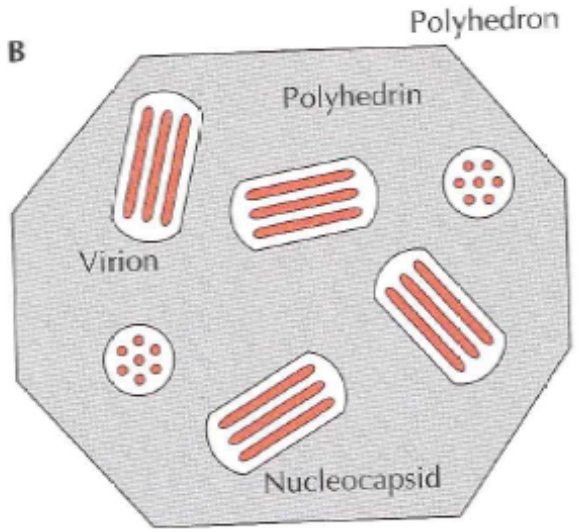
Integration of DNA into a specific *P. pastoris* chromosome by single or double recombination

Baculovirus-Insect Cell Expression Systems

Baculoviruses infect invertebrates, including many insect species



Single nucleocapsid (virus particles) are budded of from infected cells



In infected cells clusters of nucleocapsids (virions) are trapped (occluded) in a protein matrix.

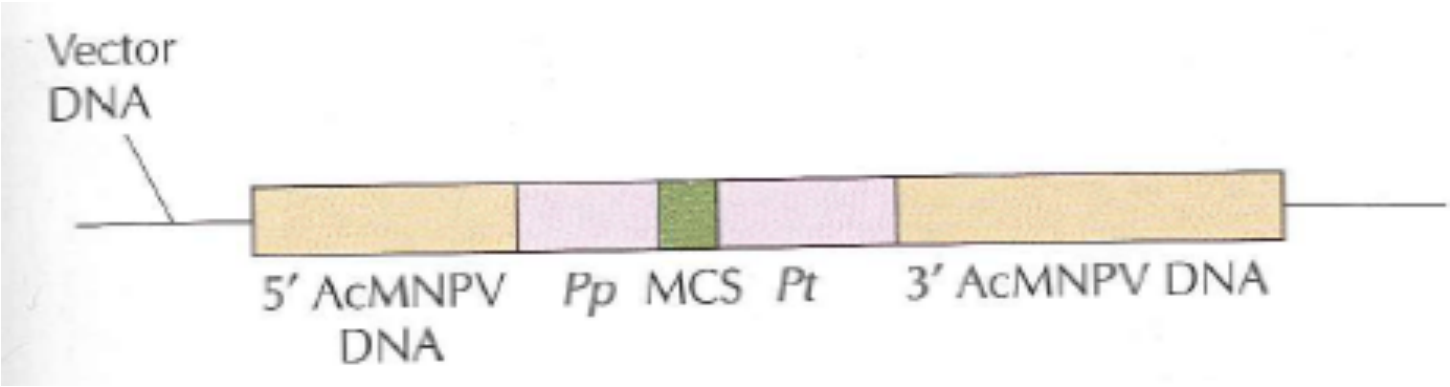
The protein matrix is called polyhedrin and the entire package is called a polyhedron.

The promoter for the polyhedrin gene is exceptionally strong, and can be used to drive expression of foreign proteins in insect cells.

Two forms of baculovirus are formed during the infection cycle

Baculovirus-Insect Cell Expression Systems

Baculoviruses transfer vector based on *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV)



The expression unit of the vector contains:

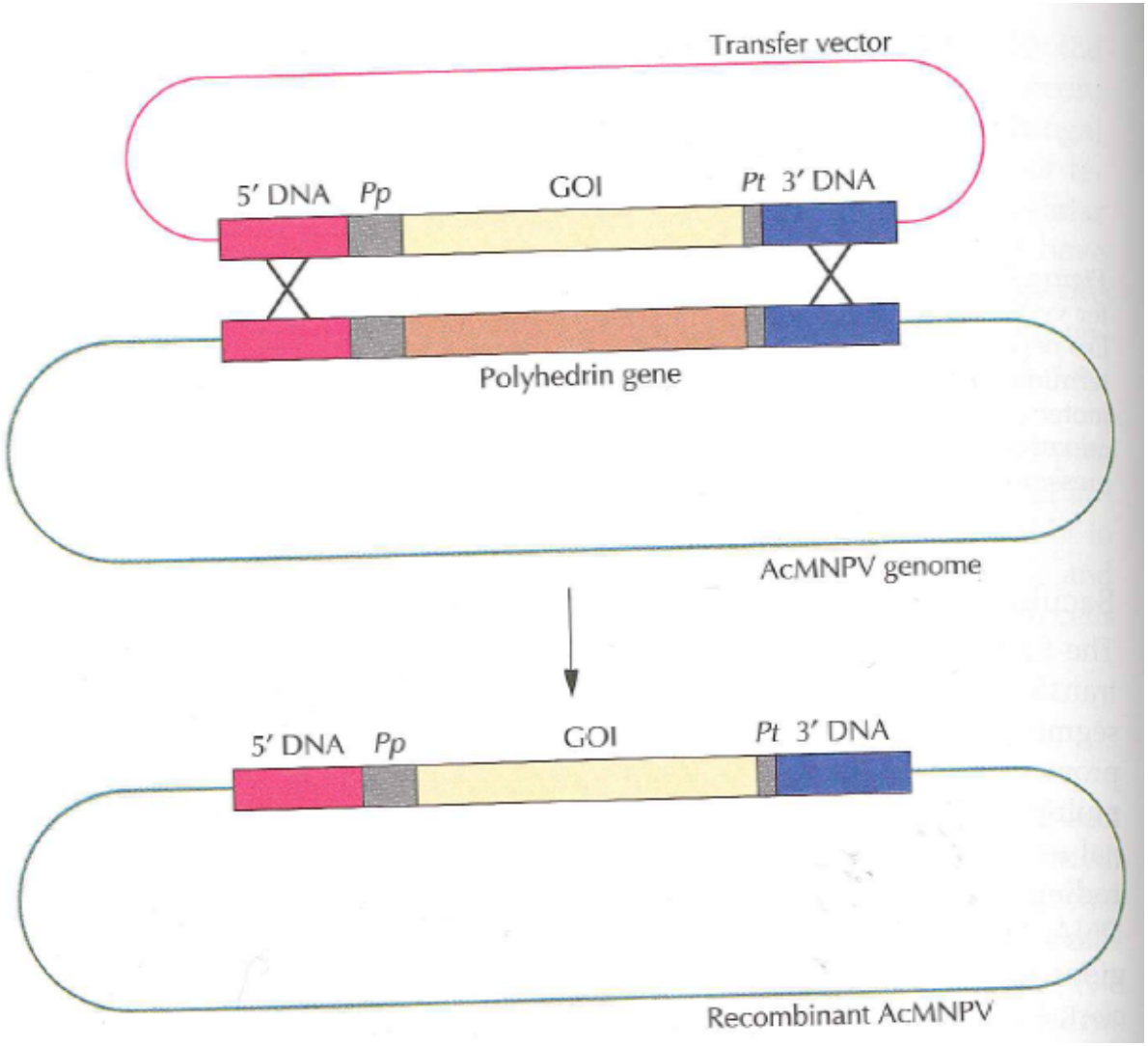
1. A multiple cloning site (MCS) in which to clone the gene to be expressed.

2. The MCS lies between the polyhedrin gene promoter (Pp) and polyhedrin gene transcription-termination sequences (Pt).

3. Flanking regions of AcMNPV DNA (5' AcMNPV and 3' AcMNPV) to allow homologous recombination into the AcMNPV genome.

Baculovirus-Insect Cell Expression Systems

Replacing the AcMNPV polyhedrin gene with an expression unit



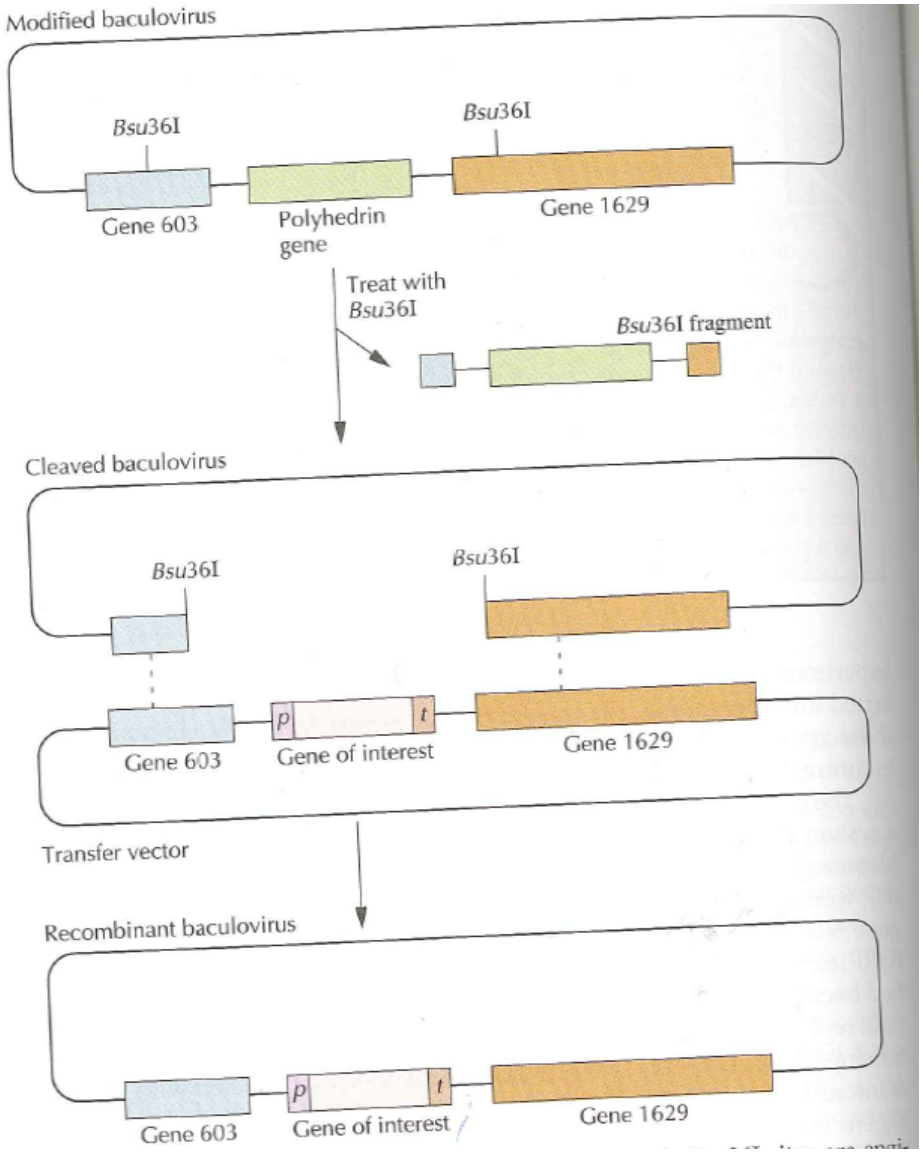
Insect cells in culture are cotransfected with AcMNPV DNA and the transfer vector containing the cloned gene.

Within some of the double infected cells a double cross-over event occurs and the expression unit is integrated into the AcMNPV genome.

Recombinant virus particles are harvested and heterologous proteins is harvested 4-5 days after infection of host insect cells with high-titre recombinant virus.

Baculovirus-Insect Cell Expression Systems

Increasing the yield of recombinant baculovirus

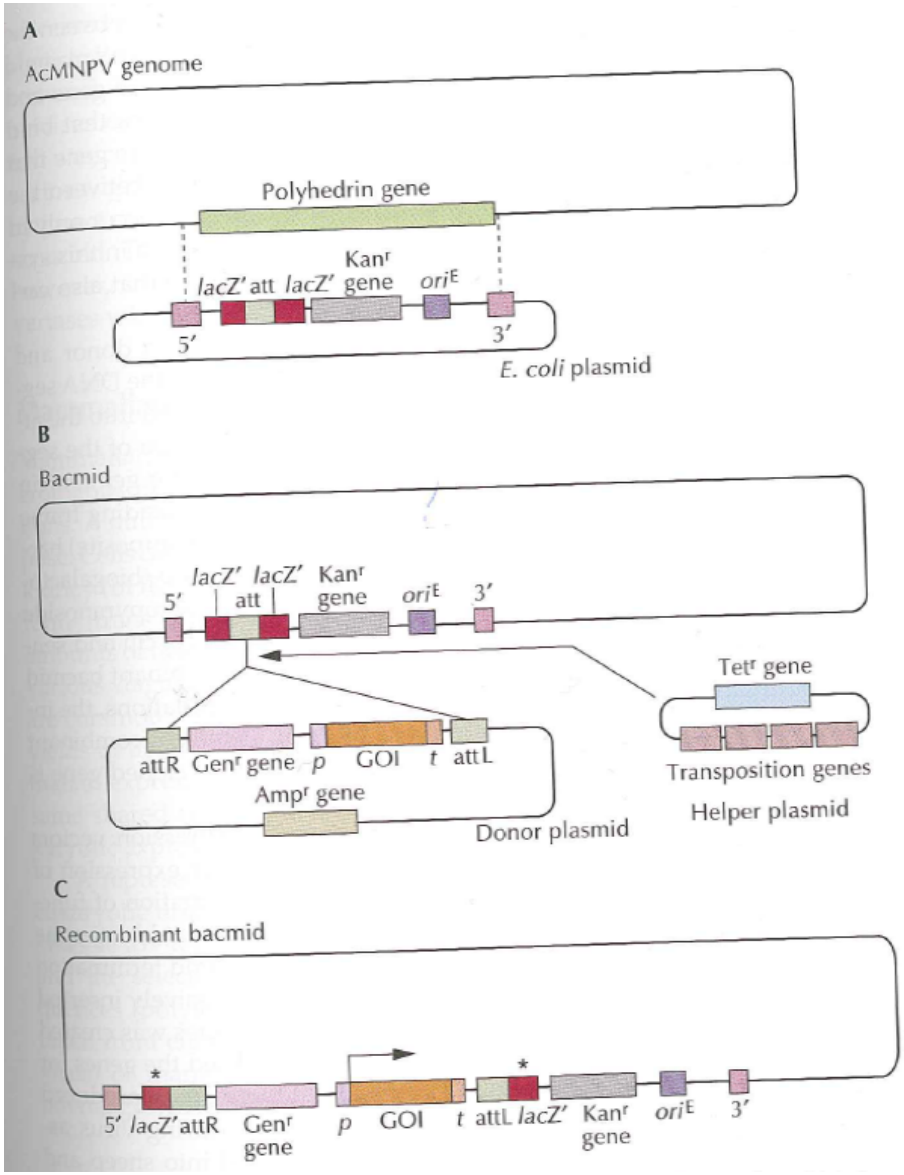


Linearisation of the AcMNPV genome before transfection into insect cells increases recombination efficiency from <1% to ~30%

To do this a baculovirus was engineered with two *Bsu*36I restriction enzyme sites on either side of the polyhedrin gene.

Baculovirus-Insect Cell Expression Systems

E. coli-insect cell baculovirus shuttle vectors (bacmids)



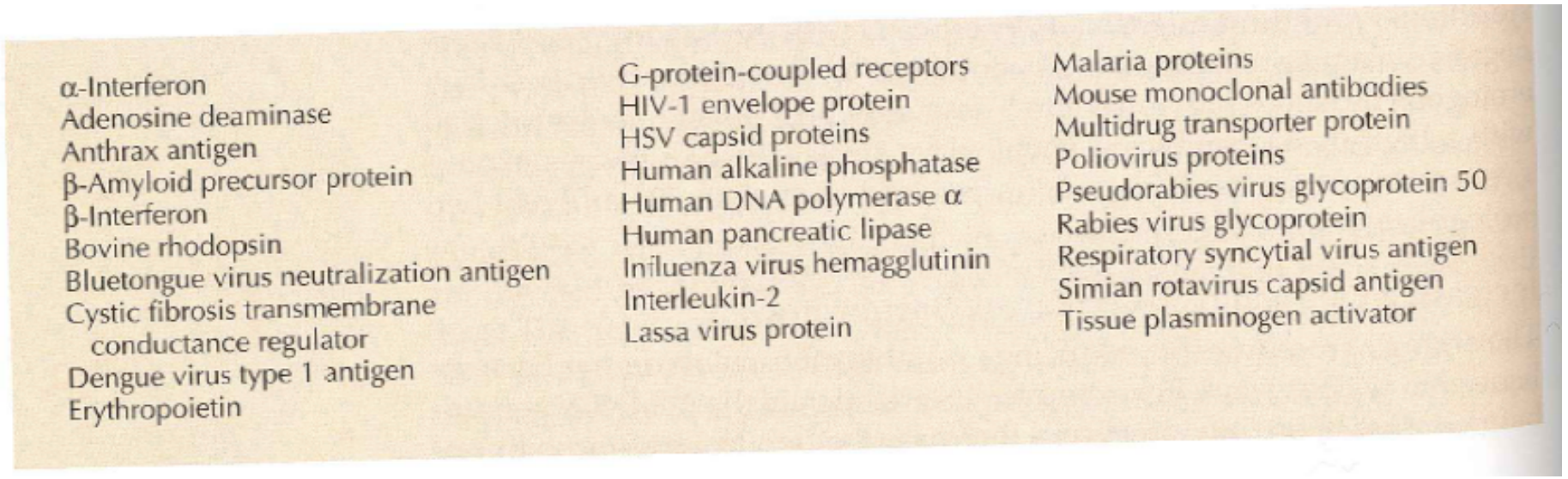
Bacmids make it possible to carry out all the genetic manipulations to generate a baculovirus expression vector in *E. coli*.

Transfection of insect cells is only required for the production of the heterologous protein

Note: the recombinant bacmid has a disrupted *lac*Z gene and cells transfected with this construct cannot produce functional β-galactosidase facilitating their identification.

Baculovirus-Insect Cell Expression Systems

Some recombinant proteins produced using baculovirus systems



α-Interferon
Adenosine deaminase
Anthrax antigen
β-Amyloid precursor protein
β-Interferon
Bovine rhodopsin
Bluetongue virus neutralization antigen
Cystic fibrosis transmembrane conductance regulator
Dengue virus type 1 antigen
Erythropoietin
G-protein-coupled receptors
HIV-1 envelope protein
HSV capsid proteins
Human alkaline phosphatase
Human DNA polymerase α
Human pancreatic lipase
Influenza virus hemagglutinin
Interleukin-2
Lassa virus protein
Malaria proteins
Mouse monoclonal antibodies
Multidrug transporter protein
Poliovirus proteins
Pseudorabies virus glycoprotein 50
Rabies virus glycoprotein
Respiratory syncytial virus antigen
Simian rotavirus capsid antigen
Tissue plasminogen activator

Problems with using baculovirus systems to produce mammalian proteins:

1. Glycosylation – insect cells do not routinely add galactose or terminal sialic acid to N-linked glycoproteins. However, a stable insect cell line has been developed with integrated mammalian α-2,6-sialyltransferase and mammalian β-1,4-galactotransferase genes.

2. Proteolytic processing – mammalian cells contain a number of enzymes (not found in insect cells) to convert larger inactive proproteins into active proteins by proteolysis (proprotein convertases).

Mammalian Cell Expression Systems

Advantages

Mammalian cell expression systems are important for producing mammalian proteins with a full complement of posttranslational modifications.

Can be achieved by:

Stable gene expression

Plasmid integration in the host genome or replication of the transferred genes as an artificial chromosome. Expression is long-term (stable).

Cell lines include:

- Chinese hamster ovary (CHO) cells

Transient gene expression

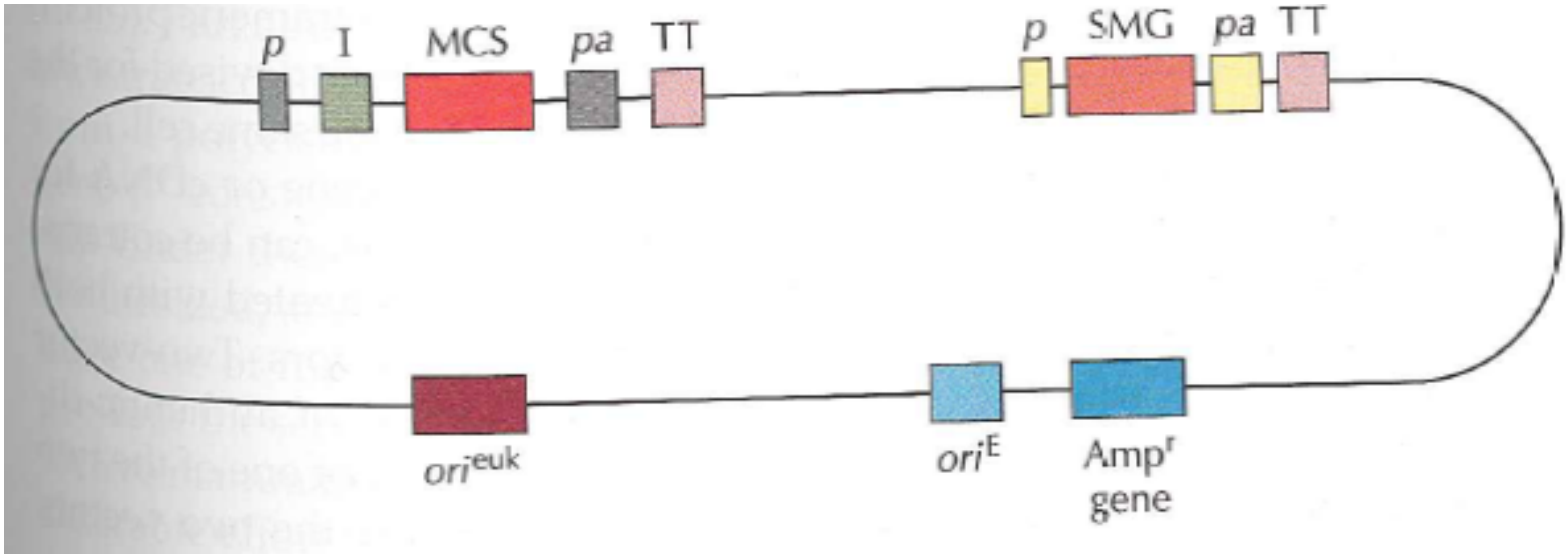
The foreign DNA is not integrated into the genome or replicated and is degraded or diluted on cell division. Expression lasts for a limited period of time (transient).

Cell lines include:

- African green monkey kidney (COS) cells
- Baby hamster kidney (BHK) cells
- Human embryonic kidney (HEK-293)

Mammalian Cell Expression Systems

A generalized mammalian expression vector



The vector contains:

1. A multiple cloning site (MCS) in which to clone the gene to be expressed.

2. The ampicillin resistance gene (Amp^r) – a bacterial selectable marker.

3. The gene for a eukaryotic selectable market (SMG)

4. An *E. coli* origin of replication (ori^E) – to allow replication of the vector in bacteria.

5. A eukaryotic origin of replication (ori^{euk}) – to allow replication of the vector in mammalian cells.

6. Eukaryotic promoter (p), polyadenlyation (pa) and termination of transcription sequences (TT)

Mammalian Cell Expression Systems

Translational control elements

The gene to be expressed can be fitted with a variety of sequences that enhance translation and facilitate secretion and purification, including:

1. A Kozak sequence (K) to allow efficient initiation of translation – CC(A/G)CCATGG (where ATG is the start codon).

2. A signal sequence (S) to assure that the protein is secreted (if required).

3. A protein affinity tag (T) to facilitate purification of the protein.

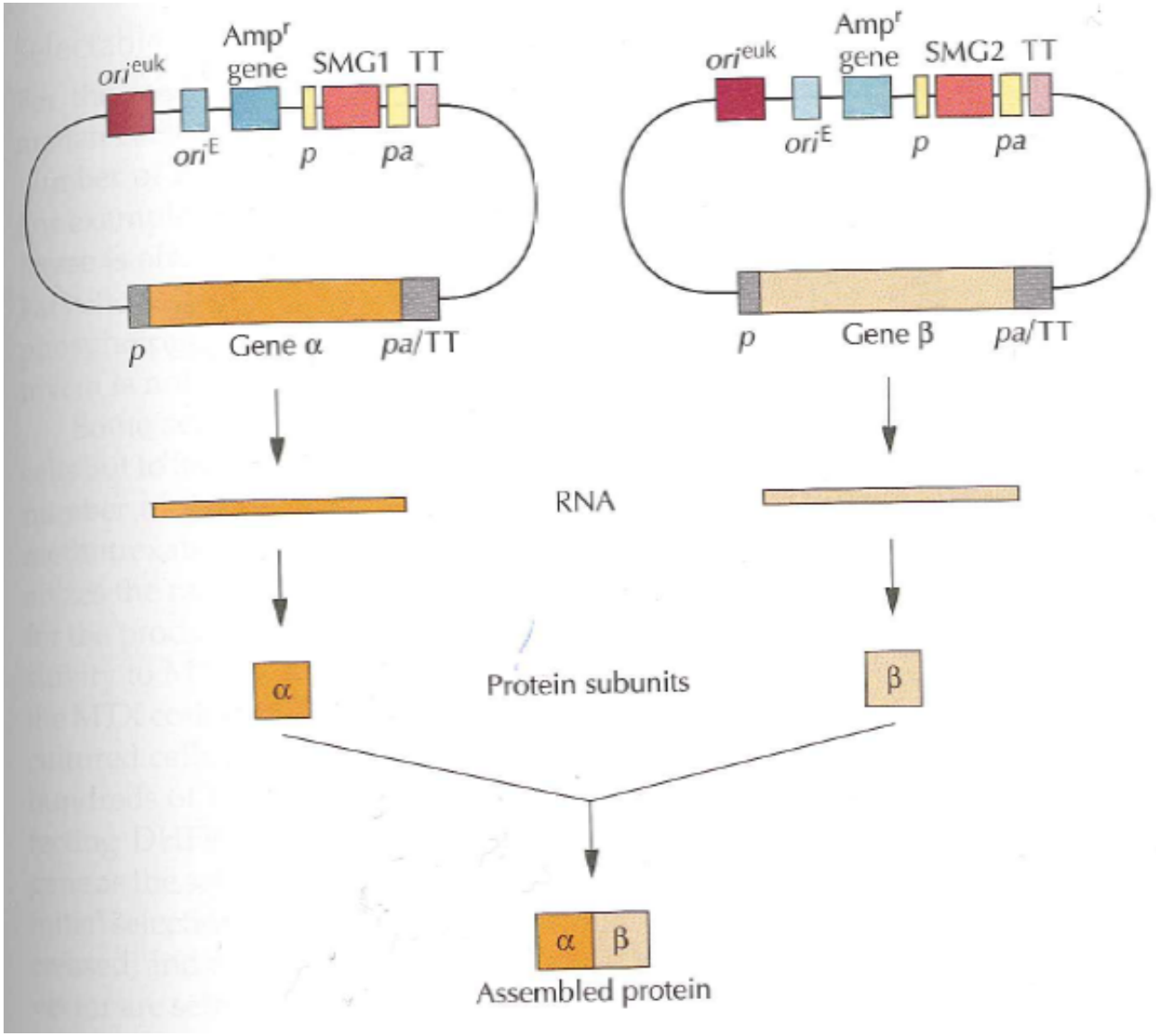
4. A proteolytic cleavage site (P) to allow the tag (T) to be removed from the purified protein.

5. A stop codon (SC) to terminate translation.

6. 5’ and 3’ untranslated regions (5’UTR and 3’UTR) to increase the efficiency of translation and improve mRNA stability.

Mammalian Cell Expression Systems

Expressing proteins made up of two different polypeptides



Some very important proteins (for example antibodies) contain two different polypeptides (the heavy and light chain in antibodies).

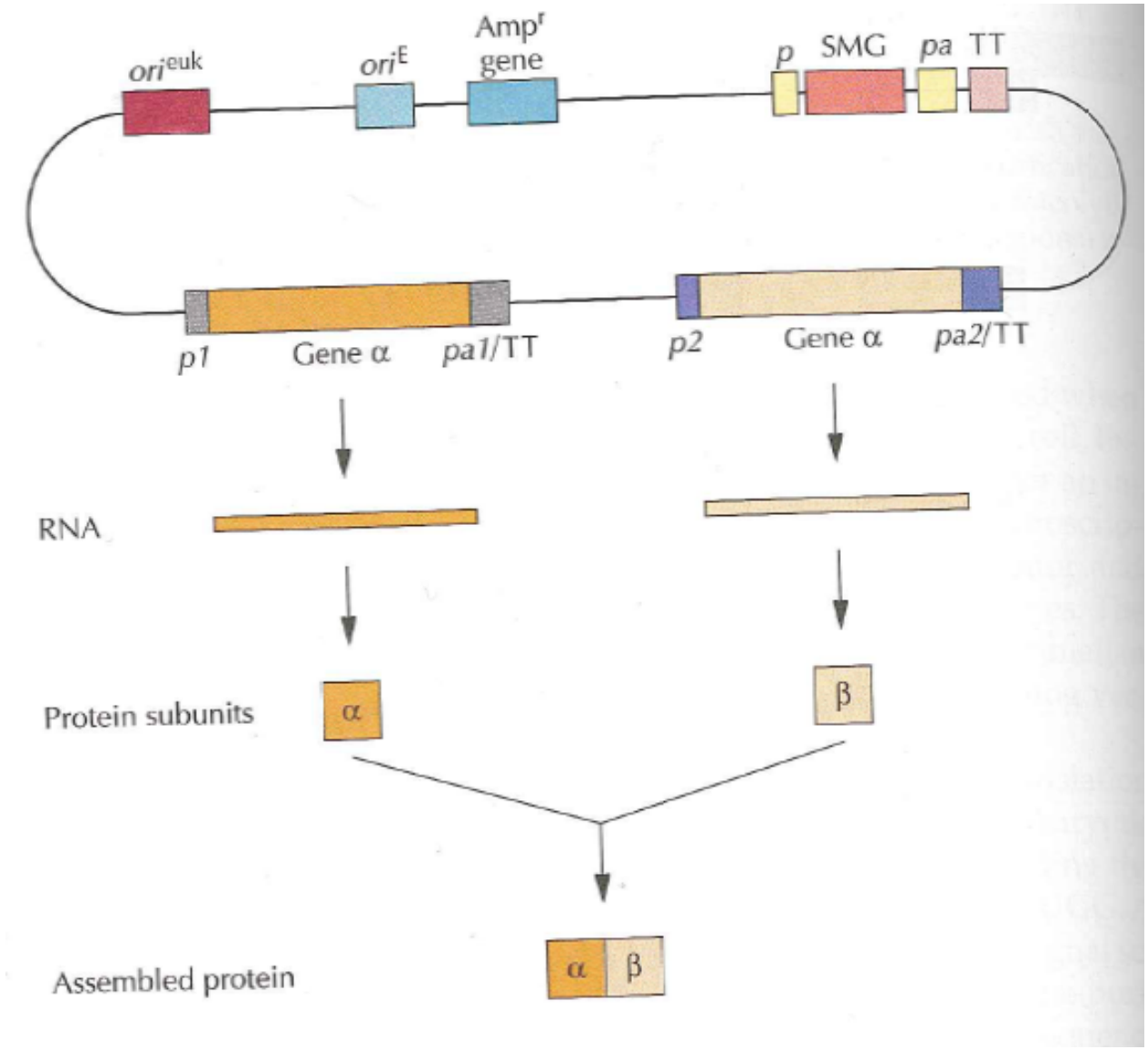
The genes for the two polypeptides (Gene α and Gene β) are cloned in expression vectors with different selectable marker genes (SMG1 and SMG2).

After cotransfection and selection with the two markers, the two polypeptides are co-expressed and the protein subunits can assemble to form the functional protein.

However, loss of one of the vectors from doubly transfected cells is common and the two vectors do not always have the same copy number, causing an imbalance in the expression of the two subunits.

Mammalian Cell Expression Systems

Expressing proteins made up of two different polypeptides

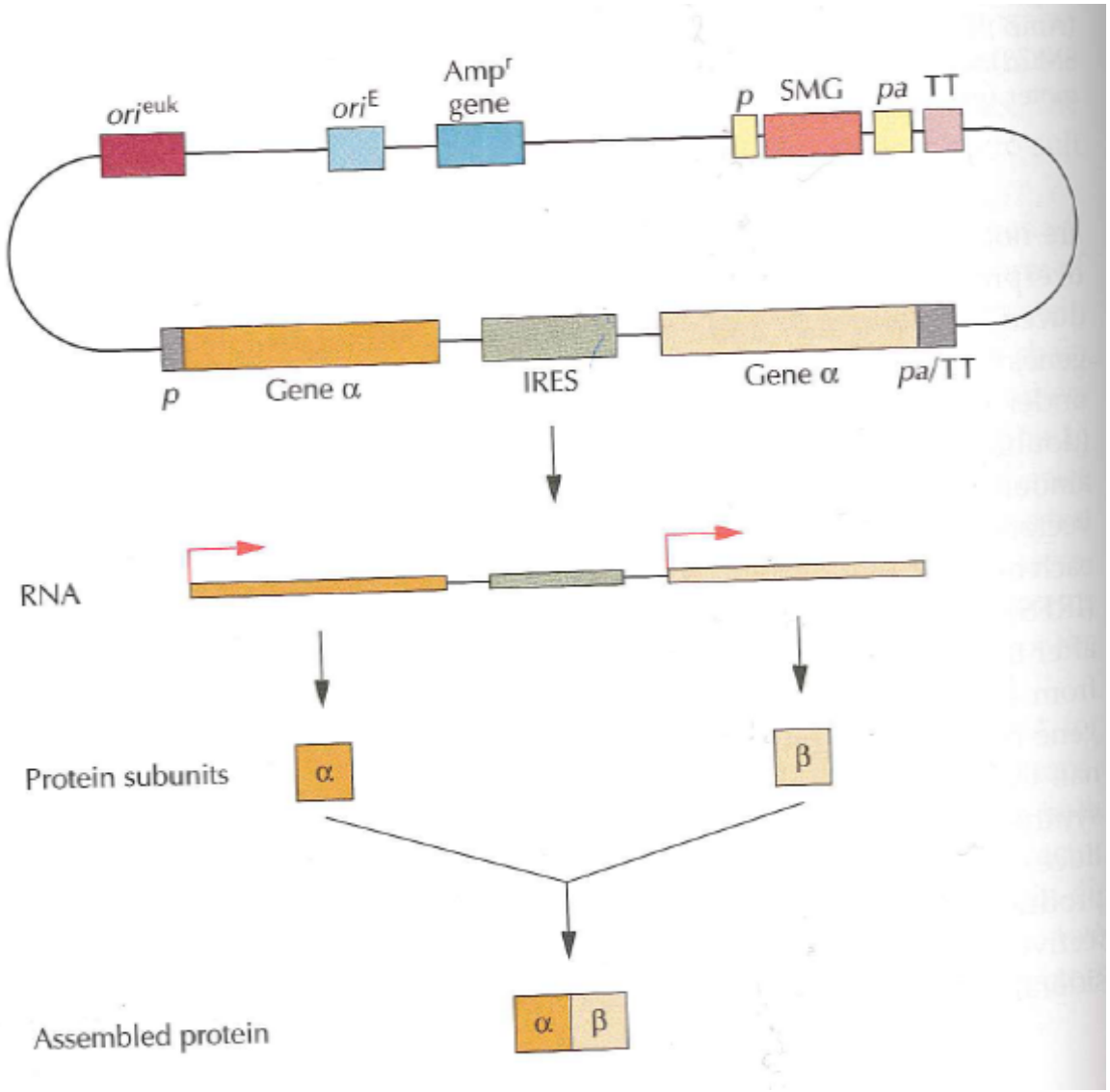


To overcome the problem of two vector systems two-gene expression vectors have been developed.

The gene for each subunit each has its own promoter (p1 and p2) and polyadenylation (pa1 and pa2) and transcription termination (TT) regions.

Mammalian Cell Expression Systems

Expressing proteins made up of two different polypeptides



Alternatively bicistronic expression vectors can be used.

There is a single promoter (p), polyadenylation (pa) and transcription termination region (TT) for both genes. Hence, there is only a single mRNA.

The mRNA contains an internal ribosome entry site (IRES) from a mammalian virus which allows intiation of translation of the downstream gene.

Mammalian Cell Expression Systems

Selectable marker systems for mammalian expression vectors

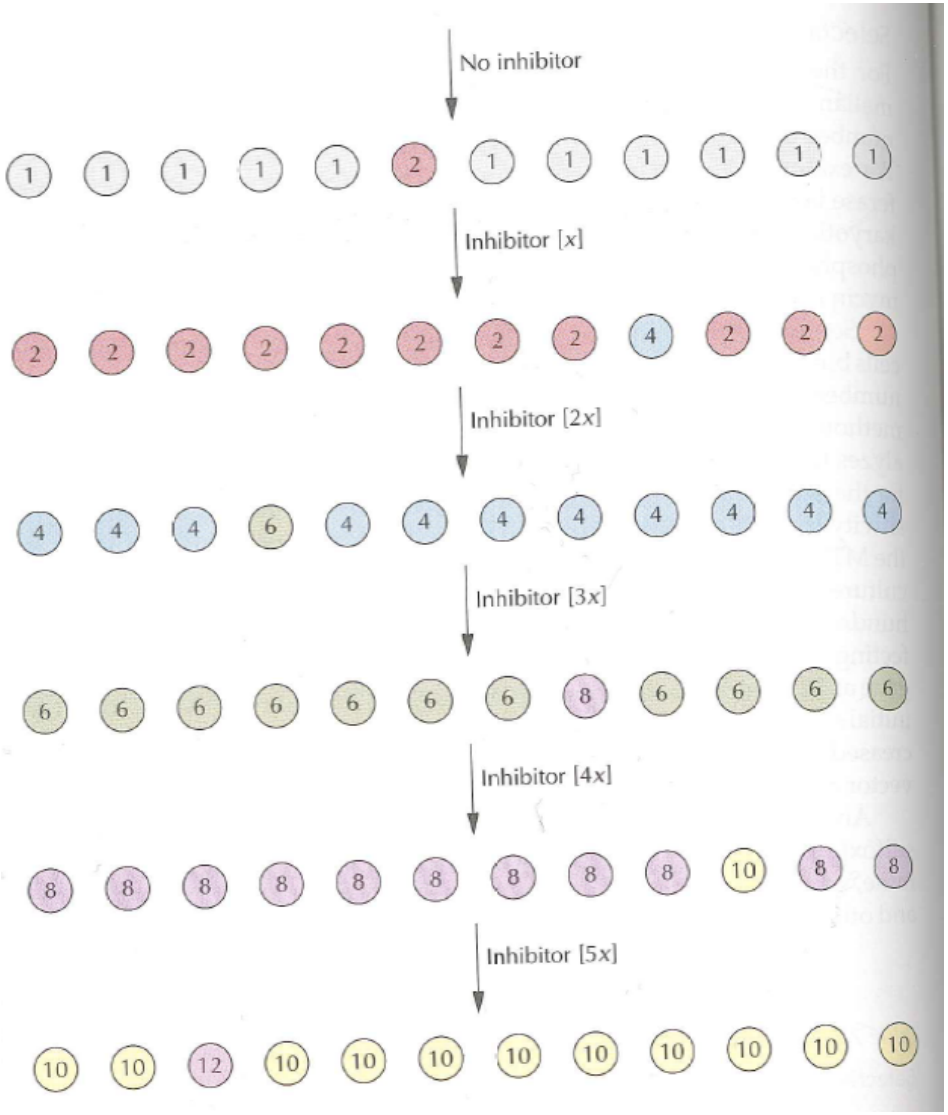
Table 7.2 Selective marker gene systems for mammalian cells

Selective agent	Action of selective agent	Marker gene	Action of marker gene protein
Xyl-A	Damages DNA	Adenine deaminase (*ada*)	Deaminates Xyl-A
Blasticidin S	Inhibits protein synthesis	Blasticidin S deaminases (*Bsr*, *BSD*)	Deaminates blasticidin S
Bleomycin	Breaks DNA strands	Bleomycin-binding protein (*Ble*)	Binds to bleomycin
G-418 (Geneticin)	Inhibits protein synthesis	Neomycin phosphotransferase (*Neo*)	Phosphorylates G-418
Histidinol	Produces cytotoxic effects	Histidinol dehydrogenase (*hisD*)	Oxidizes histidinol to histidine
Hygromycin B	Inhibits protein synthesis	Hygromycin B phosphotransferase (*Hph*)	Phosphorylates hygromycin B
MSX	Inhibits glutamine synthesis	Glutamine synthetase (*GS*)	Cells that produce excess glutamine synthetase survive
MTX	Inhibits DNA synthesis	Dihydrofolate reductase (*dhfr*)	Cells that produce excess dihydrofolate reductase survive
PALA	Inhibits purine synthesis	Cytosine deaminase (*codA*)	Lowers cytosine levels in the medium by converting cytosine to uracil
Puromycin	Inhibits protein synthesis	Puromycin *N*-acetyltransferase (*Pac*)	Acetylates puromycin

MSX, methionine sulfoximine; MTX, methotrexate; PALA, *N*-(phosphoacetyl)-L-aspartate; Xyl-A, 9-β-D-xylofuranosyl adenine.

Mammalian Cell Expression Systems

Selectable markers that allow amplification of the copy number of the vector



The dihydrofolate reductase-methotrexate (DHFR-MTX) system allows selection for increased vector copy number and enhanced protein yield.

DHFR catalyses the reduction of dihydrofolate to tetrahydrofolate, which is required for the production of purines.

MTX is a competitive inhibitor of DHFR.

Sensitivity of the cells to MTX can be overcome by making more DHFR, which can be achieved by increasing the copy number of vectors carrying the DHFR gene.

The concentration of MTX is gradually increased over time and eventually cells with very high vector copy number and protein expression level are selected.