Vectors for Recombinant DNA Technology

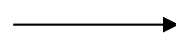
Plasmids
Autonomous Replicaton
Integration into genome
Shuttle Plasmids
E.Coli → Target host

Cosmids, Bacmids
Plasmid – Bacteriophage Hybrids

Phages
Bacteriophage Lambda

Artificial Chromosomes
YAC,

Viruses
Baculovirus – Insect Cells
Retroviruses – Mammalian Cells

Autonomous Replication  Plasmids Viruses ARS

Integration into genome Site specific -- Ectopic

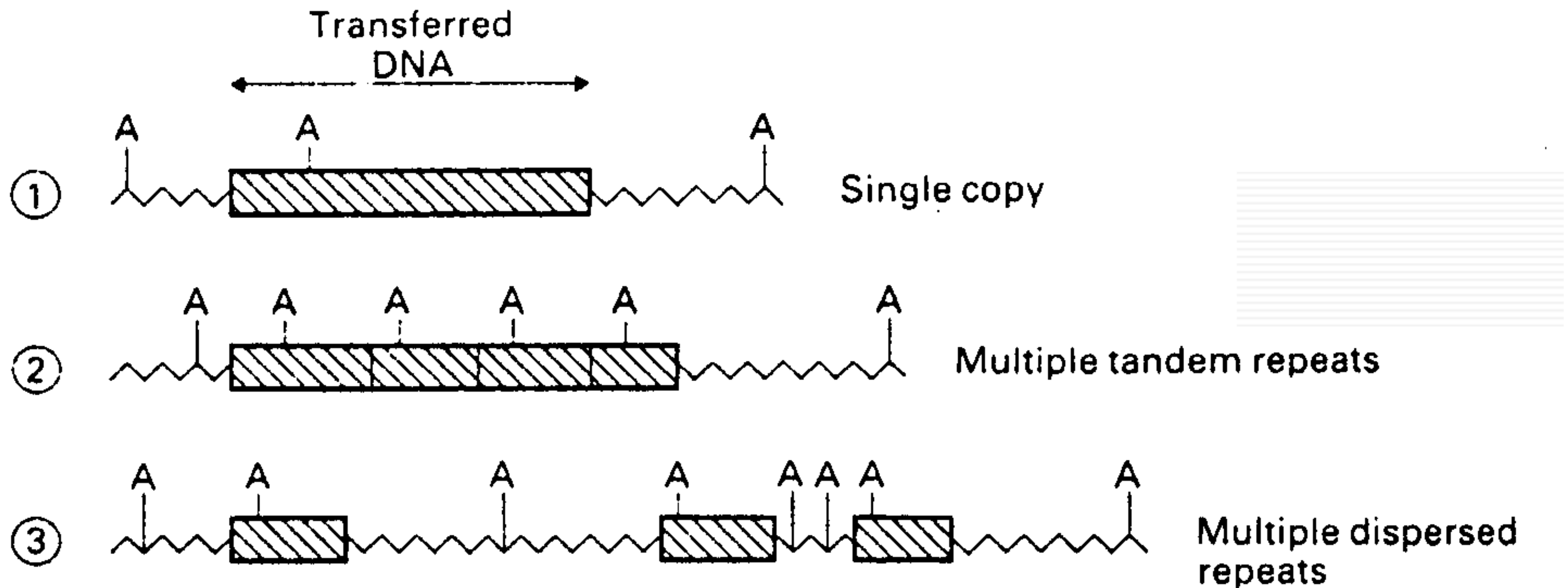
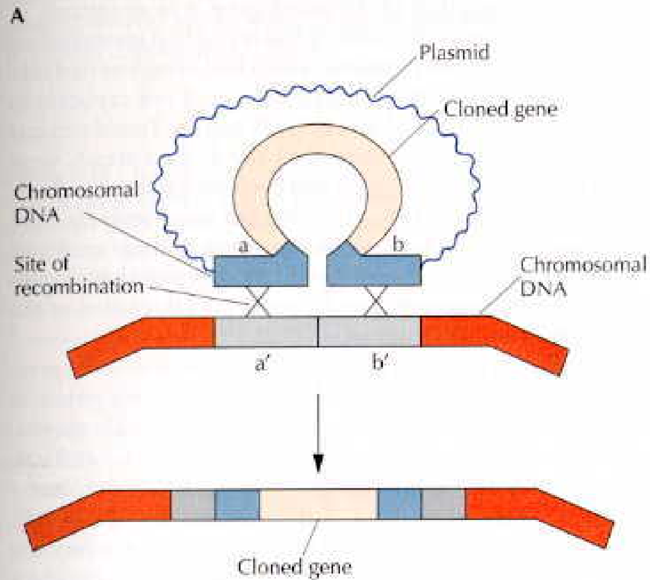


Figure 6.15 Two ways to integrate a cloned gene into a chromosomal site. **A.** On a plasmid, the cloned gene has been inserted in the middle of a cloned segment of DNA (ab) from the host chromosome. Homologous DNA pairing occurs between plasmid-borne DNA regions a and b and host chromosome DNA regions a′ and b′, respectively. A double-crossover event (X—X) results in the integration of the cloned gene. **B.** The cloned gene is inserted adjacent to the cloned DNA (c) from the host chromosome. Homologous DNA pairing occurs between plasmid DNA region c and host chromosome DNA region c′. A single recombination event (X) within the paired c-c′ DNA region results in the integration of the entire plasmid, including the cloned gene.

A



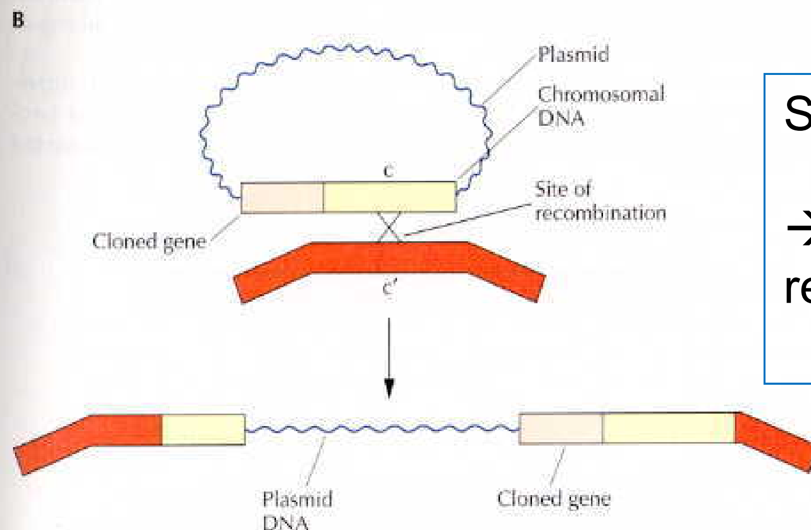
Gene Replacement

→ Double Cross-over at regions showing sufficient homology
Linearized DNA

Ectopic Integration

→ Recombination at regions of no (low ?) Homology

B

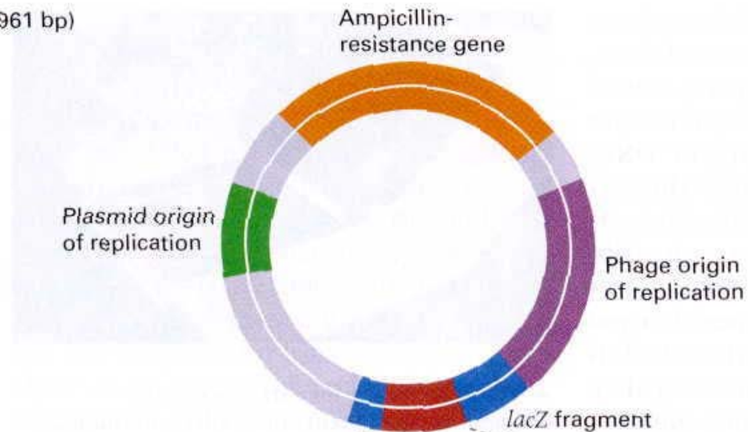


Site specific Insertion

→ → Single Cross-over at regions showing sufficient homology

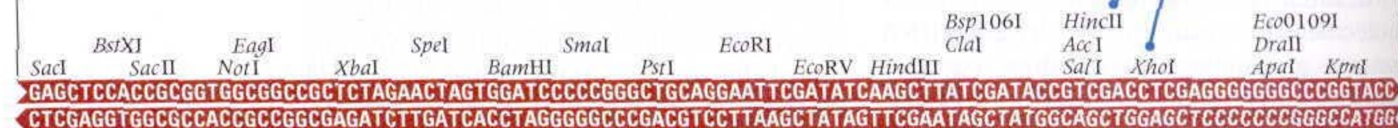
Plasmid vector

(A) pBluescript plasmid (2961 bp)



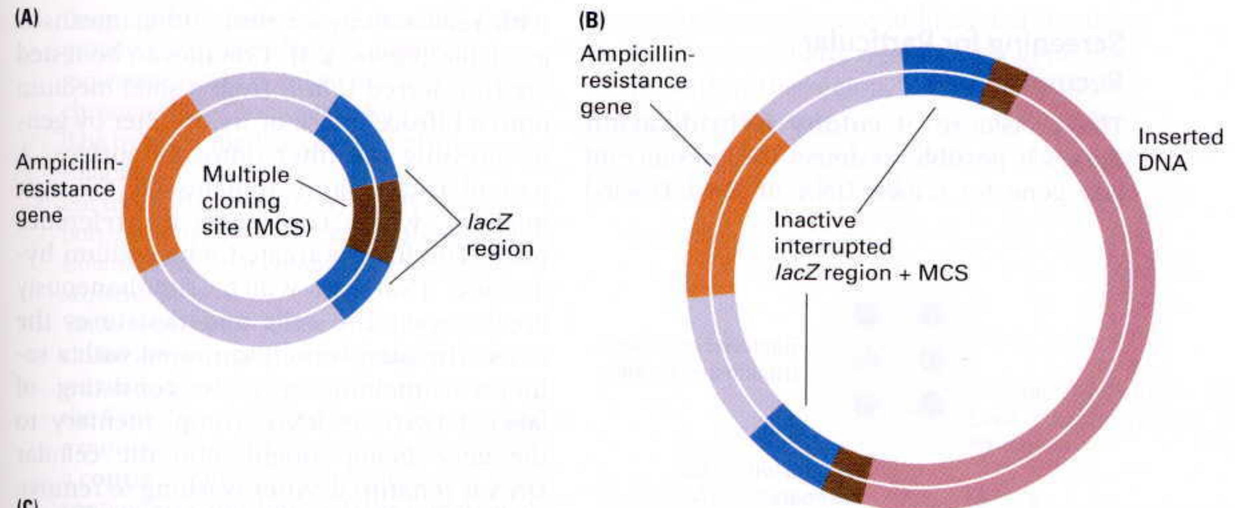
Restriction sites present in polylinker

(B)



Polylinker comprises nucleotides numbered 759 through 657 in plasmid DNA sequence

Figure 13.9 (A) Diagram of the cloning vector pBluescript II. It contains a plasmid origin of replication, an ampicillin-resistance gene, a multiple cloning site (polylinker) within a fragment of the *lacZ* gene from *E. coli*, and a bacteriophage origin of replication. (B) Sequence of the multiple cloning site showing the unique restriction sites at which the vector can be opened for the insertion of DNA fragments. The numbers 657 and 759 refer to the position of the base pairs in the complete sequence of pBluescript. [Courtesy of Stratagene Cloning Systems, La Jolla, CA.]



(C)

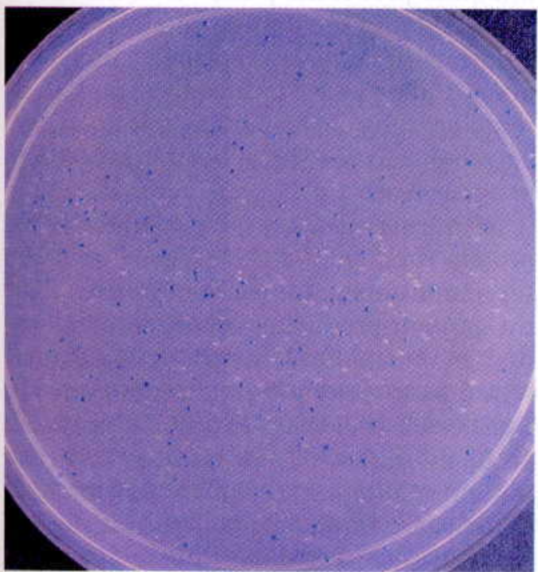


Figure 13.10 Detection of recombinant plasmids through insertional inactivation of a fragment of the *lacZ* gene from *E. coli.* (A) Nonrecombinant plasmid containing an uninterrupted *lacZ* region. The multiple cloning site (MCS) within the region (not drawn to scale) is sufficiently small that the plasmid still confers β-galactosidase activity. (B) Recombinant plasmid with donor DNA inserted into the multiple cloning site. This plasmid confers ampicillin resistance but not β-galactosidase activity, because the donor DNA interrupting the *lacZ* region is large enough to render the region nonfunctional. (C) Transformed bacterial colonies. Cells in the white colonies contain plasmids with inserts that disrupt the lacZ region; those in the blue colonies do not. [C courtesy of Elena R. Lozovsky.]

Bakteriophage Lambda Vectors

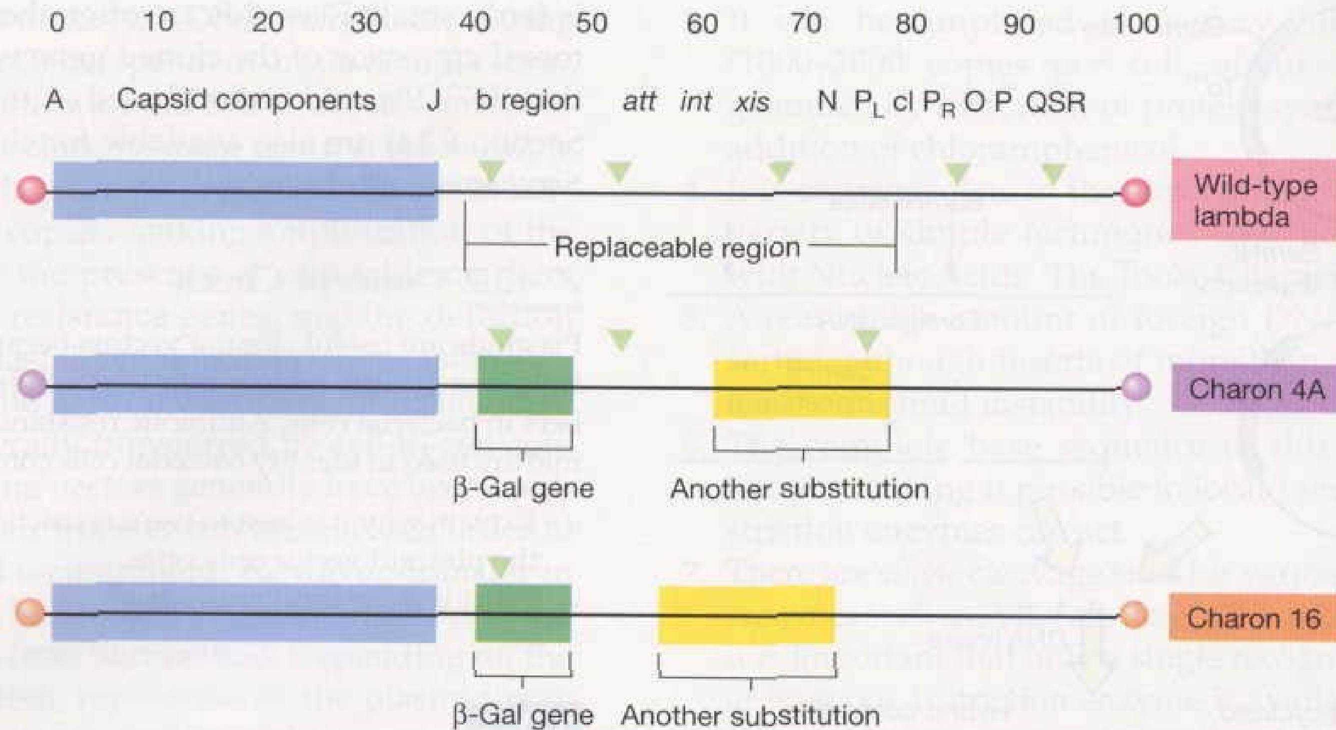


FIGURE 10.3 Molecular cloning with lambda. Abbreviated genetic map of bacteriophage lambda showing the cohesive ends as circles (Figure 8.26). Charon 4A and 16 are both derivatives of lambda, which have various substitutions and deletions in the nonessential region. One of the substitutions in each case is a gene (β-Gal) that codes for the enzyme β-galactosidase, which permits detection of clones containing this phage. Whereas the wild-type lambda genome is 48.5 kilobase pairs, that for Charon 4A is 45.4 and that for Charon 16 is 41.7 kilobase pairs. The arrows (▼) shown above the maps of each phage indicate the sites recognized by the restriction enzyme *Eco*RI.

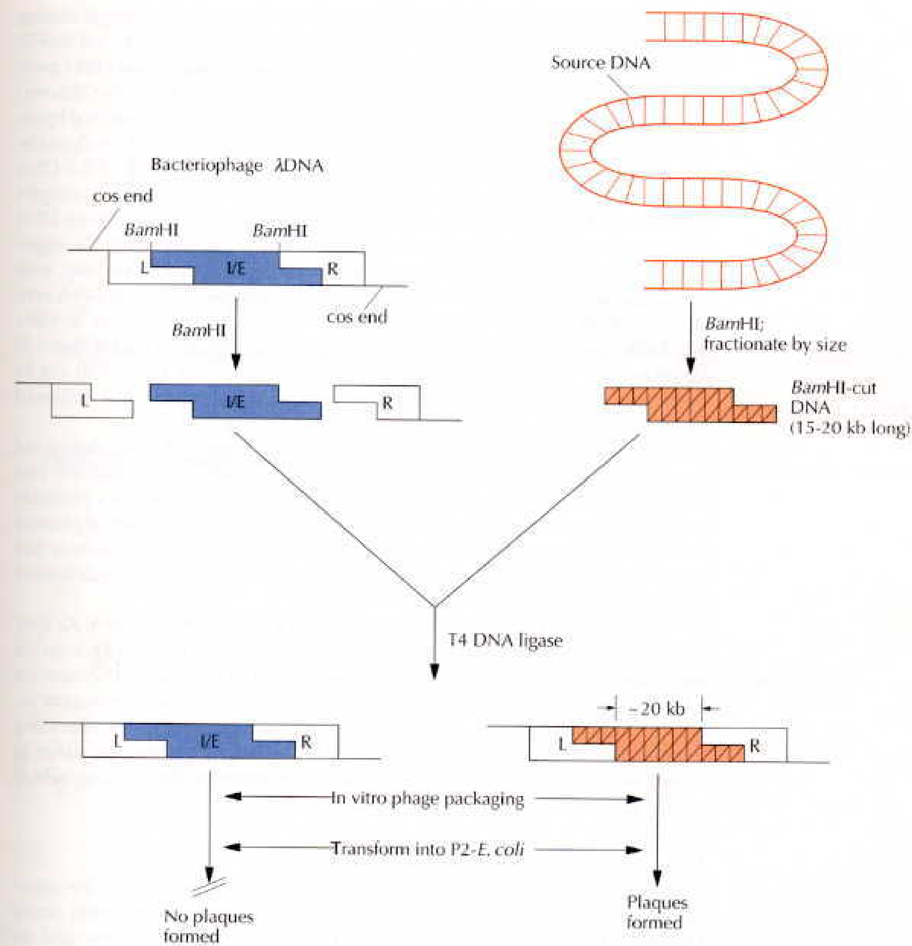
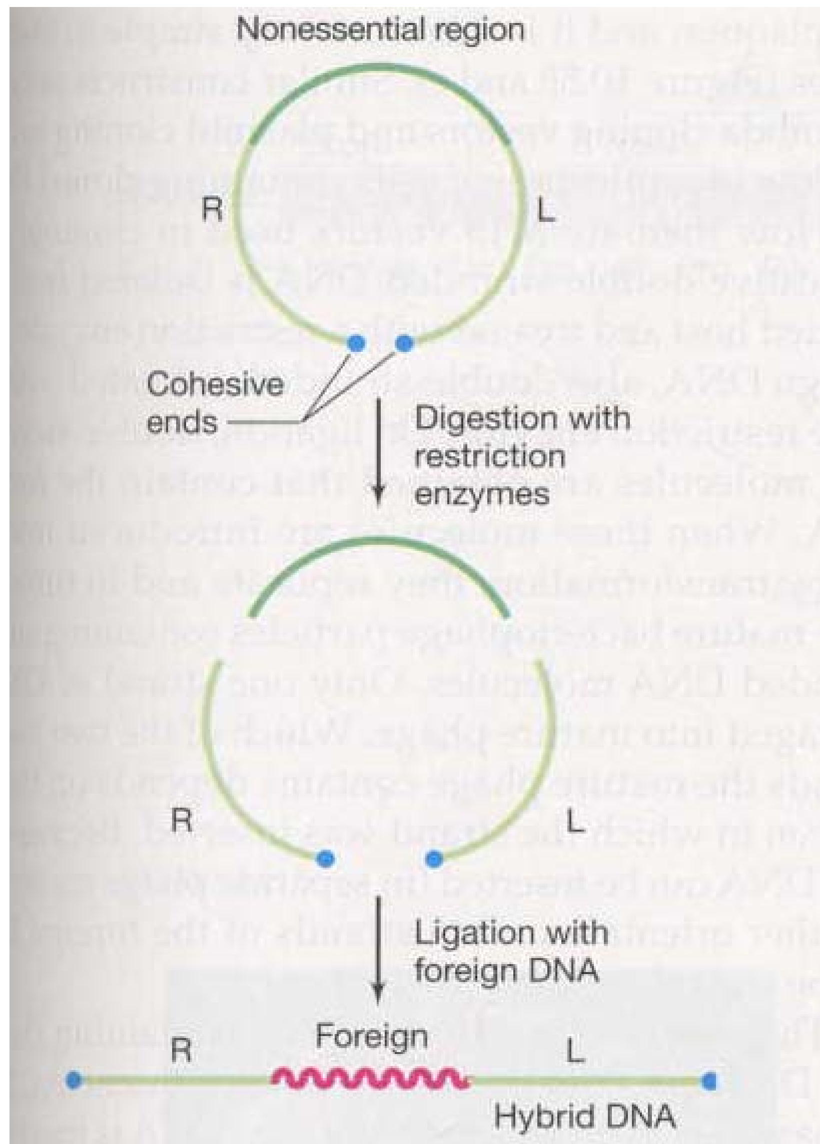
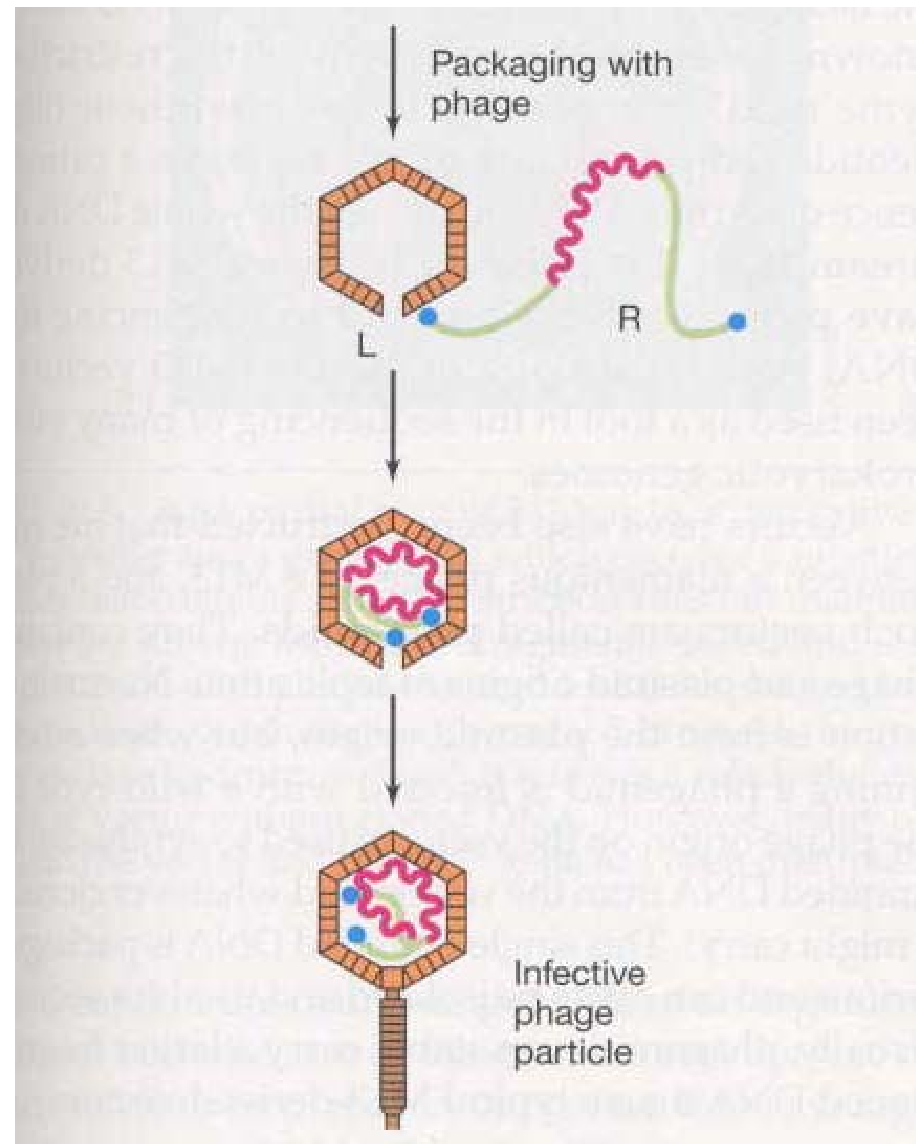


Figure 4.17 Bacteriophage λ cloning system. Bacteriophage λ is engineered to have two *Bam*HI sites that flank the I/E region of the bacteriophage λ genome. The extensions indicate the cos ends of the λ DNA. For cloning, the source DNA is cut with *Bam*HI and fractionated by size to isolate pieces that are 15 to 20 kb long. The bacteriophage λ DNA is also cut with *Bam*HI. The two DNA samples are mixed and treated with T4 DNA ligase. The ligation reaction mixture will contain a number of different DNA molecules, including (1) reconstituted bacteriophage λ and (2) the bacteriophage λ L and R regions with a 20-kb piece of DNA from the source DNA instead of the I/E region. These molecules are packaged into bacteriophage λ heads in vitro, and infective particles are formed after the addition of tail assemblies. After infection of *E. coli* cells that have P2 bacteriophage DNA integrated in their chromosomes, only the molecules with the R and L regions and a cloned ~20-kb piece of DNA can replicate and form infectious bacteriophage λ. In this way, only the bacteriophage λ containing a DNA insert are perpetuated.

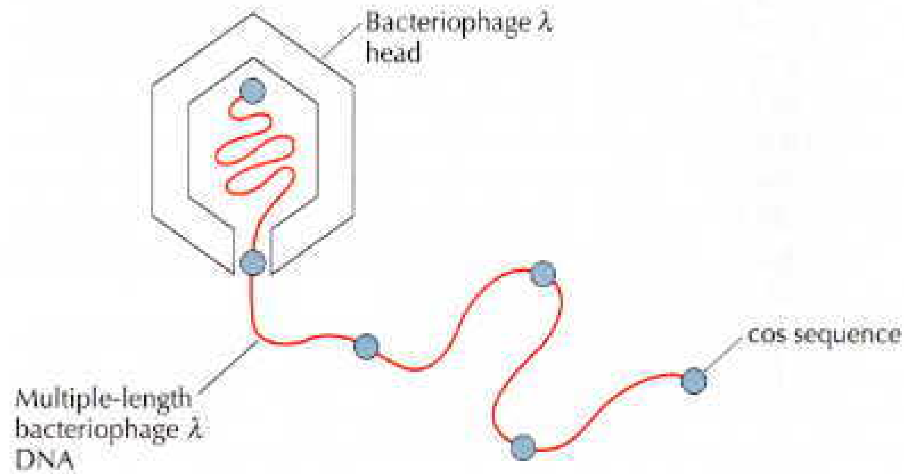
Cloning in Lambda Vectors





In vitro packaging into phage vesicles

A



B

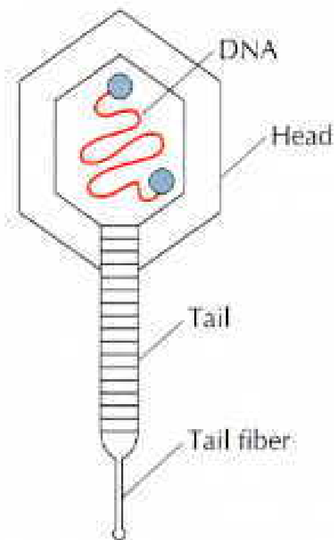
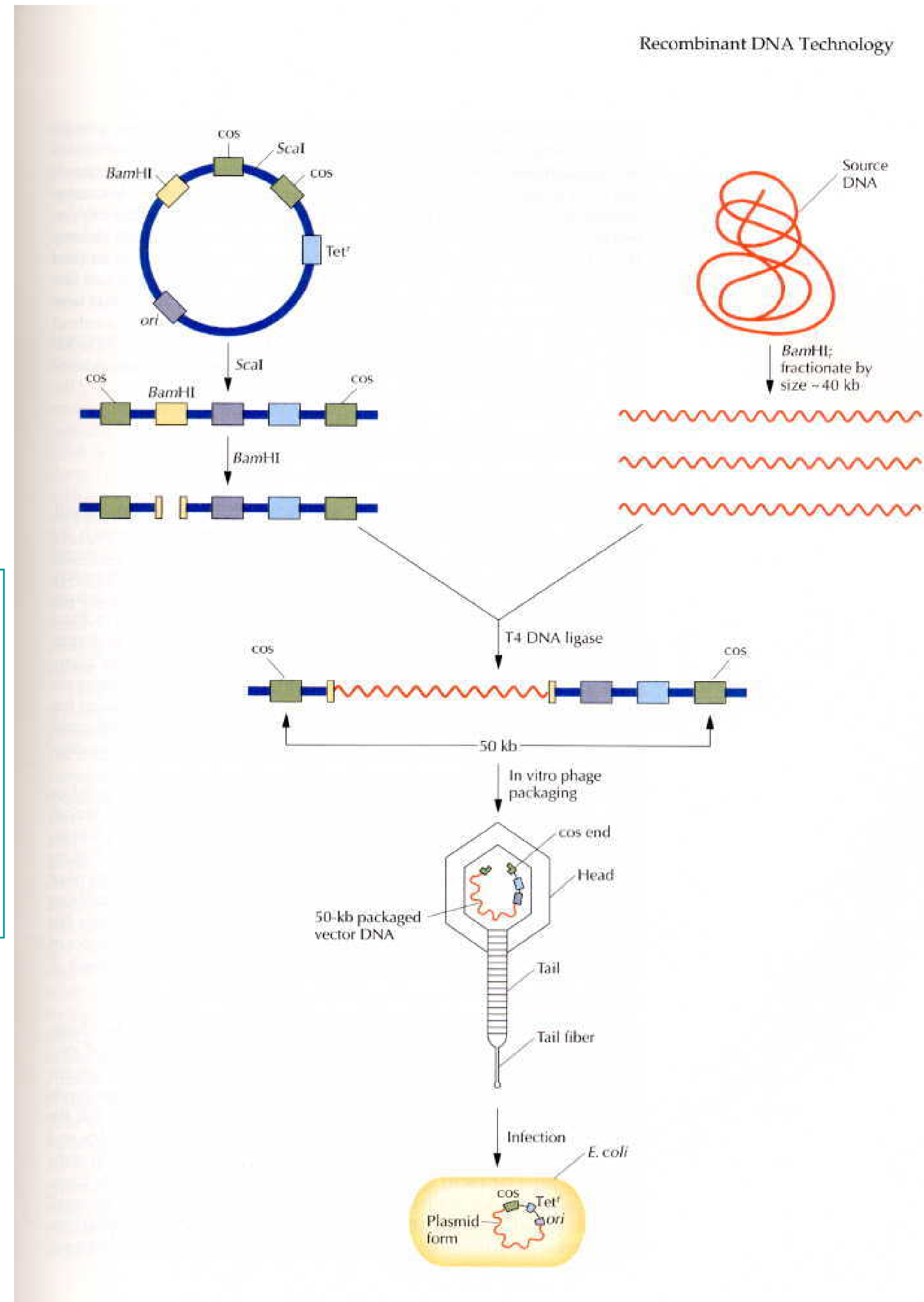


Figure 4.16 Packaging of bacteriophage λ DNA into heads during the lytic cycle. **A.** DNA replication from the circular form of bacteriophage λ creates a linear form that is composed of contiguous, multiple lengths of bacteriophage DNA of approximately 50 kb each. **B.** Each newly assembled head is filled with a 50-kb unit of λ DNA before the tail assembly is attached.

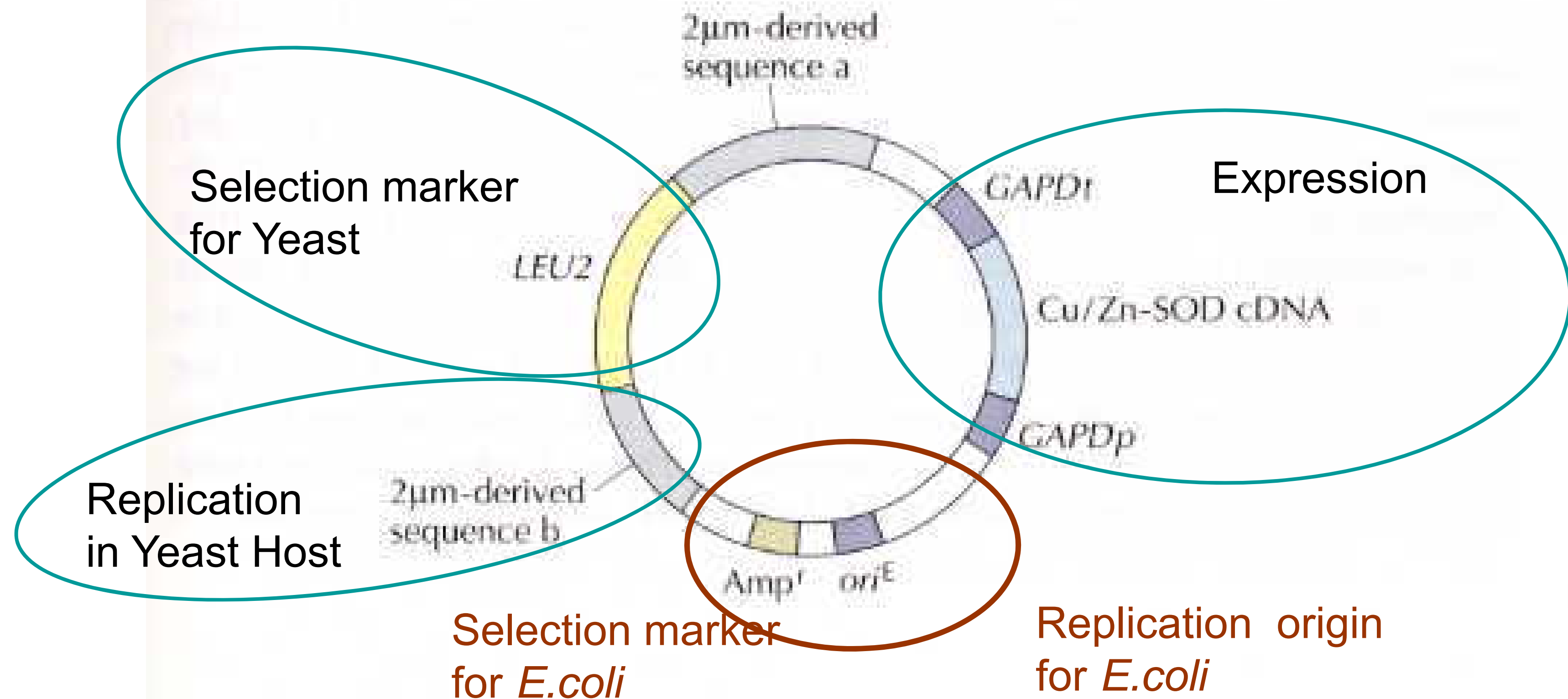
Cosmid vectors

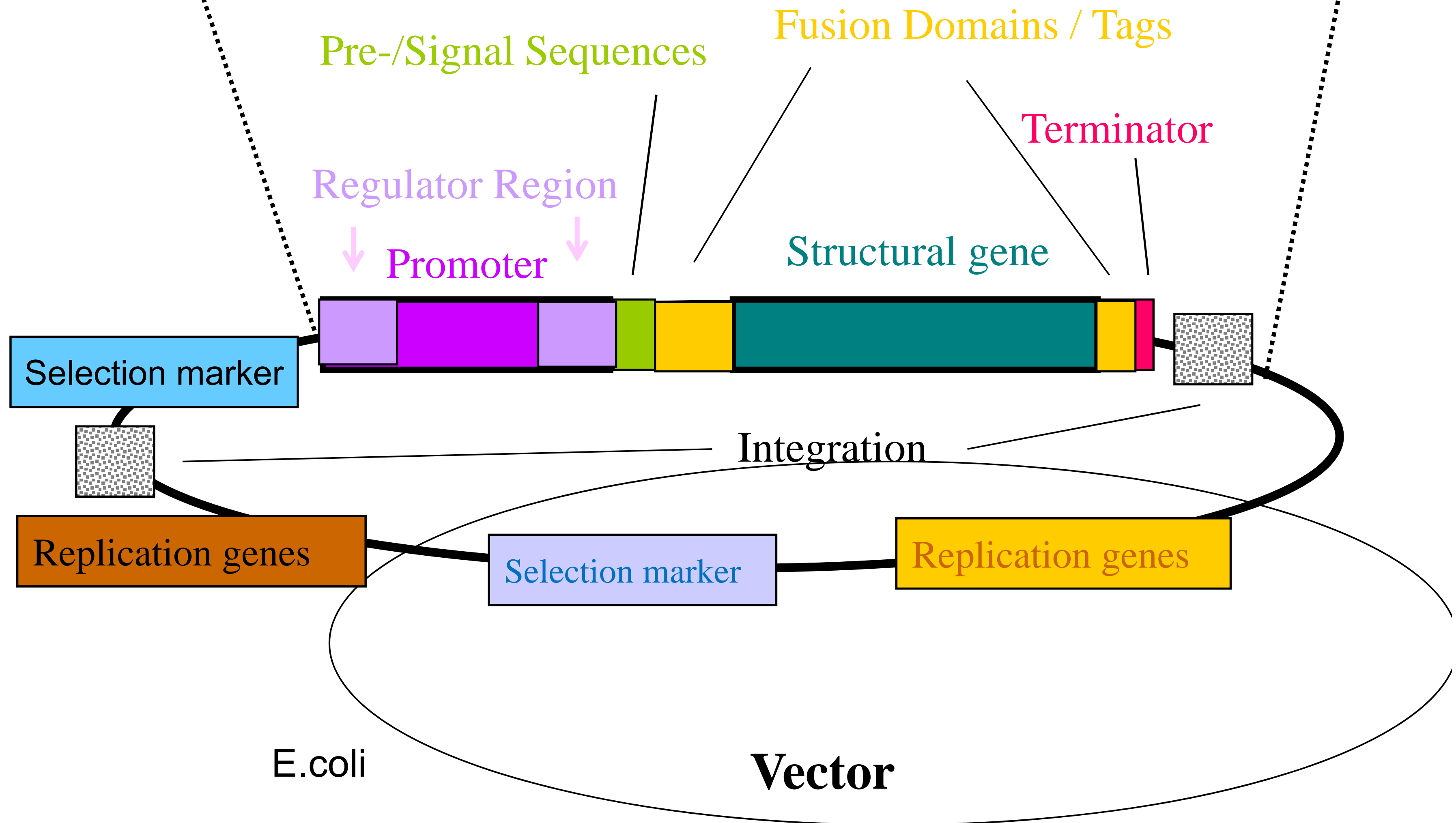
Principle: Plasmid
DNA Transfer via
Phage infection

Resulting recombinant clone
Contains self-replicating plasmid

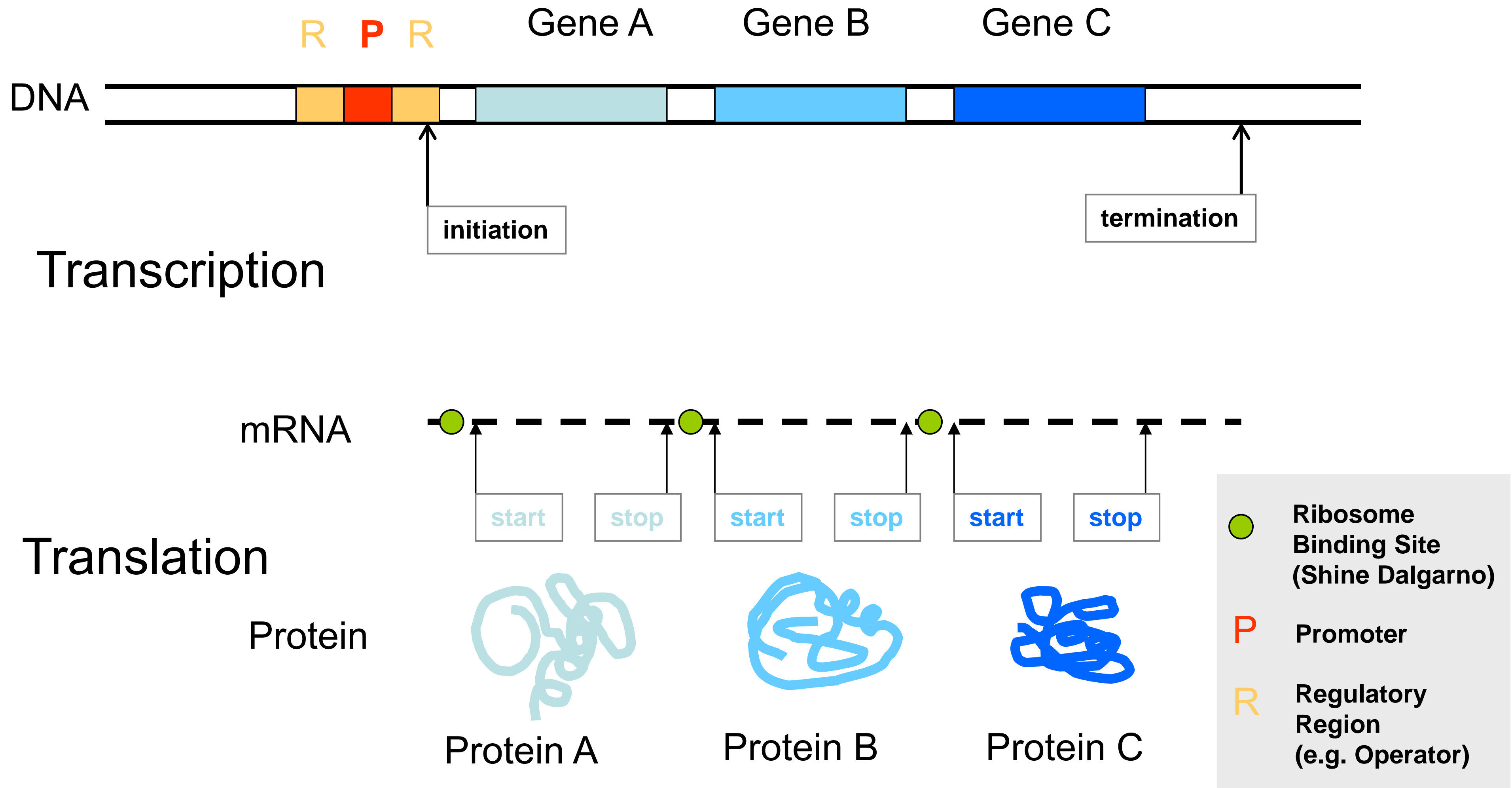


E.coli – *Saccharomyces cerevisiae* Shuttle Vector





Expression Cassette
Pre-/Signal Sequences
Fusion Domains / Tags
Terminator
Regulator Region
Promoter
Structural gene
Selection marker
Integration
Replication genes
Selection marker
Replication genes
E.coli
Vector


Gene Expression in Prokaryotes
R
P
R
Gene A
Gene B
Gene C
DNA
initiation
termination
Transcription
mRNA
start
stop
start
stop
start
stop
Translation
Protein
Protein A
Protein B
Protein C
Ribosome Binding Site (Shine Dalgarno)
P Promoter
R Regulatory Region (e.g. Operator)
Post-translational processing

9.10.14

Gene Expression – Points to consider

Location in Genome → Autonomous replication, Integration

Transkription Initiation → Promoters **Transkription Termination**

Regulatory Systems → positive/negative regulatory systems

Transkript Processing

Translation Initiation

RNA Structure

- Codon usage
- mRNA Stability

Post-translational modifications

- Modification of AA-side chains: Glycosylation, Phosphorylation, etc
- Proteolytic Processing

Protein Folding

- Disulfide bond formation

Assembly of subunits

Toxicity of gene producs

Protein Degradation

Localization

- Intracellular
- Periplasmic
- Extracellular
- Membrane associated
- Organelle specific
- Surface display

Heterologous expression in prokaryotes – *E.coli*

Transcription

- **constitutive promoters**
- **regulated promoters**
  - **lambda p_L, p_R**
  - ***lac, trp, tac. trc***
  - **T7**
- **termination**
  - **rrnB (T1,T2), trpt**
  - **Lambda N gene (premature termination)**

m-RNA stability

Translation

- **Initiation – SD sequence ...AGGAG....**
- **elongation – codon usage**

Proteolysis

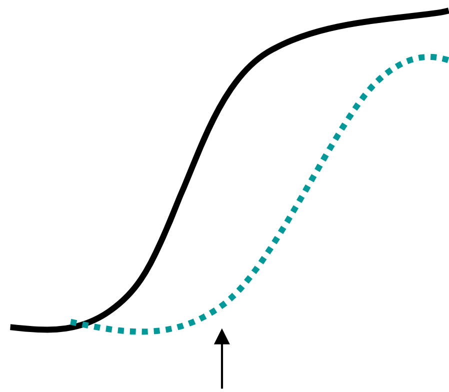
- **Lon, Clp, *htpR* (heat shock regulatory protein)**

Plasmid copy number and segregation

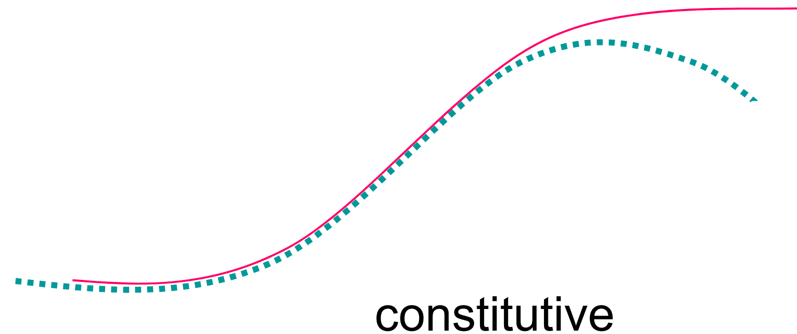
Regulated Promoters ←→ Constitutive Promoters

Both systems are used

Preferred Combination: strong Promoters – tightly regulated



induction



constitutive

Constitutive promoters: weak to medium activity

Regulated Expression in Prokaryotes

Gene Repression

negative Control

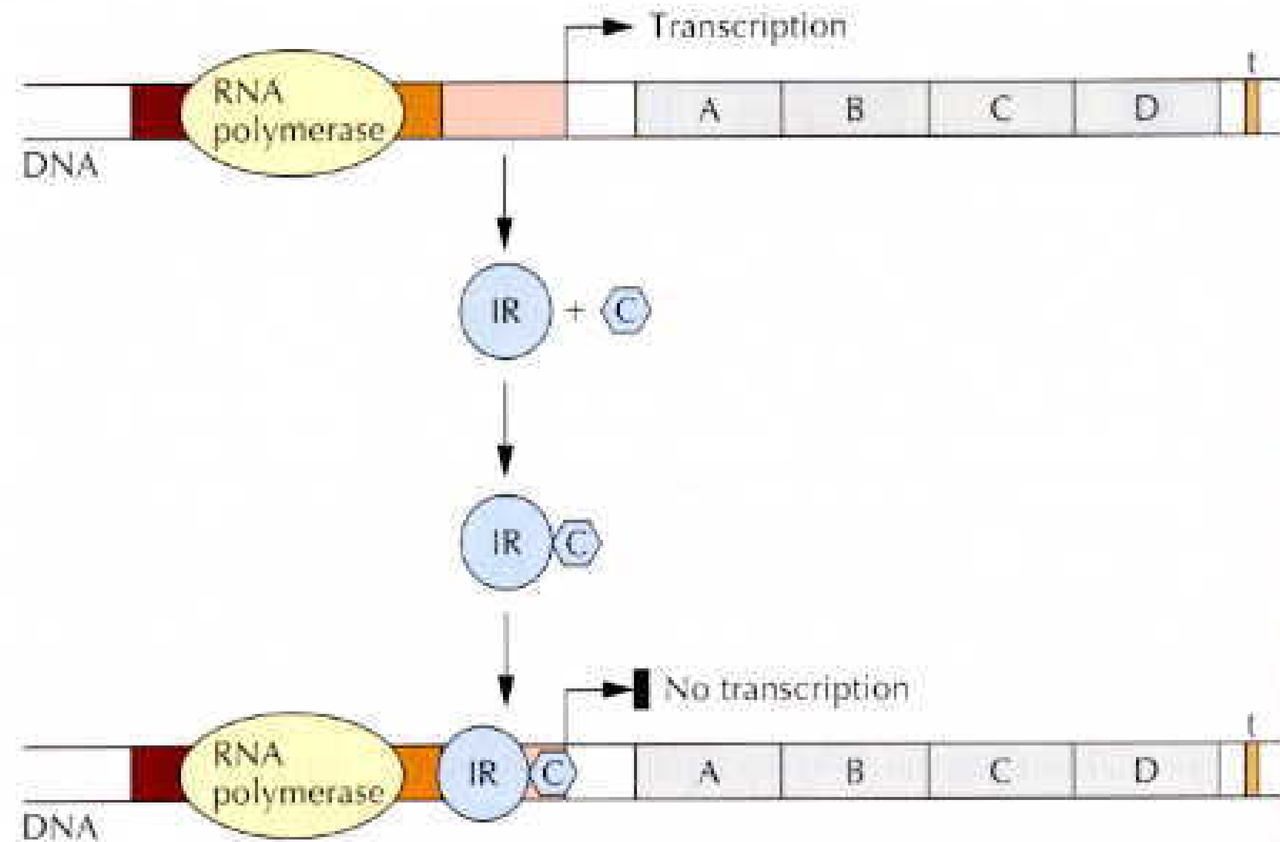
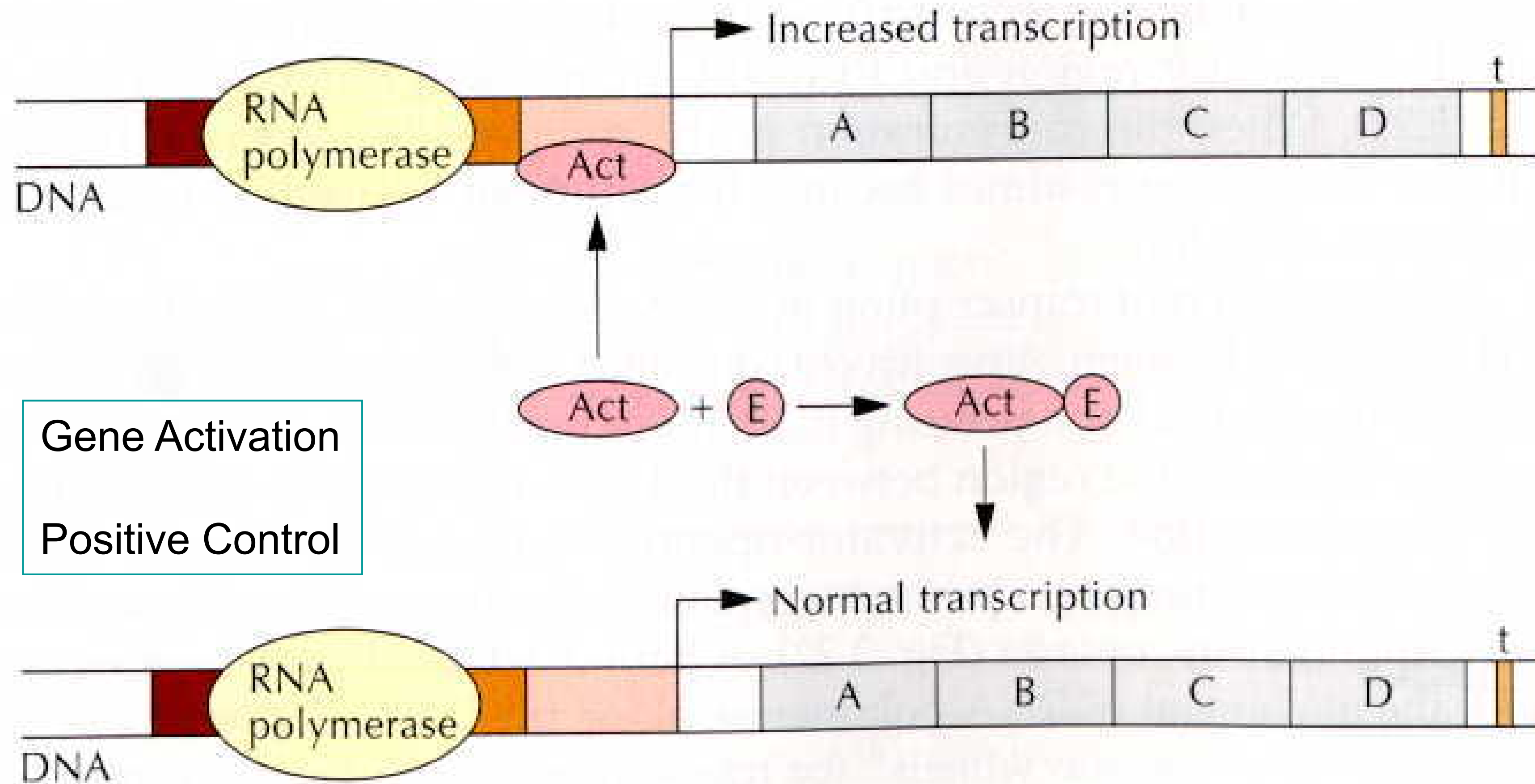


Figure 3.21 Induction of the off state for transcription of a bacterial operon. The binding of a corepressor molecule (C) to an inactive repressor protein (IR) changes the conformation of the repressor protein. The corepressor–repressor protein complex (IR-C) binds to the operator region and blocks transcription of the operon by RNA polymerase.

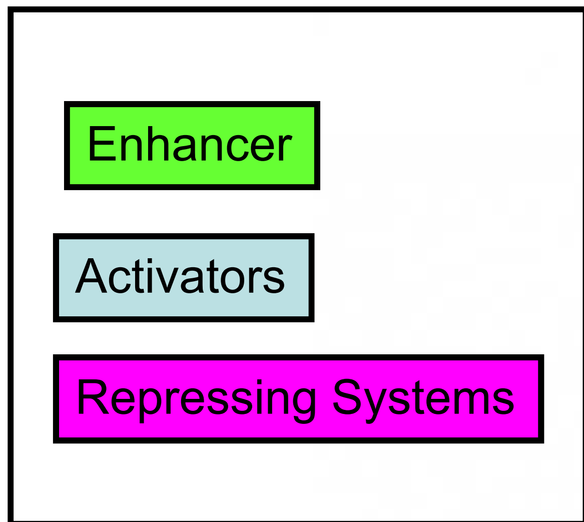
Regulated Expression in Prokaryotes

Figure 3.22 Activation and deactivation of a bacterial operon. An activator protein (Act) binds to an activating site and enhances the rate of transcription of the operon. An effector molecule (E) binds to the activator protein. The Act–E complex does not bind to the activating site. The rate of transcription of the operon is diminished when the activating site is not occupied by the activating protein.



Regulated Expression in Eukaryotes

Complex Initiation System



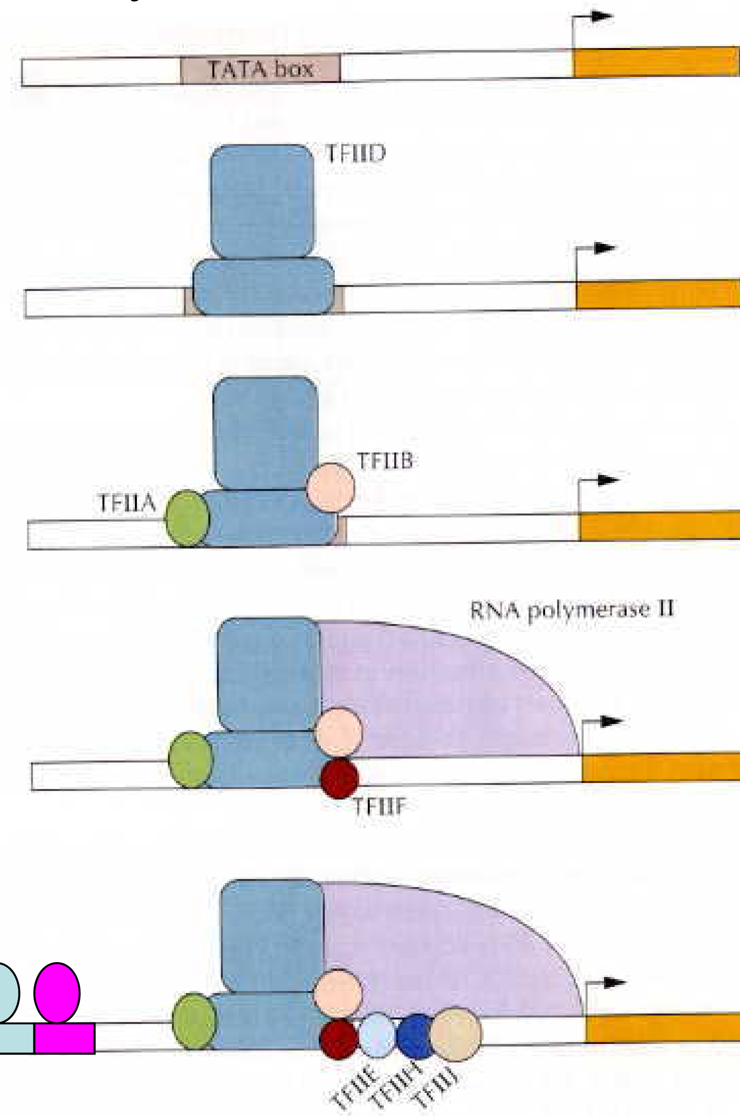
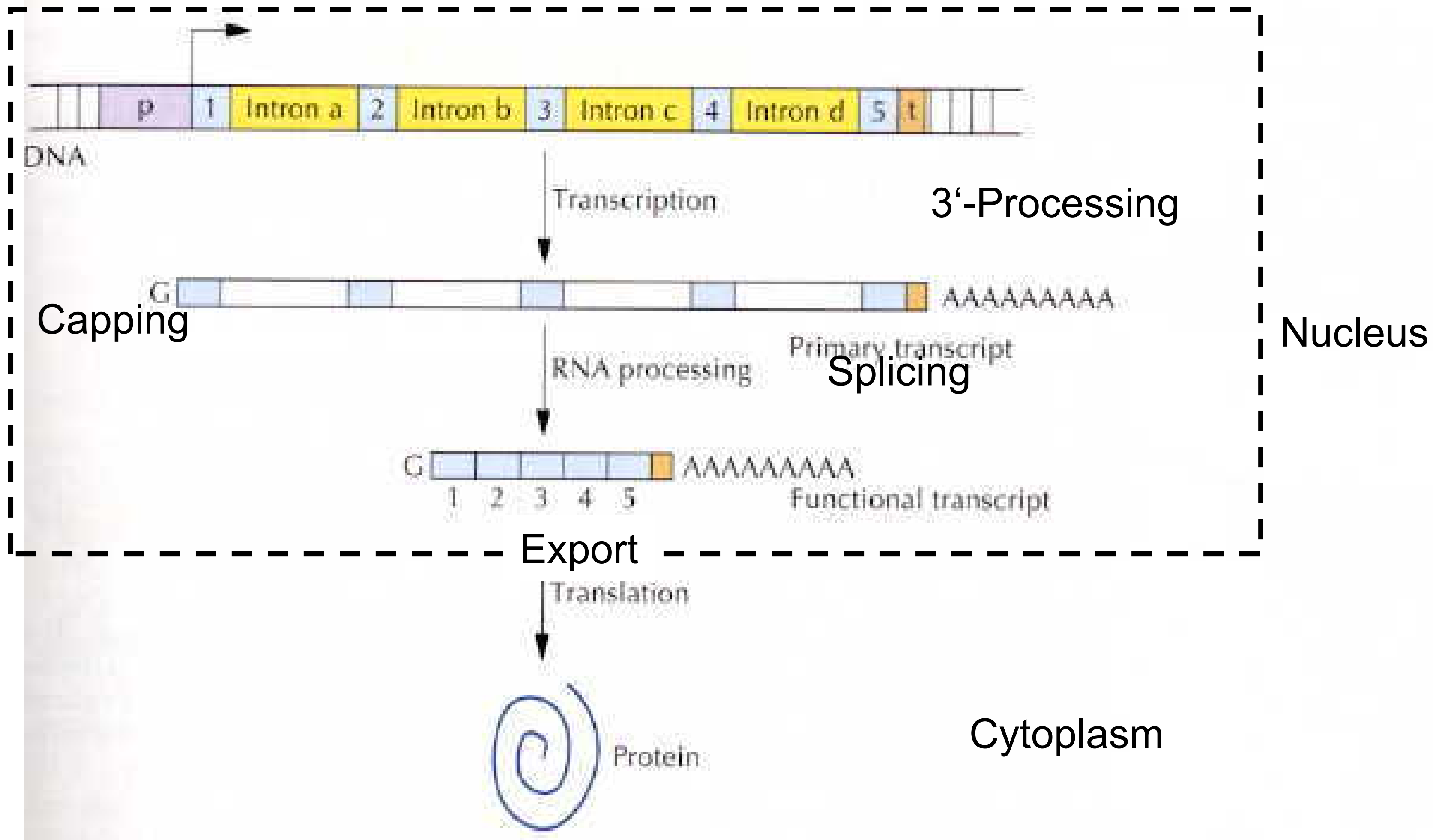


Figure 3.24 Formation of an RNA polymerase II transcription initiation complex at a TATA box. Transcription factor TFIID binds to a TATA box, and, in sequence, other transcription factors and RNA polymerase II bind to form a protein aggregate that is responsible for initiating transcription. The right-angled arrow designates the site of initiation and direction of transcription.

RNA Processing → Complex Mechanisms



Alternative Splicing

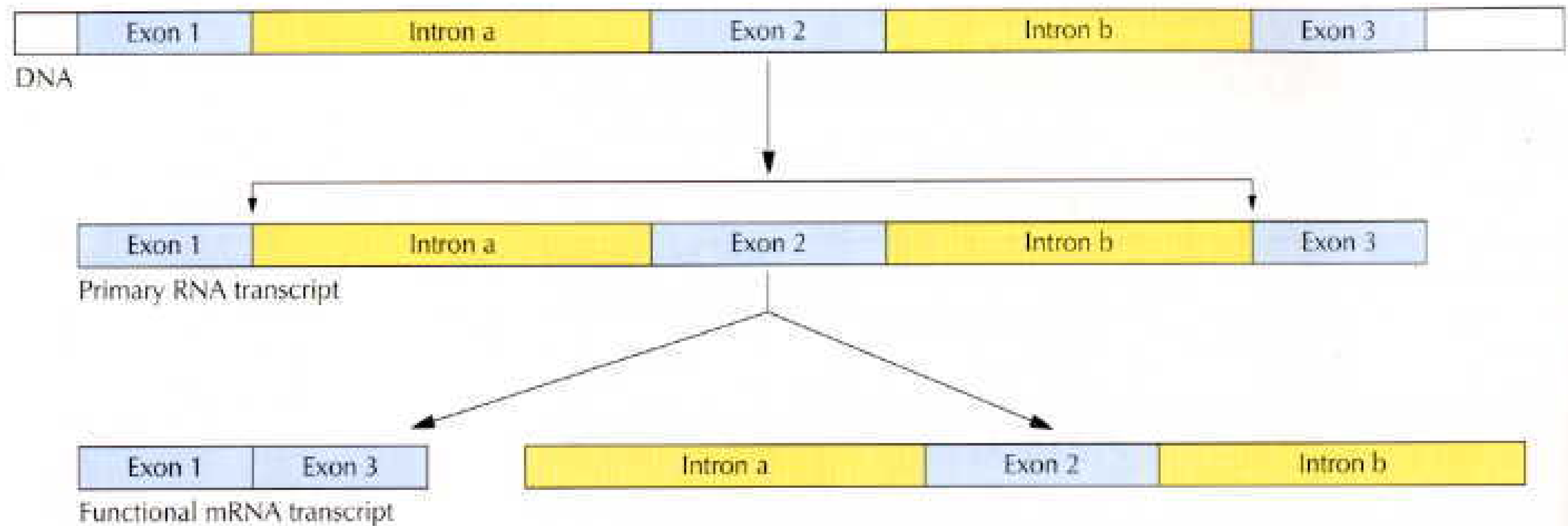


Figure 3.13 Alternative splicing of a eukaryotic primary RNA transcript. The bracketing arrows mark the sites that are spliced together after the removal of the intervening DNA region. In this example, exon 2 flanked by introns 1 and 2 is spliced out of the primary transcript and exons 1 and 3 are spliced together to form a functional mRNA transcript.

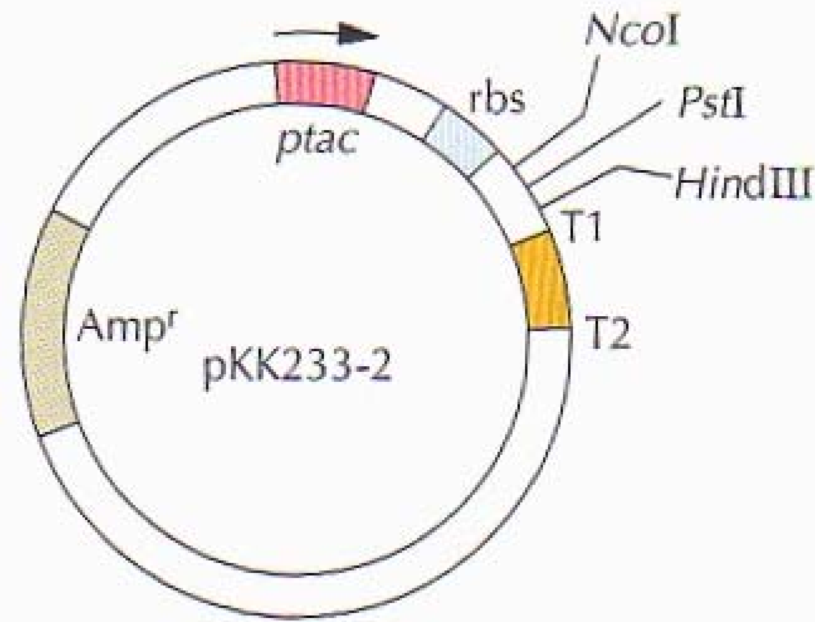
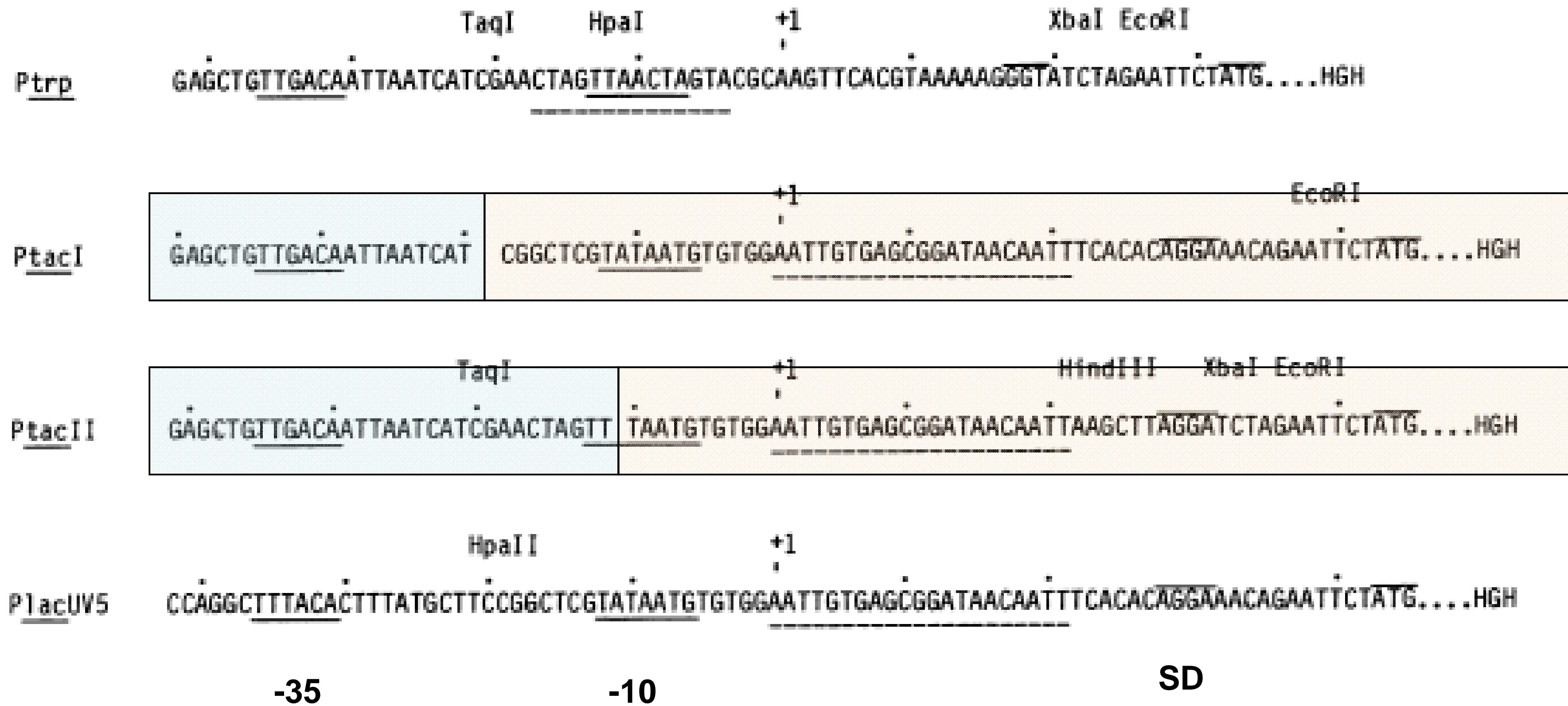
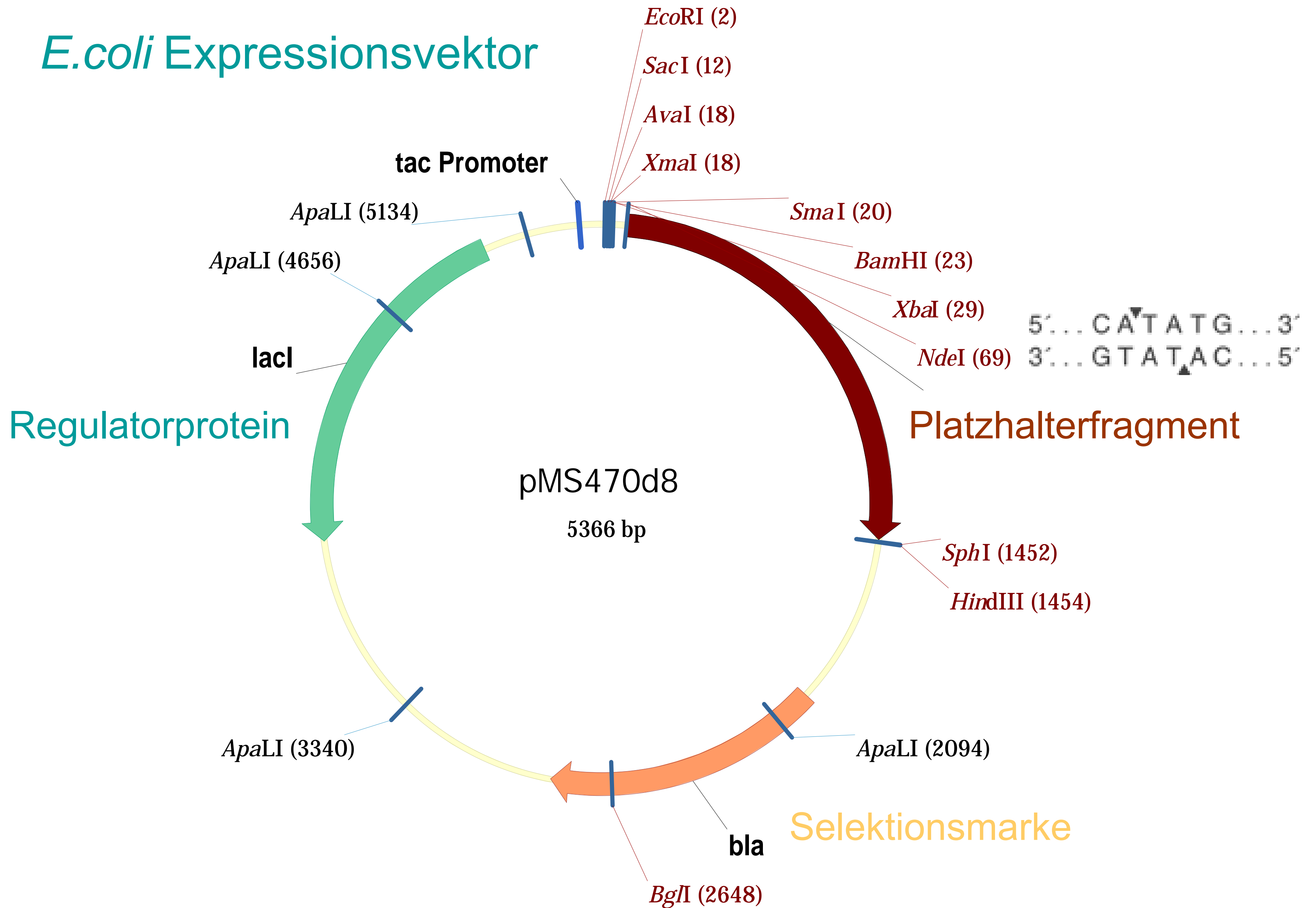


Figure 6.14 The expression vector pKK233-2. The plasmid pKK233-2 codes for the ampicillin resistance gene (Amp^r) as a selectable marker gene, the *tac* promoter (*ptac*), the *lacZ* ribosome binding site (rbs), three restriction endonuclease cloning sites (*Nco*I, *Pst*I, and *Hin*dIII), and two transcription termination sequences (T1 and T2). The arrow indicates the direction of transcription. The plasmid is not drawn to scale.

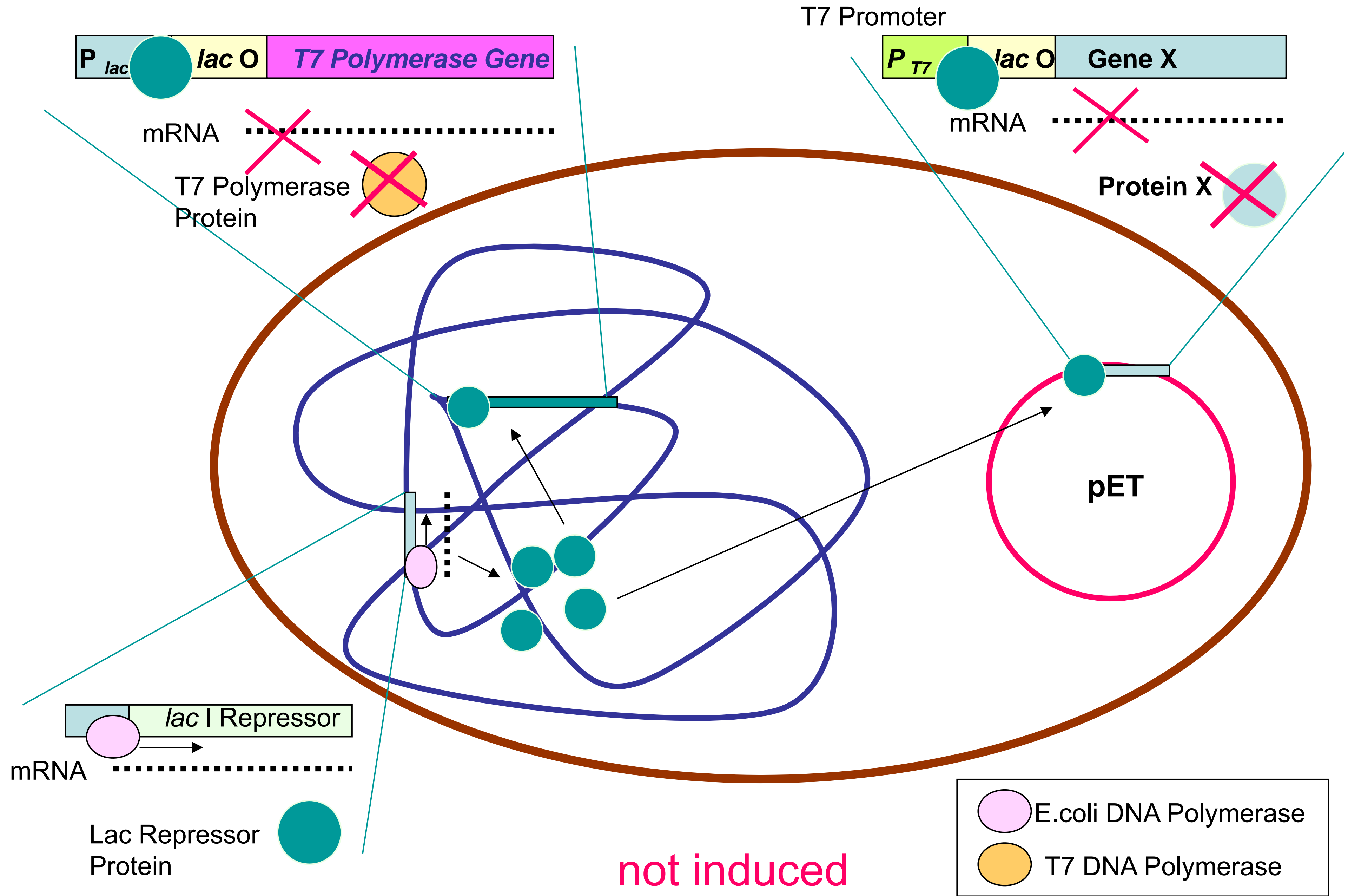
Expression Systems for E.coli

Inducible Promoters based on *lacI/lacO* repressor/operator

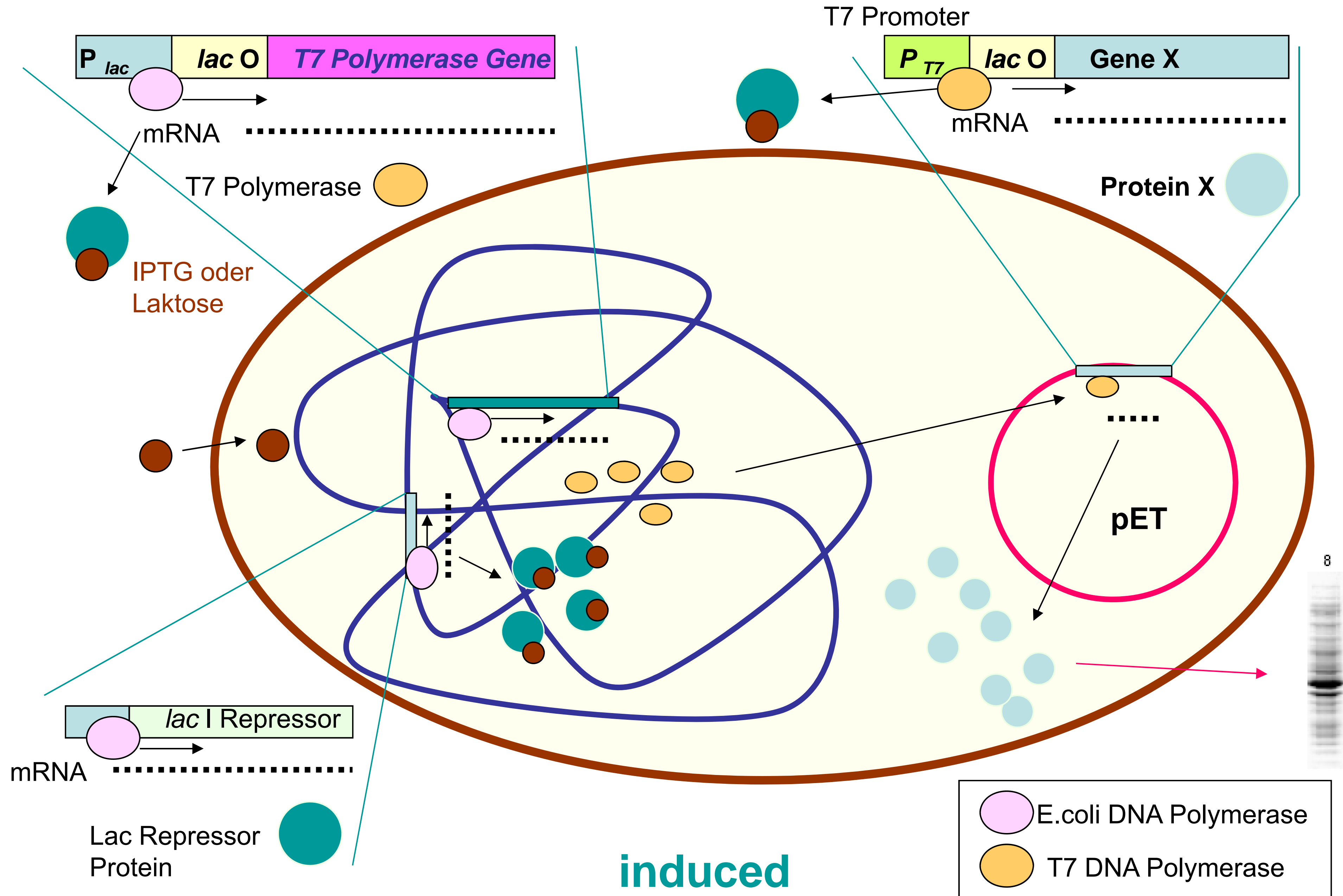


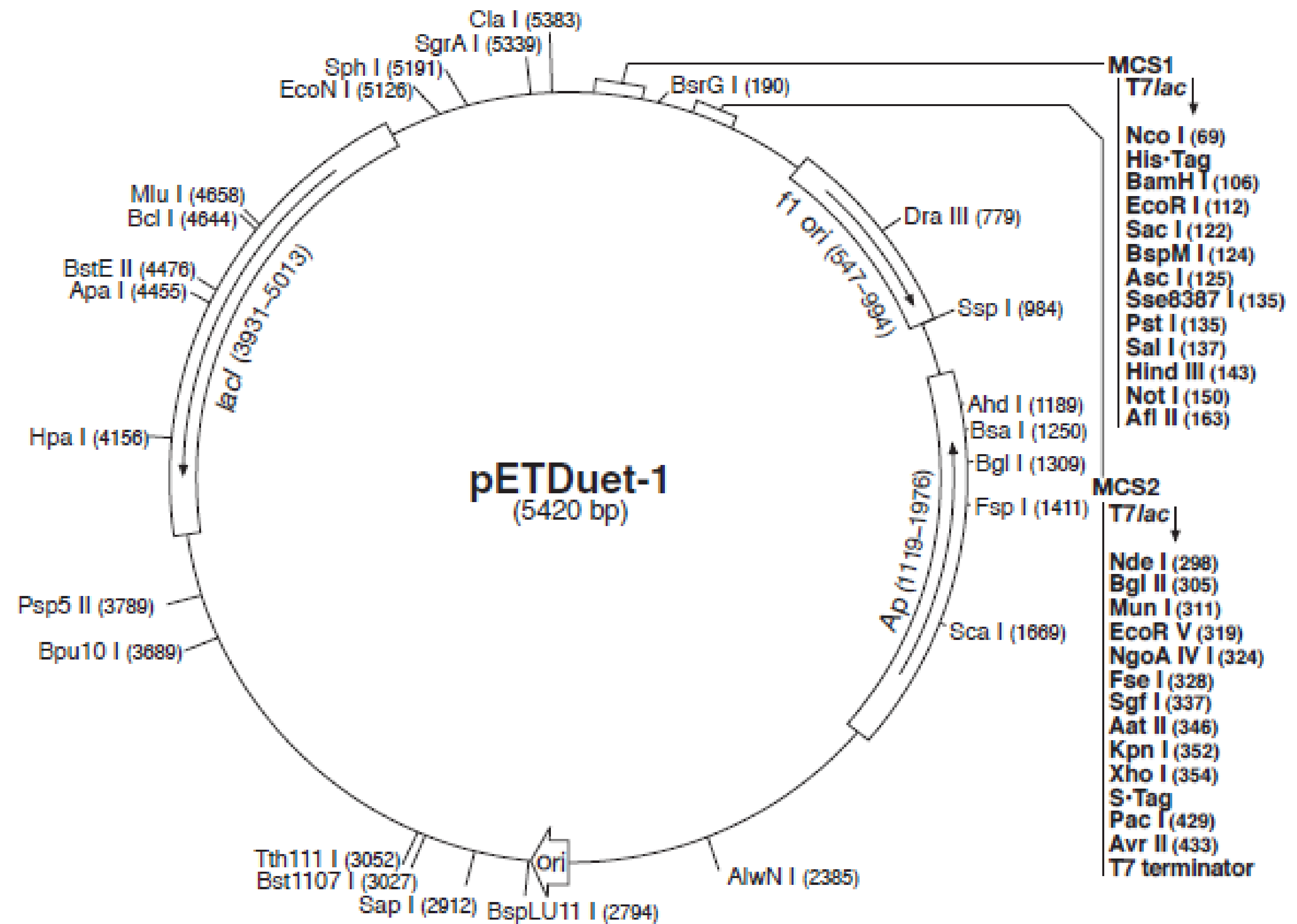

3´.5´...CATATG...3´
3´...GTATAC...5´
E.coli Expressionsvektor
EcoRI (2)
SacI (12)
AvaI (18)
XmaI (18)
SmaI (20)
BamHI (23)
XbaI (29)
NdeI (69)
tac Promoter
ApaLI (5134)
ApaLI (4656)
lacI
Regulatorprotein
5´...CATATG...3´
3´...GTATAC...5´
Platzhalterfragment
pMS470d8
5366 bp
SphI (1452)
HindIII (1454)
ApaLI (3340)
ApaLI (2094)
bla
Selektionsmarke
BglI (2648)

pET-Expression system



pET-Expression system





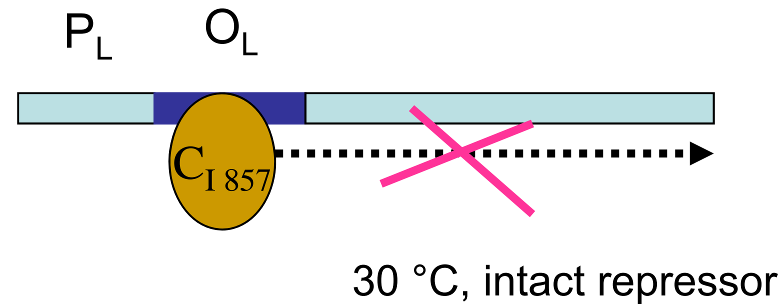
pETDuet-1 sequence landmarks

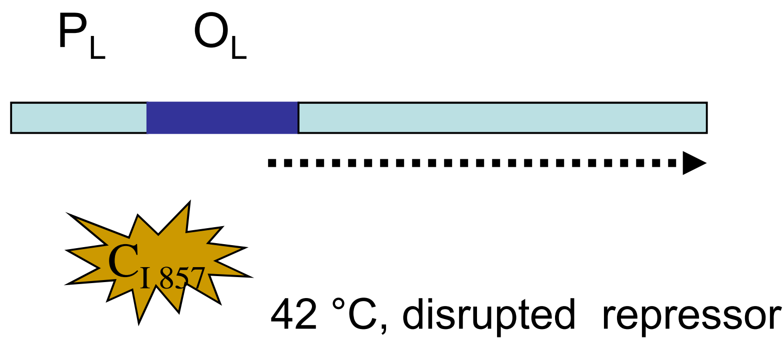
T7 promoter-1	5404–5420
T7 transcription start-1	1
His•Tag® coding sequence	83–100
Multiple cloning sites-1 (*Nco* I–*Afl* II)	69–168
T7 promoter-2	214–230
T7 transcription start-2	231
Multiple cloning sites-2 (*Nde* I–*Avr* II)	297–438
S•Tag™ coding sequence	366–410
T7 terminator	462–509
lacI coding sequence	3931–5013
pBR322 origin	2737
bla (Ap) coding sequence	1119–1976
f1 origin	547–994

pETDuet™-1 is designed for the coexpression of two target genes. The vector contains two multiple cloning sites (MCS), each of which is preceded by a T7 promoter/*lac* operator and a ribosome binding site (rbs). The vector also carries the pBR322-derived ColE1 replicon, *lacI* gene and ampicillin resistance gene.

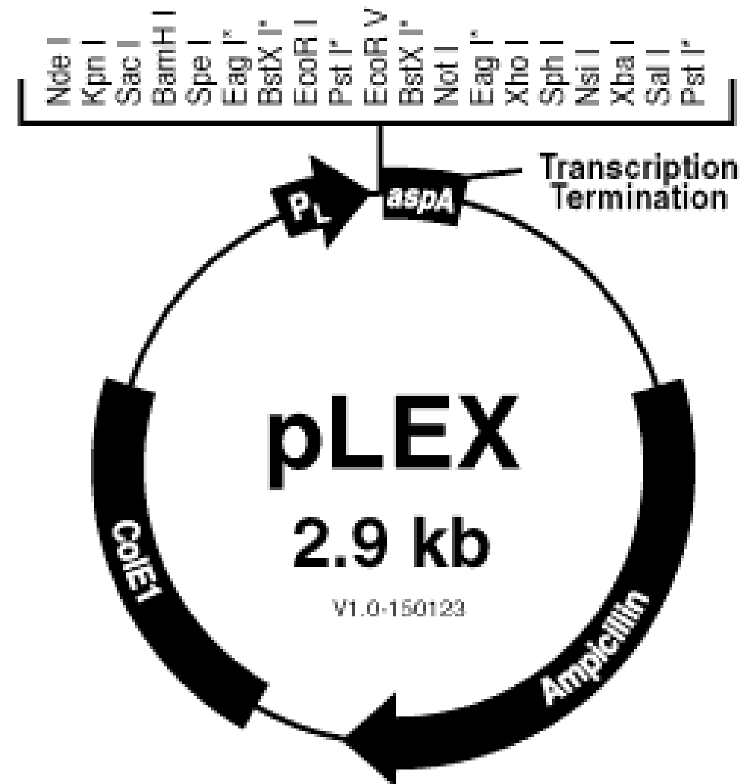
Bacteriophage Lambda Promoters P_L and P_R

$C_{I\,857}$ Lambda Repressor C_I thermosensitive mutant





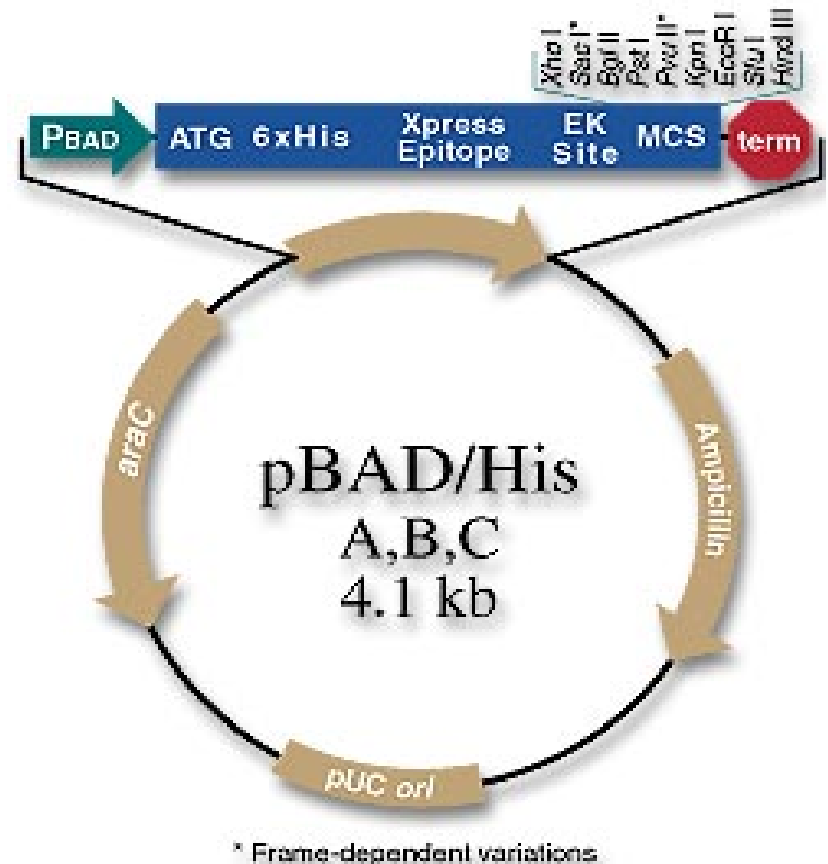
P_L based Expression Vector



* These enzymes have two recognition sites found only in the multiple cloning site.

Arabinose Operon based Expression system

The pBAD Expression System is based on the *araBAD* operon which controls the arabinose metabolic pathway in E.coli. It allows you to precisely modulate heterologous expression to levels that are optimal for recovering high yields of your protein of interest.



The pBAD/His vector offers the following key features:

The PBAD promoter and the *ara*C gene product for regulated expression of the gene of interest
N-terminal polyhistidine tag for rapid purification of fusion proteins using ProBond™ resin
Anti-Xpress™ epitope for detection of fusion proteins with the Anti-Xpress™ Antibody
Enterokinase cleavage site to facilitate removal of the fusion partner
Multiple cloning site in three reading frames to simplify subcloning in frame with the N-terminal polyhistidine tag
Ampicillin resistance gene and ColE1 origin for selection and maintenance in E. coli

16.10.14

Heterologous expression in prokaryotes – *E.coli*

Transcription

- **regulated promoters**
  - **lambda p_L, p_R**
  - ***lac, trp, tac. trc, araBAD***
  - **T7**
- **termination**
  - **rrnB (T1,T2), trpt**
  - **Lambda N gene (premature termination)**

m-RNA stability

Translation

- **Initiation – SD sequence ...AGGAG....**
- **elongation – codon usage**

Protein Folding

Proteolysis

- **Lon, Clp, *htpR* (heat shock regulatory protein)**

Posttranslational Processing

Plasmid copy number and segregation

m-RNA Stability

RNA has programmed half life

no good information available on factors determining decay

Secondary structures → Target for RNases

Sequence structure → determines secondary structure and accessibility to RNases

Heterologous expression in prokaryotes – *E.coli*

Transcription

- **regulated promoters**
  - **lambda p_L, p_R**
  - ***lac, trp, tac. trc, araBAD***
  - **T7**
- **termination**
  - **rrnB (T1,T2), trpt**
  - **Lambda N gene (premature termination)**

m-RNA stability

Translation

- **Initiation – SD sequence ...AGGAG....**
- **elongation – codon usage**

Protein Folding

Proteolysis

- **Lon, Clp, *htpR* (heat shock regulatory protein)**

Posttranslational Processing

Plasmid copy number and segregation

Translation Initiation

- SD sequence ...AGGAG....

- Secondary structures

Translation elongation

- Codon usage

- Secondary structures

- Codon structure – translational frameshifting

AAA	AAA	AAA	UCA
Lys	Lys	Lys	Ser

AAAAAAAAAUCA
Lys
Lys Lys Ile

Translation - Prokaryotes

Shine-Dalgarno (SD) Sequence

rRNA 5'-GAUACCAUCCUCCUUA-3'

mRNA ....G**GAGG**..(5-7bp)...**AUG**

Influences:

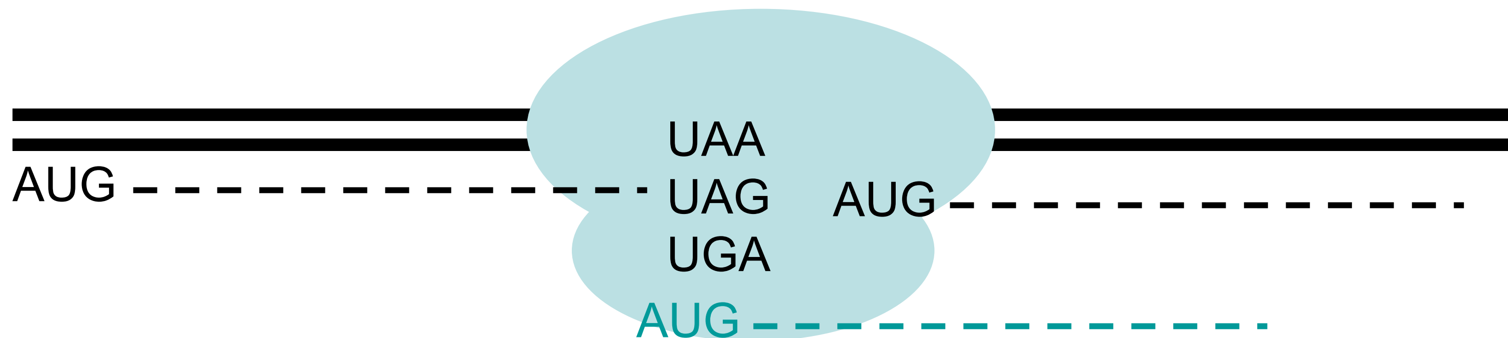
Secondary structure!! SD and AUG in unstructured region

Surrounding of SD and AUG!!!

Start

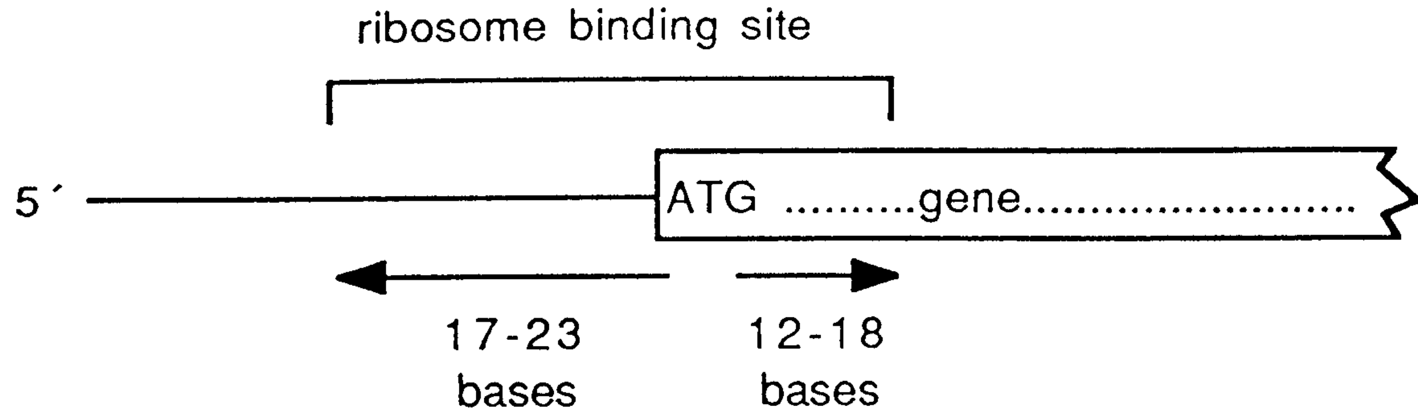
AUG	91%
GUG	8
UUG	1

Translational coupling

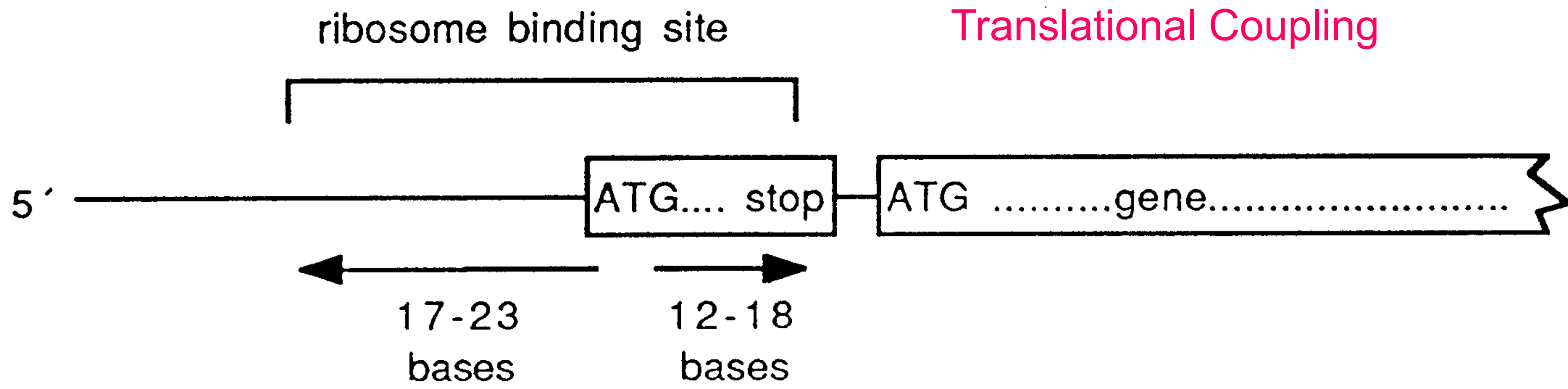


T

one-cistron mRNA



two-cistron mRNA



Translation - Eukaryotes

Start Codon

mRNA 5'-CAP......**AUG**

CAP structure essential for efficient translation initiation

Influences on Translation efficiency:

Surrounding of AUG!!!

Kozak Consensus

.........CC$^{A}/_{G}$CCAUGG...... **mammalian**

.... $^{A}/_{T}$A$^{A}/_{C}$A$^{A}/_{C}$AAUGTC$^{T}/_{C}$........ **yeast**

Translation Initiation

- **SD sequence ...AGGAG....**
- **Secondary structures**

Translation elongation

- **Codon usage**
- **Secondary structures**
- **Codon structure – translational frameshifting**

AAAAAAAAAUCA
LysLysLysSer

AAAAAAAAAUCA
LysLysLysSer
LysLysIle

Table 3.2 The genetic code and codon usage in *E. coli* and humans

Codon	Amino acid	Frequency of use in:	
		E. coli	Human
GGG	Glycine	0.13	0.23
GGA	Glycine	0.09	0.26
GGU	Glycine	0.38	0.18
GGC	Glycine	0.40	0.33
GAG	Glutamic acid	0.30	0.59
GAA	Glutamic acid	0.70	0.41
GAU	Aspartic acid	0.59	0.44
GAC	Aspartic acid	0.41	0.56
GUG	Valine	0.34	0.48
GUA	Valine	0.17	0.10
GUU	Valine	0.29	0.17
GUC	Valine	0.20	0.25
GCG	Alanine	0.34	0.10
GCA	Alanine	0.22	0.22
GCU	Alanine	0.19	0.28
GCC	Alanine	0.25	0.40
AAG	Lysine	0.24	0.60
AAA	Lysine	0.76	0.40
AAU	Asparagine	0.39	0.44
AAC	Asparagine	0.61	0.56
AUG	Methionine	1.00	1.00
AUA	Isoleucine	0.07	0.14
AUU	Isoleucine	0.47	0.35
AUC	Isoleucine	0.46	0.51
ACG	Threonine	0.23	0.12
ACA	Threonine	0.12	0.27
ACU	Threonine	0.21	0.23
ACC	Threonine	0.43	0.38
UGG	Tryptophan	1.00	1.00
UGU	Cysteine	0.43	0.42
UGC	Cysteine	0.57	0.58
UGA	Stop	0.30	0.61
UAG	Stop	0.09	0.17
UAA	Stop	0.62	0.22
UAU	Tyrosine	0.53	0.42
UAC	Tyrosine	0.47	0.58
UUU	Phenylalanine	0.51	0.43
UUC	Phenylalanine	0.49	0.57
UCG	Serine	0.13	0.06
UCA	Serine	0.12	0.15
UCU	Serine	0.19	0.17
UCC	Serine	0.17	0.23
AGU	Serine	0.13	0.14
AGC	Serine	0.27	0.25
CGG	Arginine	0.08	0.19
CGA	Arginine	0.05	0.10
CGU	Arginine	0.42	0.09
CGC	Arginine	0.37	0.19
AGG	Arginine	0.03	0.22
AGA	Arginine	0.04	0.21
CAG	Glutamine	0.69	0.73
CAA	Glutamine	0.31	0.27
CAU	Histidine	0.52	0.41
CAC	Histidine	0.48	0.59
CUG	Leucine	0.55	0.43
CUA	Leucine	0.03	0.07
CUU	Leucine	0.10	0.12
CUC	Leucine	0.10	0.20
UUG	Leucine	0.11	0.12
UUA	Leucine	0.11	0.06
CCG	Proline	0.55	0.11
CCA	Proline	0.20	0.27
CCU	Proline	0.16	0.29

Codon	Amino acid	*E. coli*	Human
GUA	Valine	0.17	0.10
GUU	Valine	0.29	0.17
GUC	Valine	0.20	0.25
GCG	Alanine	0.34	0.10
GCA	Alanine	0.22	0.22
GCU	Alanine	0.19	0.28
GCC	Alanine	0.25	0.40
AAG	Lysine	0.24	0.60
AAA	Lysine	0.76	0.40
AAU	Asparagine	0.39	0.44
AAC	Asparagine	0.61	0.56
AUG	Methionine	1.00	1.00
AUA	Isoleucine	0.07	0.14
AUU	Isoleucine	0.47	0.35
AUC	Isoleucine	0.46	0.51
ACG	Threonine	0.23	0.12
ACA	Threonine	0.12	0.27
ACU	Threonine	0.21	0.23
ACC	Threonine	0.43	0.38
UGG	Tryptophan	1.00	1.00
UGU	Cysteine	0.43	0.42
UGC	Cysteine	0.57	0.58
UGA	Stop	0.30	0.61
UAG	Stop	0.09	0.17
UAA	Stop	0.62	0.22
UAU	Tyrosine	0.53	0.42
UAC	Tyrosine	0.47	0.58
UUU	Phenylalanine	0.51	0.43
UUC	Phenylalanine	0.49	0.57
UCG	Serine	0.13	0.06
UCA	Serine	0.12	0.15
UCU	Serine	0.19	0.17
UCC	Serine	0.17	0.23
AGU	Serine	0.13	0.14
AGC	Serine	0.27	0.25
CGG	Arginine	0.08	0.19
CGA	Arginine	0.05	0.10
CGU	Arginine	0.42	0.09
CGC	Arginine	0.37	0.19

Table 3.2 The genetic code and codon usage in *E. coli* and humans

Codon	Amino acid	Frequency of use in:	
		E. coli	Humans
GGG	Glycine	0.13	0.23
GGA	Glycine	0.09	0.26
GGU	Glycine	0.38	0.18
GGC	Glycine	0.40	0.33
GAG	Glutamic acid	0.30	0.59
GAA	Glutamic acid	0.70	0.41
GAU	Aspartic acid	0.59	0.44
GAC	Aspartic acid	0.41	0.56
GUG	Valine	0.34	0.48
GUA	Valine	0.17	0.10
GUU	Valine	0.29	0.17
GUC	Valine	0.20	0.25
GCG	Alanine	0.34	0.10
GCA	Alanine	0.22	0.22
GCU	Alanine	0.19	0.28
GCC	Alanine	0.25	0.40
AAG	Lysine	0.24	0.60
AAA	Lysine	0.76	0.40
AAU	Asparagine	0.39	0.44
AAC	Asparagine	0.61	0.56
AUG	Methionine	1.00	1.00
AUA	Isoleucine	0.07	0.14
AUU	Isoleucine	0.47	0.35
AUC	Isoleucine	0.46	0.51
ACG	Threonine	0.23	0.12
ACA	Threonine	0.12	0.27
ACU	Threonine	0.21	0.23
ACC	Threonine	0.43	0.38
UGG	Tryptophan	1.00	1.00
UGU	Cysteine	0.43	0.42
UGC	Cysteine	0.57	0.58

UGA	Stop	0.30	0.61
UAG	Stop	0.09	0.17
UAA	Stop	0.62	0.22
UAU	Tyrosine	0.53	0.42
UAC	Tyrosine	0.47	0.58
UUU	Phenylalanine	0.51	0.43
UUC	Phenylalanine	0.49	0.57
UCG	Serine	0.13	0.06
UCA	Serine	0.12	0.15
UCU	Serine	0.19	0.17
UCC	Serine	0.17	0.23
AGU	Serine	0.13	0.14
AGC	Serine	0.27	0.25
CGG	Arginine	0.08	0.19
CGA	Arginine	0.05	0.10
CGU	Arginine	0.42	0.09
CGC	Arginine	0.37	0.19
AGG	Arginine	0.03	0.22
AGA	Arginine	0.04	0.21
CAG	Glutamine	0.69	0.73
CAA	Glutamine	0.31	0.27
CAU	Histidine	0.52	0.41
CAC	Histidine	0.48	0.59
CUG	Leucine	0.55	0.43
CUA	Leucine	0.03	0.07
CUU	Leucine	0.10	0.12
CUC	Leucine	0.10	0.20
UUG	Leucine	0.11	0.12
UUA	Leucine	0.11	0.06
CCG	Proline	0.55	0.11
CCA	Proline	0.20	0.27
CCU	Proline	0.16	0.29
CCC	Proline	0.10	0.33

Heterologous expression in prokaryotes – *E.coli*

Transcription

- **regulated promoters**
  - **lambda p_L, p_R**
  - ***lac, trp, tac. trc, araBAD***
  - **T7**
- **termination**
  - **rrnB (T1,T2), trpt**
  - **Lambda N gene (premature termination)**

m-RNA stability

Translation

- **Initiation – SD sequence ...AGGAG....**
- **elongation – codon usage**

Protein Folding

Proteolysis

- **Lon, Clp, *htpR* (heat shock regulatory protein)**

Posttranslational Processing

Plasmid copy number and segregation

Protein Folding

Translation Conditions

- Elongation velocity
- Codon Structure – Pausing
- Domain folding

Disulide Bond Formation

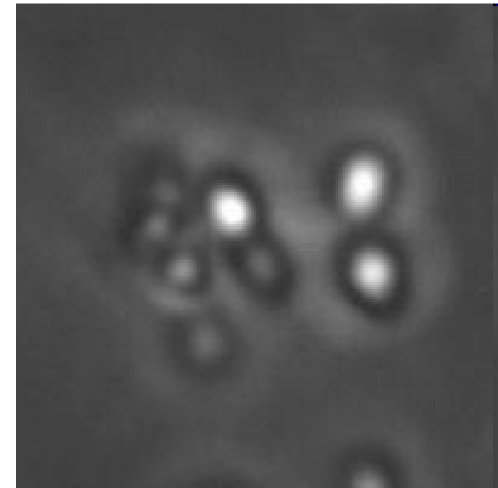
- Redox Conditions
  - E.coli Cytosol → bad conditions - reductive
  - E.coli Periplasm → optimal conditions - oxidative

Chaperones

Inclusion Body Formation

Expression velocity → Translation

Protein Folding



The Department of Surface Biotechnology with the Center for Surface Biotechnology, Box 577, BMC, 751 23 Uppsala

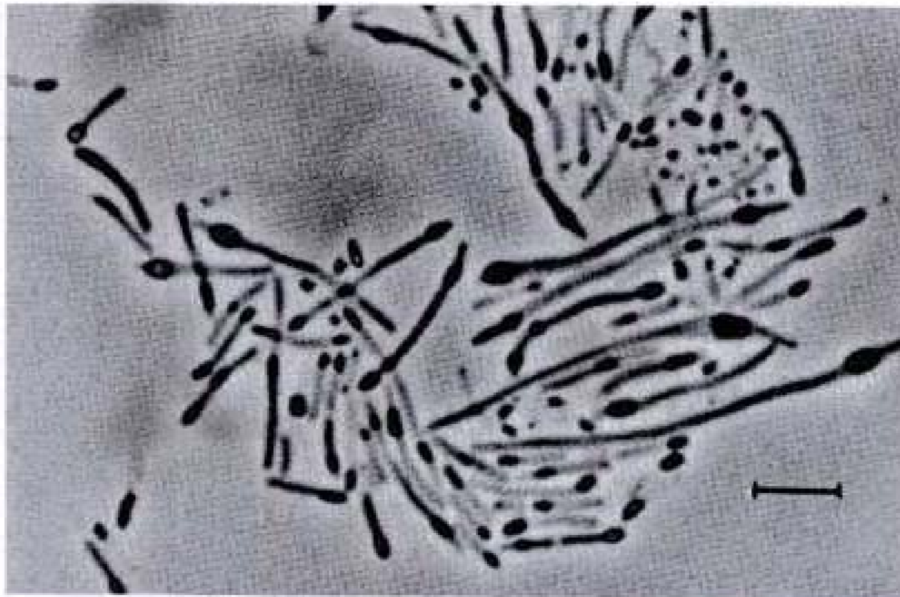
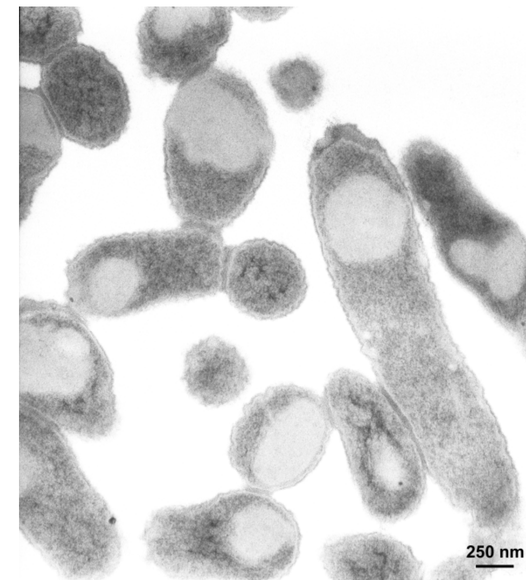


Figure 3 Phase-contrast microscopy of HB101/pBSF2-SD7 cells overproducing hIL-6 (bar equals 2 μm). (From Ref. 61)



www.boku.ac.at/IAM/dn/EM424_23.jpg

Heterologous expression in prokaryotes – *E.coli*

Transcription

- **regulated promoters**
  - **lambda p_L, p_R**
  - ***lac, trp, tac. trc, araBAD***
  - **T7**
- **termination**
  - **rrnB (T1,T2), trpt**
  - **Lambda N gene (premature termination)**

m-RNA stability

Translation

- **Initiation – SD sequence ...AGGAG....**
- **elongation – codon usage**

Protein Folding

Proteolysis

- **Lon, Clp, *htpR* (heat shock regulatory protein)**

Posttranslational Processing

Plasmid copy number and segregation

Post-translational modifications

- Side Chain Modifications
  - Glycosylation, Phosphorylation, Sulfatation, etc.
- Proteolytic Processing
  - ss Cleavage
  - Pro-protein processing
  - N/C-terminal Processing

Posttranslational Processing in prokaryotes – *E.coli*

N-terminal processing – the problem of Met

- f-Met deformylase
- methionine aminopeptidase (MAP) of *E.coli*
- peptidase M *(S. typhimurium)*
- aminopeptidase M: Exopeptidase → ...... X-Pro
- aminopeptidase P: NH_2-X-/Pro
- dipeptidylaminopeptidase I (DAP-I, Cathepsin C) → not at NH_2 Pro/Arg/Lys

protein fusion strategies

- sequence specific proteases
- tags

Table 6.5 α-Amylase gene copy number and activity in *B. subtilis*

Copies/genome	Activity (U/mL of mid-log cells)
2	500
5	2,300
7	3,100
8	3,400
9	4,400
Multicopy plasmid	700

Adapted from Kallio et al., 1987, *Appl. Microbiol. Biotechnol.* **27**:64–71.

Table 6.6 Effect of plasmid copy number on host cell growth rate

***E. coli* HB101 with plasmid:**	**Plasmid copy number**	**Relative specific growth rate**
None	0	1.00
A	12	0.92
B	24	0.91
C	60	0.87
D	122	0.82
E	408	0.77

Adapted from Seo and Bailey, 1985, *Biotechnol. Bioeng.* 27:1668–1674.

The different plasmids, designated A, B, C, D and E, encode only β-lactamase and are all the same size. The growth rates were normalized to the growth rate value for *E. coli* HB101 without a plasmid.

Metabolic load

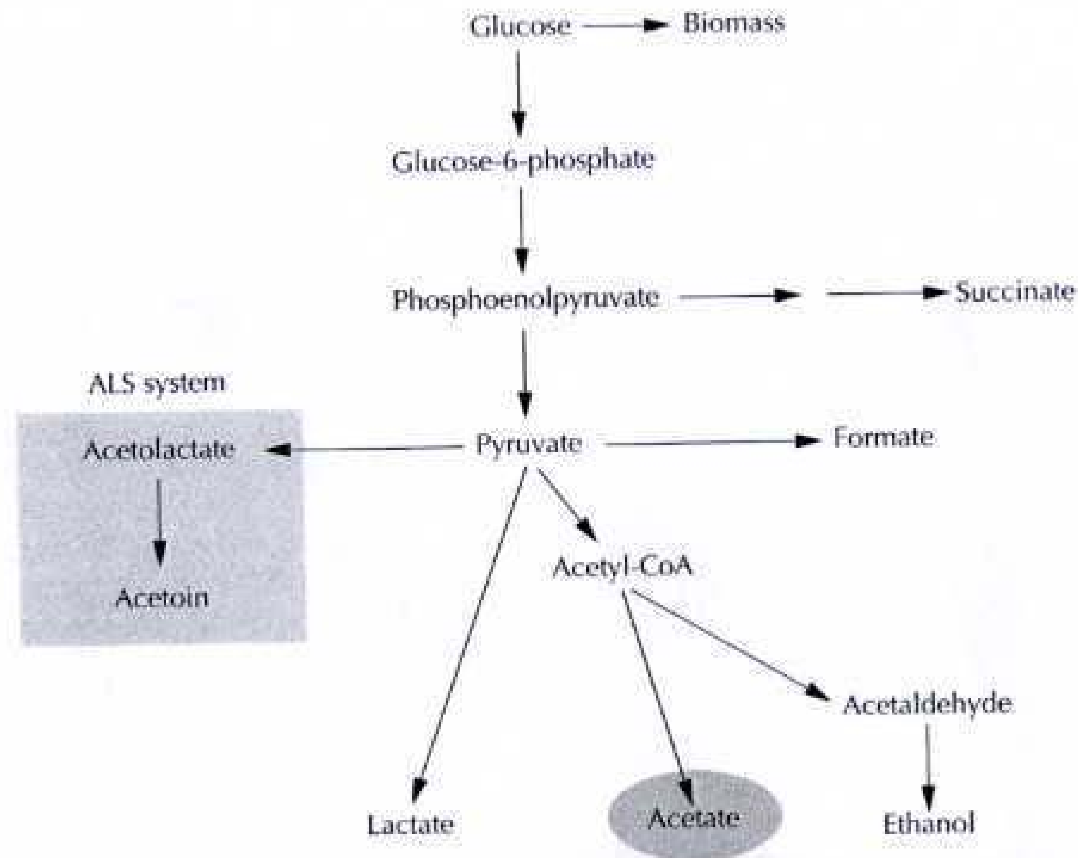


Figure 6.17 Schematic representation of the pathways for glucose metabolism in an *E. coli* strain that has been transformed with a plasmid carrying the genes for acetolactate synthase (ALS).

Gene Fusion Strategies

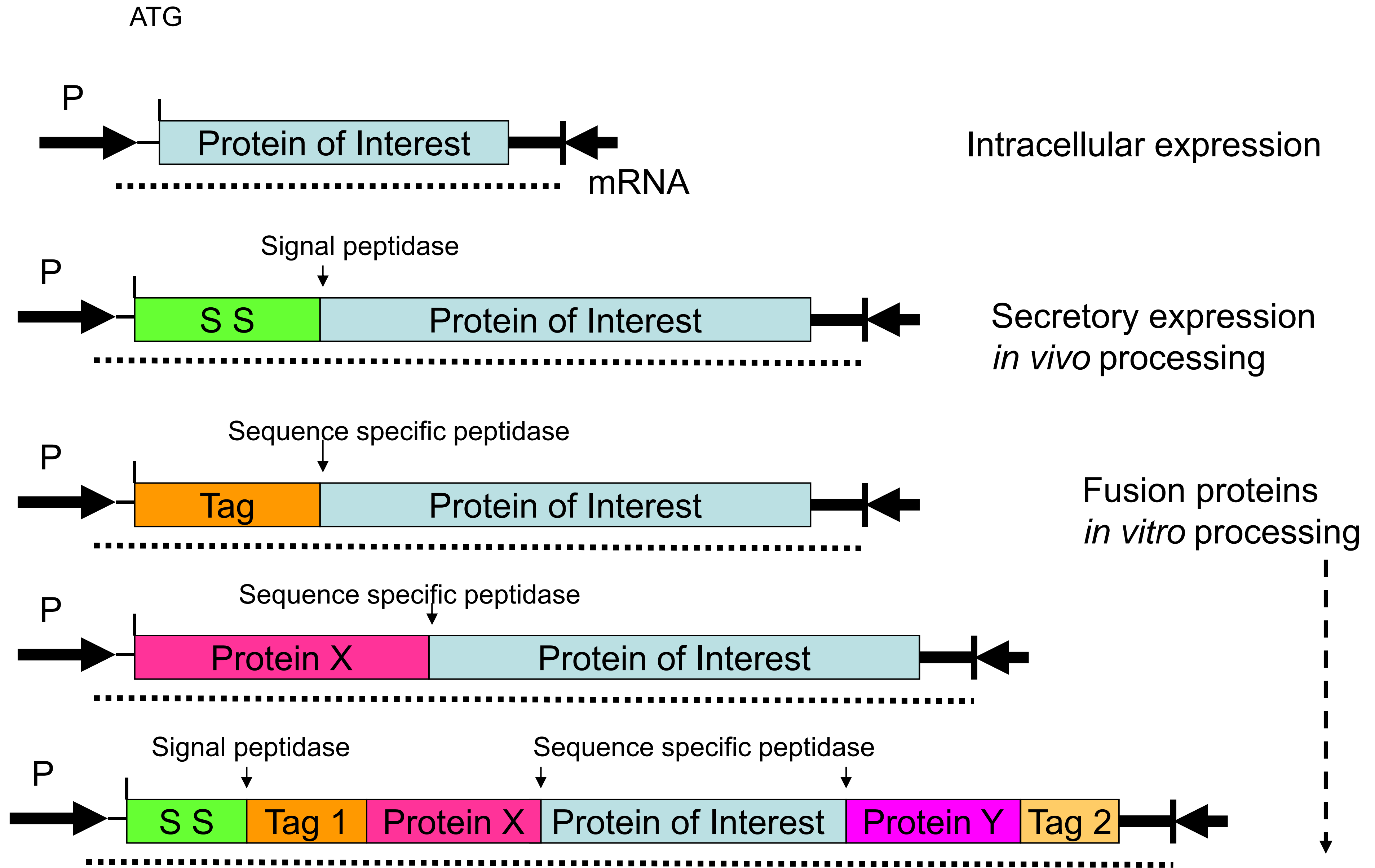


Table 6.3 Some fusion systems used to facilitate the purification of foreign proteins produced in *E. coli*

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., 1994, *Trends Biotechnol.* **12:**184–188.

Abbreviations: aa, amino acids; kDa, kilodaltons; ZZ, a fragment of *Staphylococcus aureus* protein A; His, histidine; Strep-tag, a peptide with affinity for streptavidin; PinPoint, a protein fragment which is

Table 2 Sequence and size of affinity tags

Tag	Residues	Sequence	Size (kDa)
Poly-Arg	5–6 (usually 5)	RRRRR	0.80
Poly-His	2–10 (usually 6)	HHHHHH	0.84
FLAG	8	DYKDDDDK	1.01
Strep-tag II	8	WSHPQFEK	1.06
c-myc	11	EQKLISEEDL	1.20
S-	15	KETAAAKFERQHMDS	1.75
HAT-	19	KDHLIHNVHKEFHAHAHNK	2.31
3x FLAG	22	DYKDHDGDYKDHDIDYKDDDDK	2.73
Calmodulin-binding peptide	26	KRRWKKNFIAVSAANRFKKISSSGAL	2.96
Cellulose-binding domains	27–189	Domains	3.00–20.00
SBP	38	MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP	4.03
Chitin-binding domain	51	TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ	5.59
Glutathione S-transferase	211	Protein	26.00
Maltose-binding protein	396	Protein	40.00

Table 1 Matrices and elution conditions of affinity tags

Affinity tag	Matrix	Elution condition
Poly-Arg	Cation-exchange resin	NaCl linear gradient from 0 to 400 mM at alkaline pH>8.0
Poly-His	Ni^{2+}-NTA, Co^{2+}-CMA (Talon)	Imidazole 20–250 mM or low pH
FLAG	Anti-FLAG monoclonal antibody	pH 3.0 or 2–5 mM EDTA
Strep-tag II	Strep-Tactin (modified streptavidin)	2.5 mM desthiobiotin
c-myc	Monoclonal antibody	Low pH
S	S-fragment of RNaseA	3 M guanidine thiocyanate, 0.2 M citrate pH 2, 3 M magnesium chloride
HAT (natural histidine affinity tag)	Co^{2+}-CMA (Talon)	150 mM imidazole or low pH
Calmodulin-binding peptide	Calmodulin	EGTA or EGTA with 1 M NaCl
Cellulose-binding domain	Cellulose	Family I: guanidine HCl or urea>4 M Family II/III: ethylene glycol
SBP	Streptavidin	2 mM Biotin
Chitin-binding domain	Chitin	Fused with intein: 30–50 mM dithiothreitol, β-mercaptoethanol or cysteine
Glutathione S-transferase	Glutathione	5–10 mM reduced glutathione
Maltose-binding protein	Cross-linked amylose	10 mM maltose

Cleavage of Tags:

Enterokinase

D-D-D-D-K↓-X1

TEV protease

E-X-X-Y-X-Q↓-S

α-thrombin

X4-X3-P-R[K]-X1'↓-X2
L - V-P-R- G - S

Table 4 Cleavage (%) of enterokinase through densitometry (Hosfield and Lu 1999) based on the amino acid residue X_1. The sequence....-GSDYKDDDDK-X_1-ADQLTEEQIA-... of a GST-calmodulin fusion protein was tested using 5 mg protein digested with 0.2 U of enterokinase for 16 h at 37 °C

Amino acid in position X_1	Cleavage of enterokinase (%)
Alanine	88
Methionine	86
Lysine	85
Leucine	85
Asparagine	85
Phenylalanine	85
Isoleucine	84
Aspartic acid	84
Glutamic acid	80
Glutamine	79
Valine	79
Arginine	78
Threonine	78
Tyrosine	78
Histidine	76
Serine	76
Cysteine	74
Glycine	74
Tryptophan	67
Proline	61

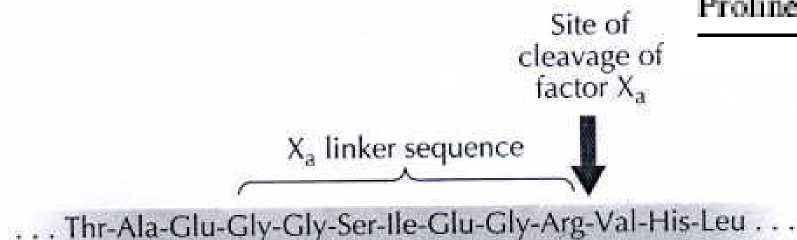


Figure 6.6 Proteolytic cleavage of a fusion protein by blood coagulation factor X_a. The factor X_a recognition sequence (X_a linker sequence) lies between the amino acid sequences of two different proteins. A functional cloned gene protein (with Val at its N terminus) is released after cleavage.

Tag purification strategies

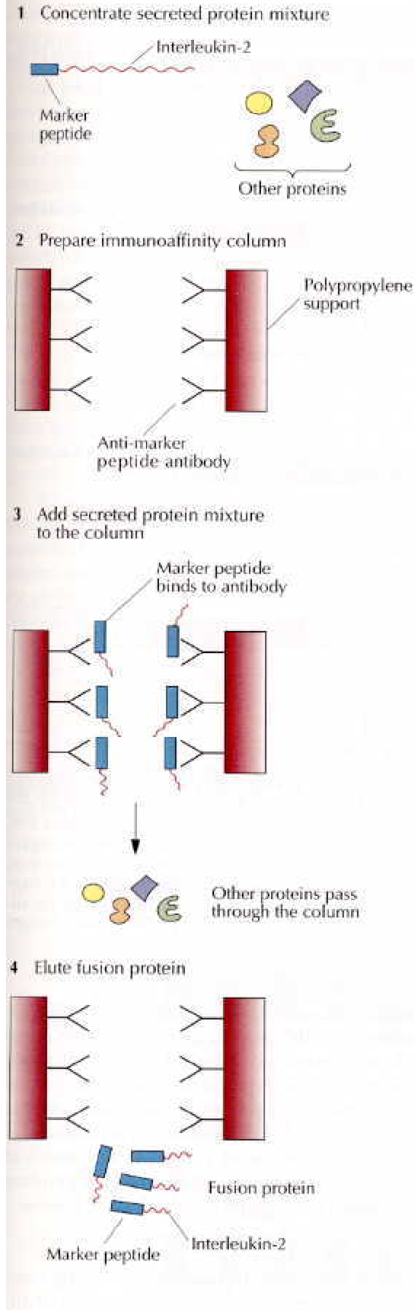
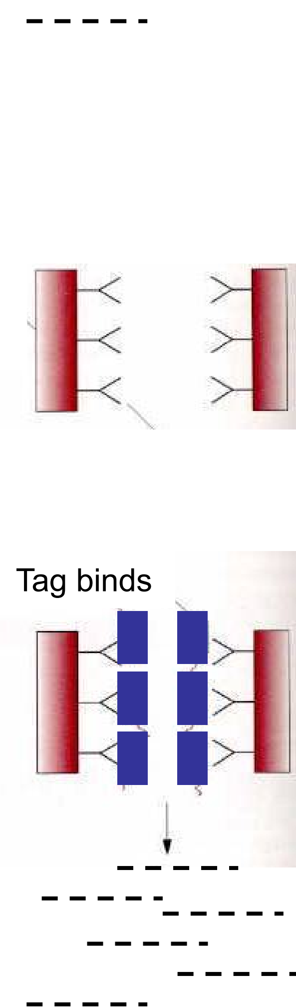


Figure 6.8 Immunoaffinity chromatographic purification of a fusion protein. An antibody that binds to the marker peptide of the fusion protein (anti-marker peptide antibody) is attached to a solid polypropylene support. The secreted proteins are passed through the column containing the bound antibody. The marker peptide portion of the fusion protein is bound to the antibody, and the other proteins pass through. The immunopurified fusion protein can then be selectively eluted from the column.

Removal of Tag

Proteolytic cleavage of Tag



23.10.14

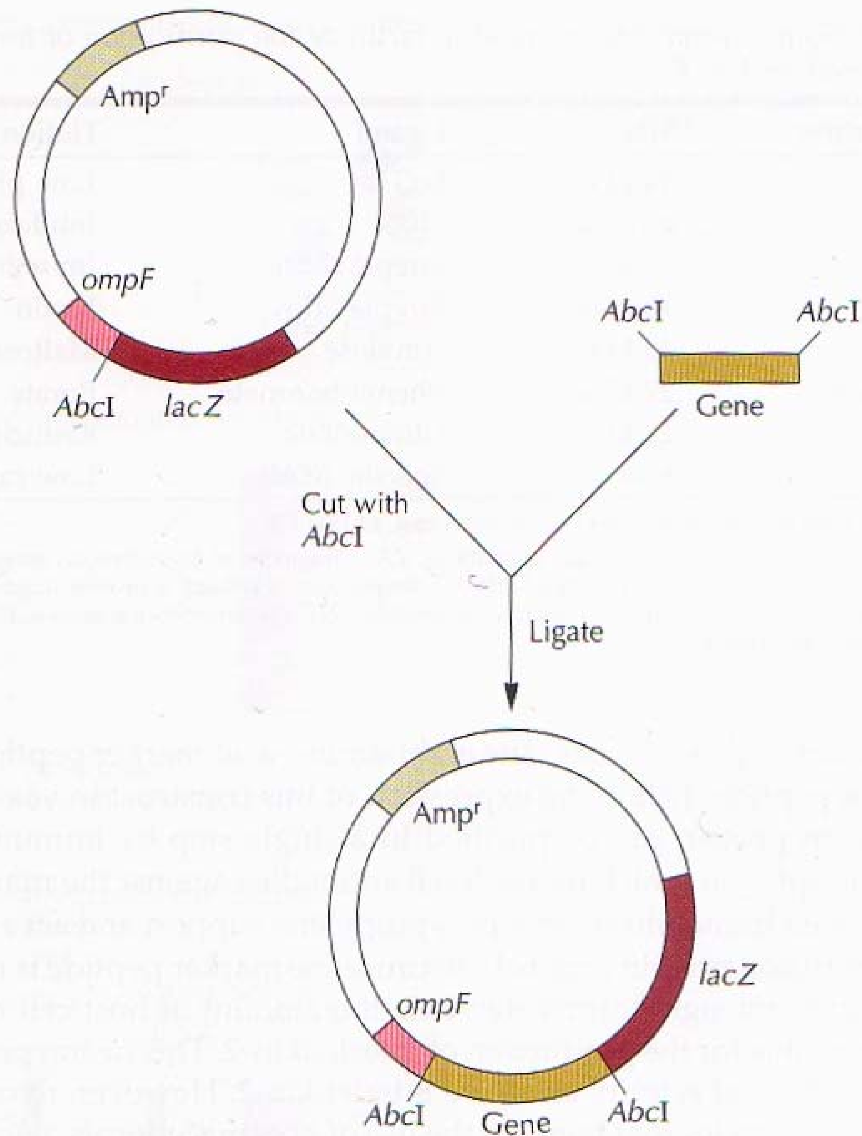


Figure 6.7 Fusion protein cloning vector. The plasmid contains an ampicillin resistance (Amp^r) gene as the selectable marker, a DNA sequence encoding the N-terminal segment of the *E. coli* outer membrane protein (*ompF*), a restriction endonuclease site (*Abc*I) for cloning, and a truncated β-galactosidase gene (*lacZ*). The cloned gene (Gene) is inserted into the *Abc*I site. After transcription and translation, a tribrid protein is produced.

Examples for fusion strategies

For E.coli:

Maltose binding protein
Thioredoxin reductase

Generally: well soluble proteins
Well folded proteins

Fusions can hep for:

Translation initiation
Folding
Protein detection: Antibodies against
Fusion partner (also with small tags)

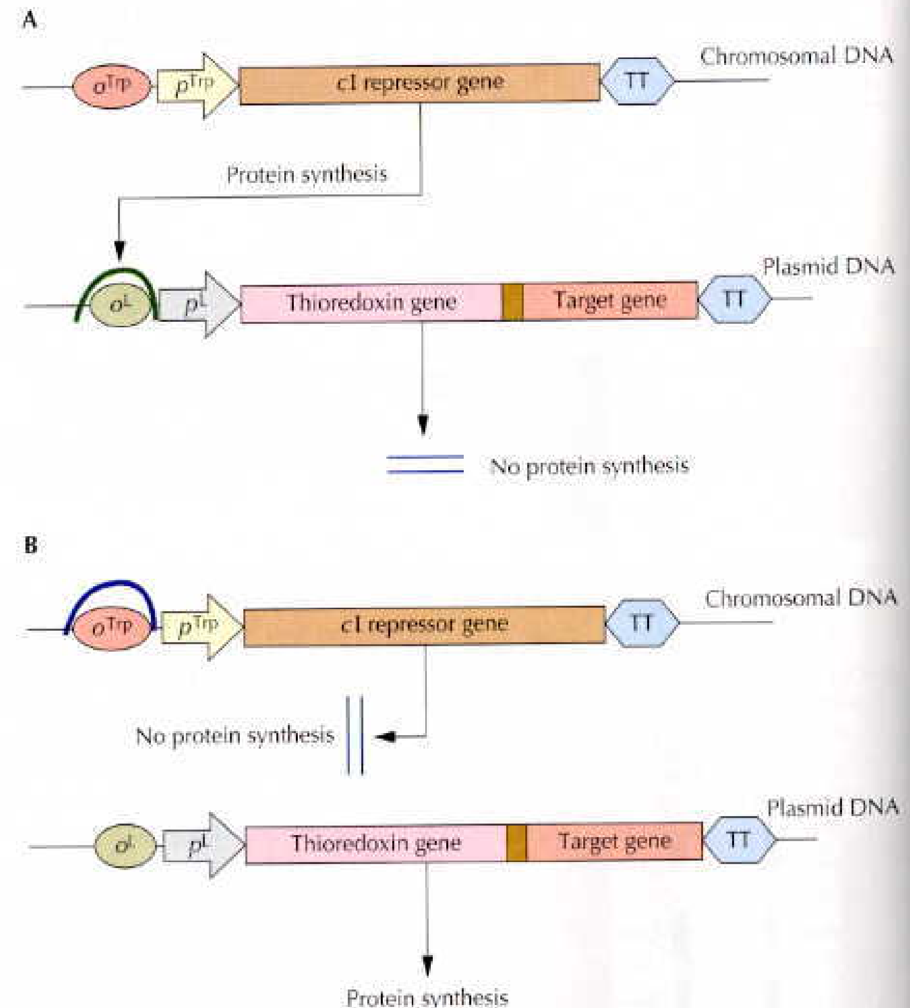


Figure 6.9 Synthesis of a thioredoxin–target protein fusion in the absence (**A**) and in the presence (**B**) of tryptophan in the growth medium. The arrows labeled p^{Trp} and p^{L} indicate the direction of transcription. Abbreviations and designations: o^{Trp}, the operator region where the *trp* repressor protein binds; o^{L}, the operator region where the cI repressor binds; p^{Trp}, the *trp* promoter; p^{L}, the leftward promoter from bacteriophage lambda; TT, transcription termination region. The box with the diagonal lines indicates the DNA region that codes for the peptide that acts as the enterokinase cleavage site; the horseshoes indicate the binding of a repressor protein to its operator region.

Eukaryotic Expression Systems

Fungi – Yeasts

Insect Cells

Plant Cells

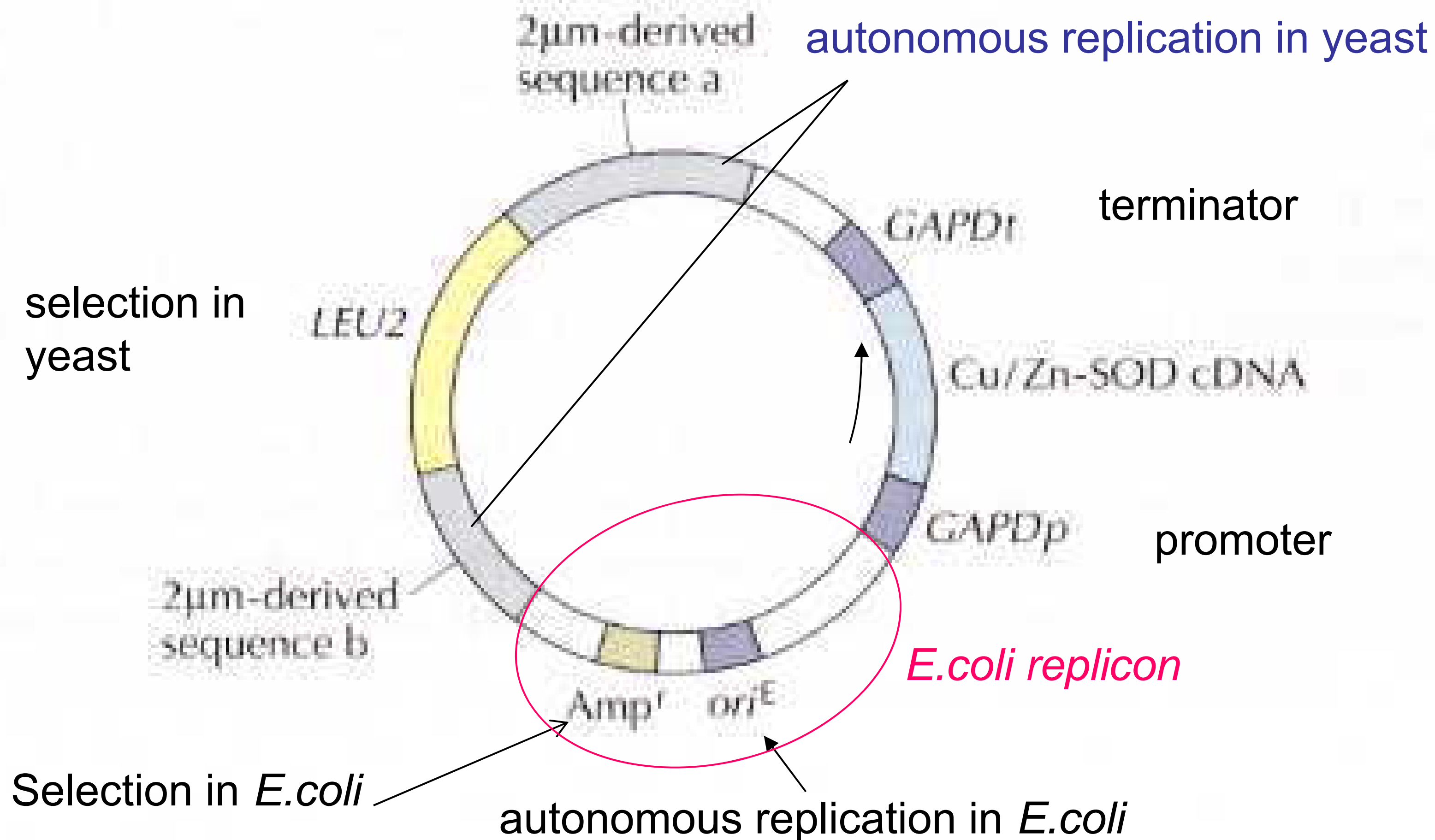
Mammalian Cells

- Mouse
- Hamster
- Avian
- Human

Transgenic Plants

Transgenic Animals

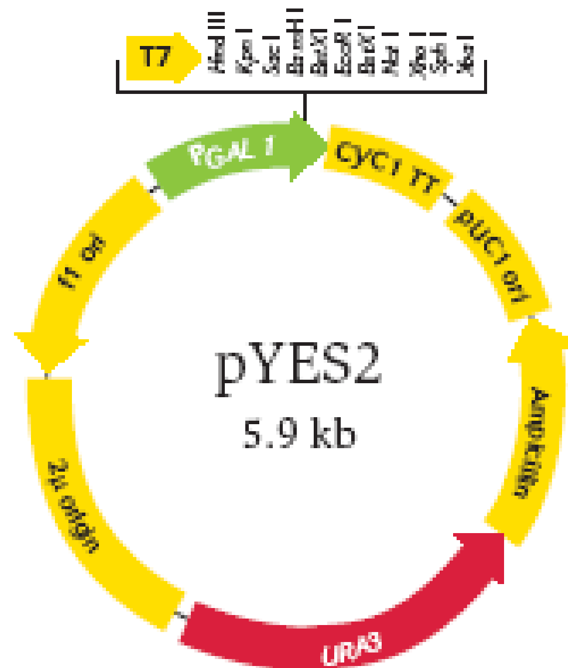
Figure 7.4 *S. cerevisiae* expression vector. The cDNA for human Cu/Zn-SOD has been cloned between the promoter (*GAPDp*) and termination–polyadenylation sequence (*GAPDt*) of the *S. cerevisiae* glyceraldehyde phosphate dehydrogenase gene. The *LEU2* gene, cloned into the middle of the yeast 2μm plasmid DNA, encodes an enzyme of the leucine biosynthesis pathway. The yeast origin of replication is included in the 2μm plasmid sequences. The ampicillin resistance gene (Amp^r) and the *E. coli* origin of replication (ori^E) are derived from plasmid pBR322.

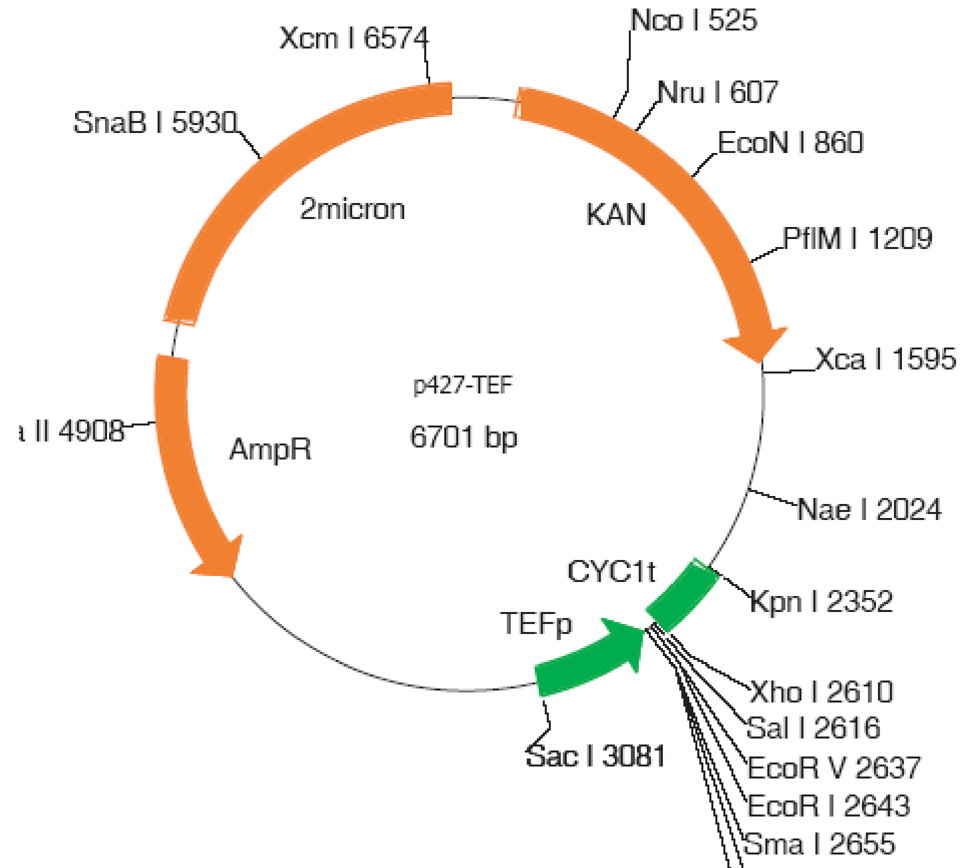


S.cerevisiae
Expression vectors

2μ-based multicopy vector

Figure 3 - pYES2 vector





Vector features

TEFp TEF1 promoter (nt 2673-3081)

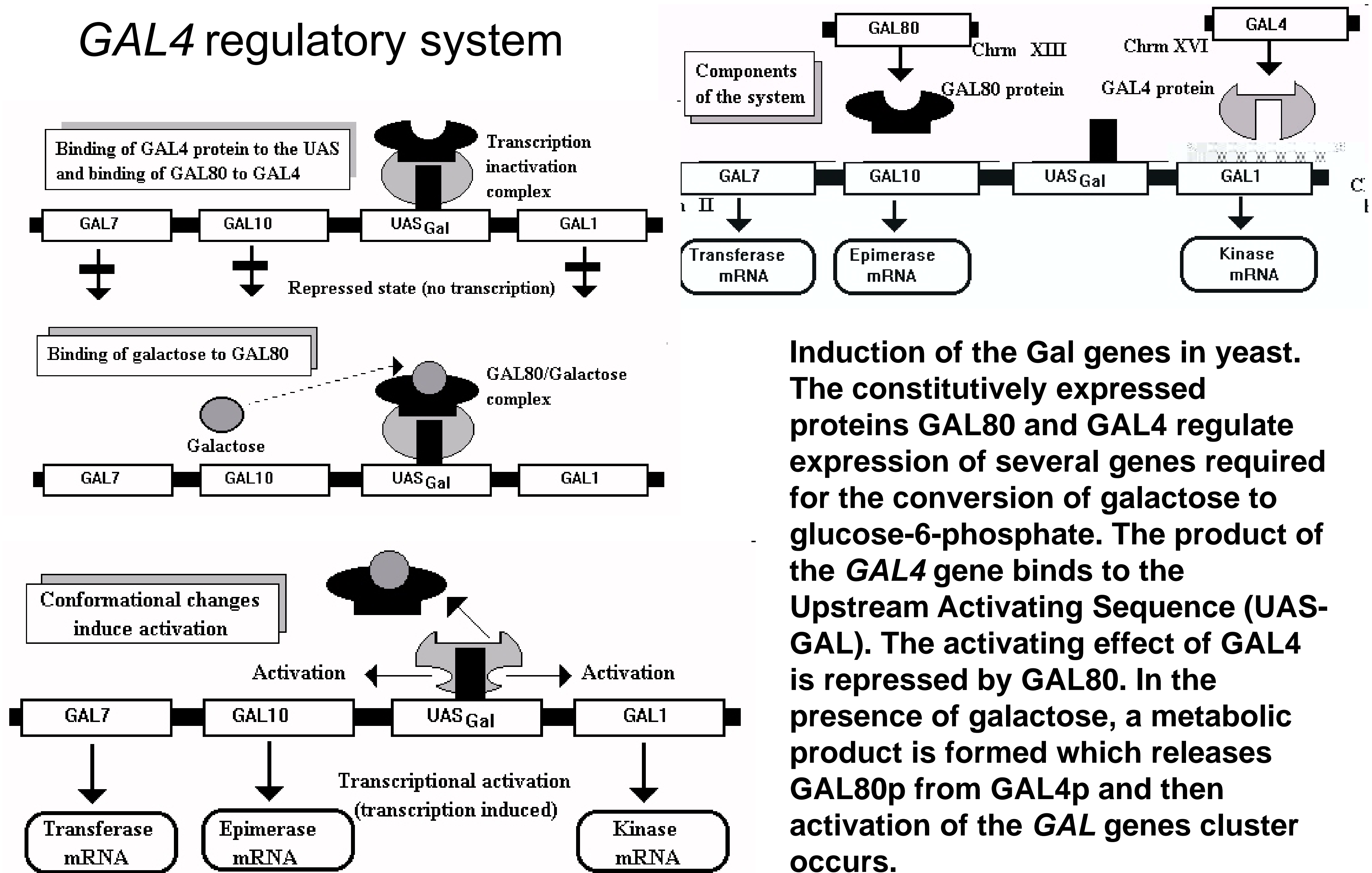
CYC1t S. cerevisiae CYC1 terminator (nt 2352-2610)

KAN Kanamycine resistance gene (aminoglycoside phosphotransferase),
allows selection in yeast using 200 mg/ml G418 (nt 190-1571)

2micron Origin of replication derived from the endogenous yeast 2m circle.
Allows propagation of plasmids in yeast at high copy numbers (10-50 copies/cell, nt 5291-6637)

AmpR Ampicillin resistance gene (nt 4300-5158)

GAL4 regulatory system



Induction of the Gal genes in yeast. The constitutively expressed proteins GAL80 and GAL4 regulate expression of several genes required for the conversion of galactose to glucose-6-phosphate. The product of the *GAL4* gene binds to the Upstream Activating Sequence (UAS-GAL). The activating effect of GAL4 is repressed by GAL80. In the presence of galactose, a metabolic product is formed which releases GAL80p from GAL4p and then activation of the *GAL* genes cluster occurs.

Protein Expression in *Pichia pastoris*

- Methylotrophic yeast

  Two alcohol oxidase genes: *AOX1, AOX2*

  AOX1: 5 % of total mRNA, 30 % of total protein
- Well established commercial expression system
- More than 300 proteins successfully expressed

  (bacterial, virusal, fungal, plant, protozoan, invertebrate, vertebrate → 120 human proteins)
- High cell density fermentation (>100 g/L) on simple media
- No switch to anaerobic fermentation (ethanol problem)
- Stable integration into host chromosome
- Intracellular and secretory production capacities
- Advantages of a eukaryotic host cell – but simple system

  Glycosylation (N-linked, high-mannose type)

  Post-translational processing

P.pastoris
Expression system

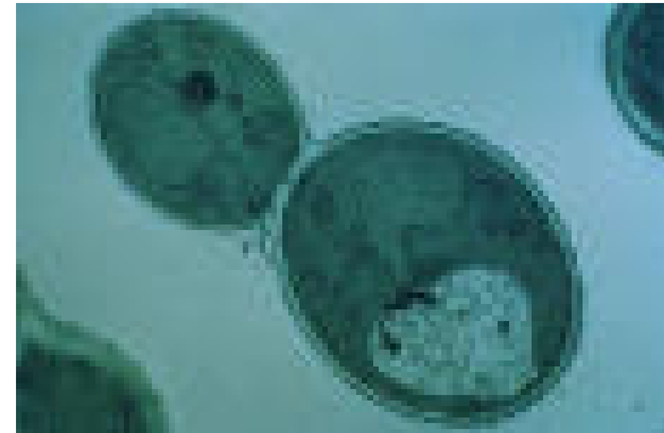
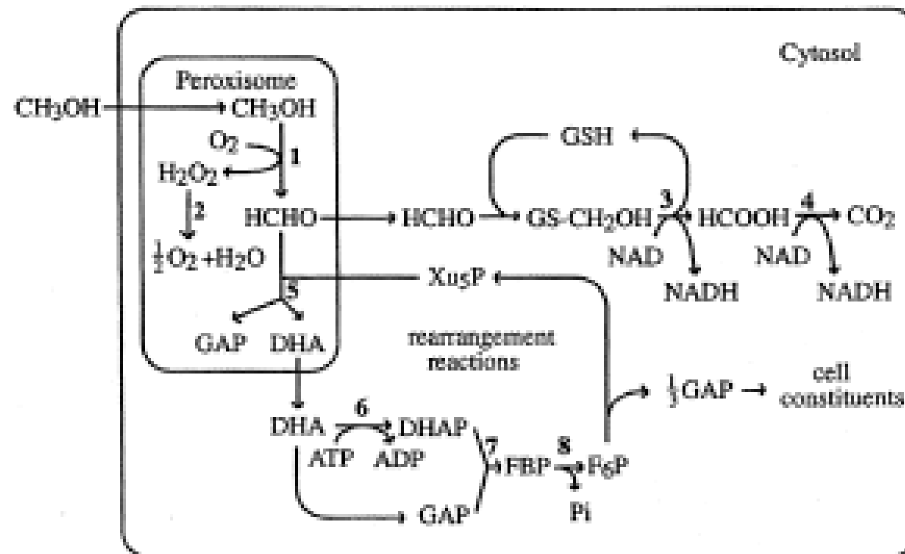


Figure 1 - High Biomass of *Pichia pastoris*



AOX1: strong expression
AOX2: weak expression

S. cerevisiae   *P. pastoris*

Pichia expression tools

- **Promoters**

  AOX1, GAP
- **Selection marker**

  ***HIS4*, *ARG4,* Zeocin^R, Blasticidin^R, Kanamycin^R (G418)**

- **Signal sequences**

  ***PHO1*, alpha-Factor**

- **Host strains**

  X-33 (wt), GS115 (*his4*), KM 71 (*aox1*::*arg4 his4*),

  KM7IH (*aox1::arg4*),SMD1168 (pep4 his4), SMD1168H (*pep4*)

  CBS 7435 (WZ or *Δaox1* or *Δ his4* knockouts)

Integration in *Pichia pastoris*

Gene replacement at AOX1

phenotype: Mut^S

Single cross-over integration of circular molecules

AOX1 (5‘ and 3‘ regions)

HIS4

GAP

Tandem repeat multicopy integration

Ectopic integration events

Integration vector for *Pichia pastoris*

Gene Replacement

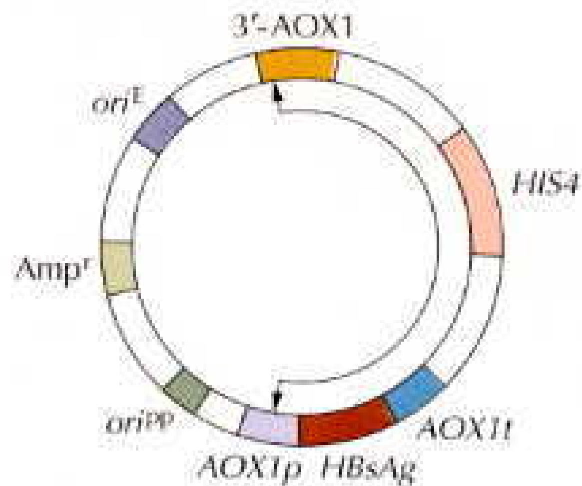
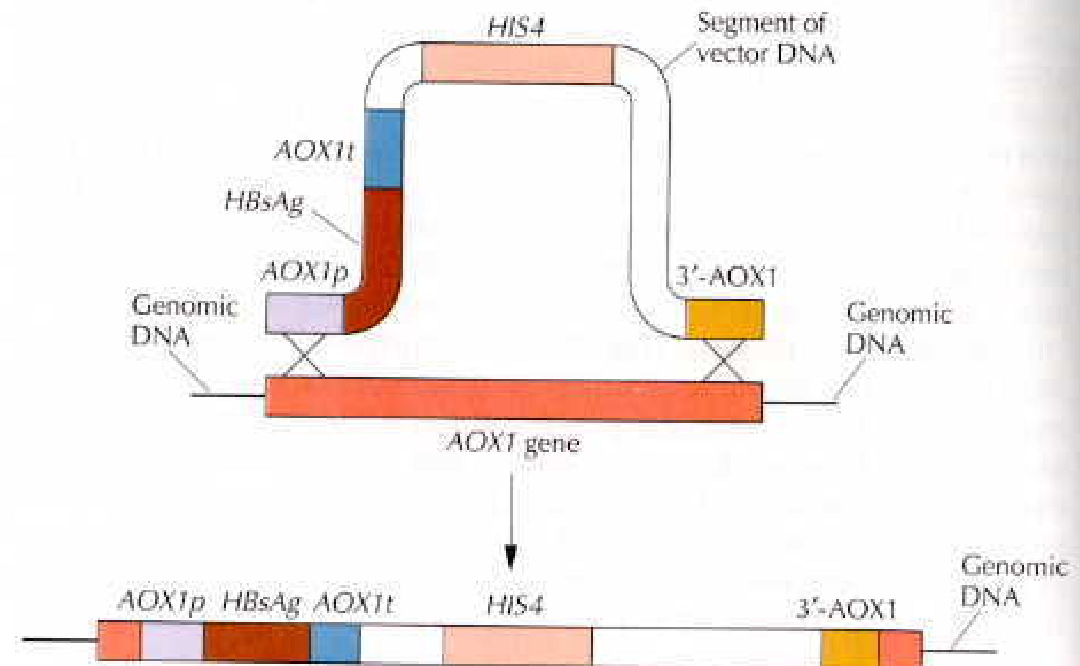


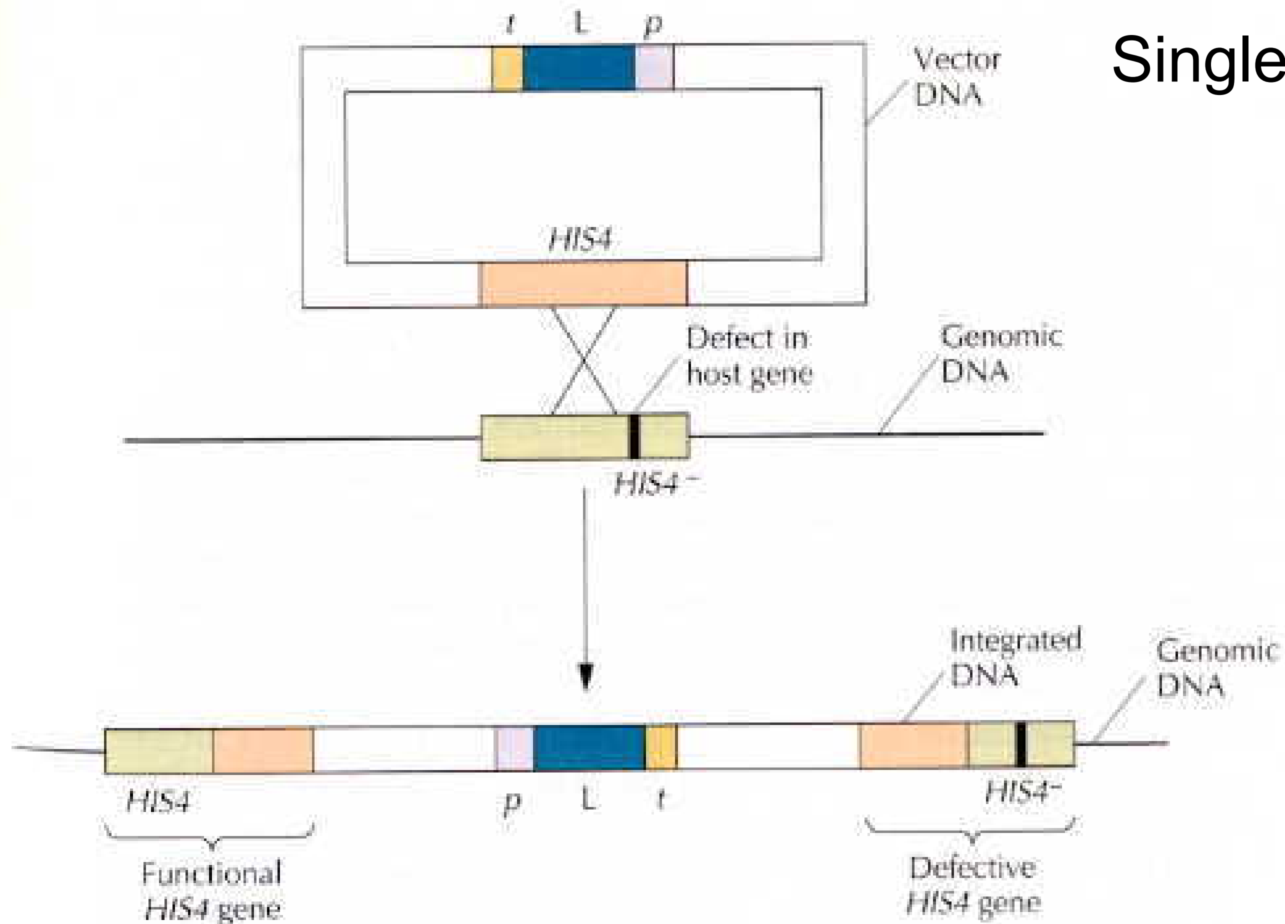
Figure 7.5 *P. pastoris* integrating expression vector. The *HBsAg* gene is cloned between the promoter (*AOX1p*) and termination-polyadenylation sequence (*AOX1t*) of the *P. pastoris* alcohol oxidase 1 gene. The *HIS4* gene encodes histidinol dehydrogenase, which is an enzyme in the histidine biosynthesis pathway. An origin of replication from *P. pastoris* (*ori*ᴾᴾ) is included, as are both the ampicillin resistance gene (Ampʳ) and an origin of replication (*ori*ᴱ) that function in *E. coli*. The segment marked 3′-AOX1 is a piece of DNA from the 3′ end of the alcohol oxidase 1 gene of *P. pastoris*. The joined right-angled arrows indicate the DNA region that will be integrated into the *P. pastoris* genome.

Figure 7.6 Integration of part of an expression vector into the alcohol oxidase 1 gene of *P. pastoris*. The double crossover event occurs within the *AOX1p* and 3′-AOX1 DNA segments (shown at the top). This event results in the integration of the input DNA into the genomic DNA and the loss of most of the alcohol oxidase 1 gene (*AOX1*) from the host chromosome (shown at the bottom). The *HIS4* gene product enables cells with integrated DNA to grow on medium lacking histidine. In the presence of methanol, the *AOX1p* region drives the transcription of the *HBsAg* gene. The *AOX1t* segment provides transcription termination and polyadenylation signals for the *HBsAg* gene.



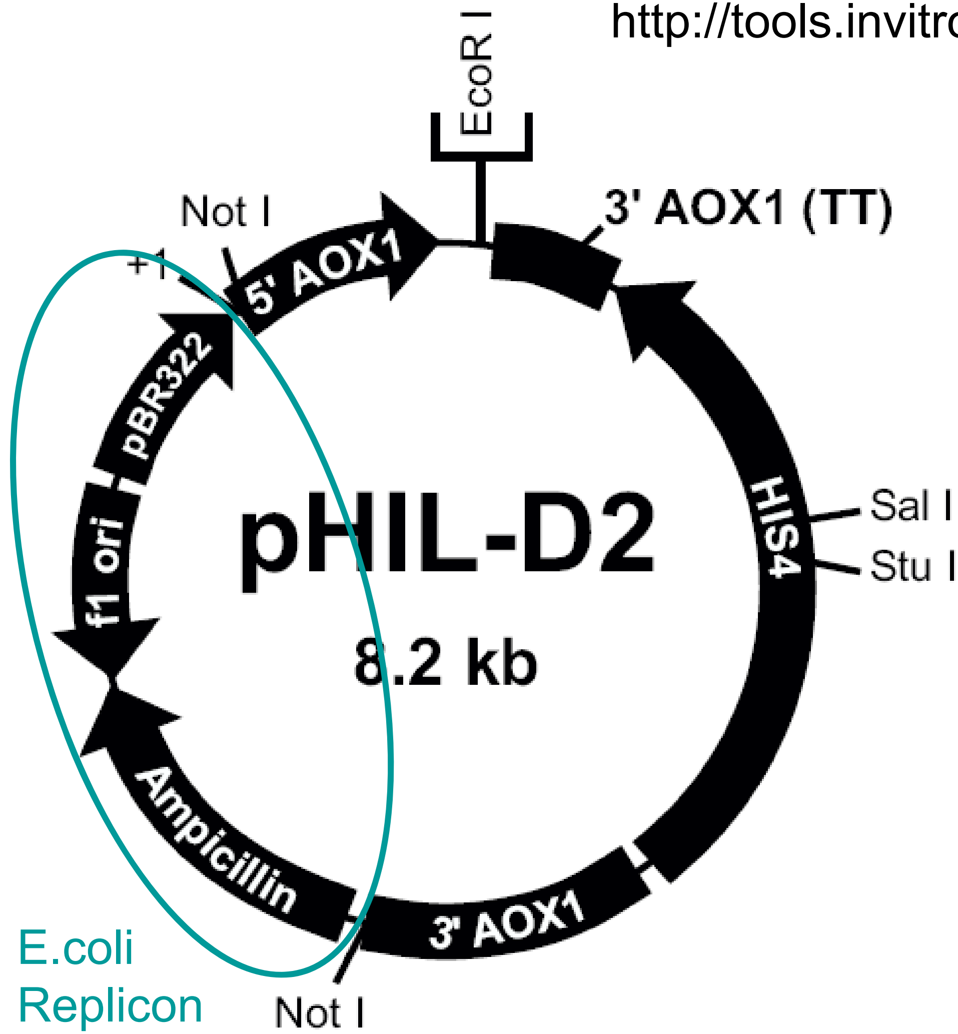
Single-site Integration

Figure 7.7 Integration of an expression vector into the defective chromosomal *HIS4*⁻ gene of *P. pastoris*. The input DNA is a plasmid that is first introduced by transformation into a histidine-requiring *P. pastoris* strain. A single crossover within the *HIS4* gene of the plasmid and the *HIS4*⁻ gene of the host cell results in the integration of the entire plasmid, which then is flanked by the functional *HIS4* gene and the defective *HIS4*⁻ gene. The letters *p*, L, and *t* denote the *AOX1* promoter sequence, bovine lysozyme C2 cDNA, and *AOX1* termination–polyadenylation signal sequence, respectively. The black bar in the *HIS4*⁻ gene represents the defective sequence.



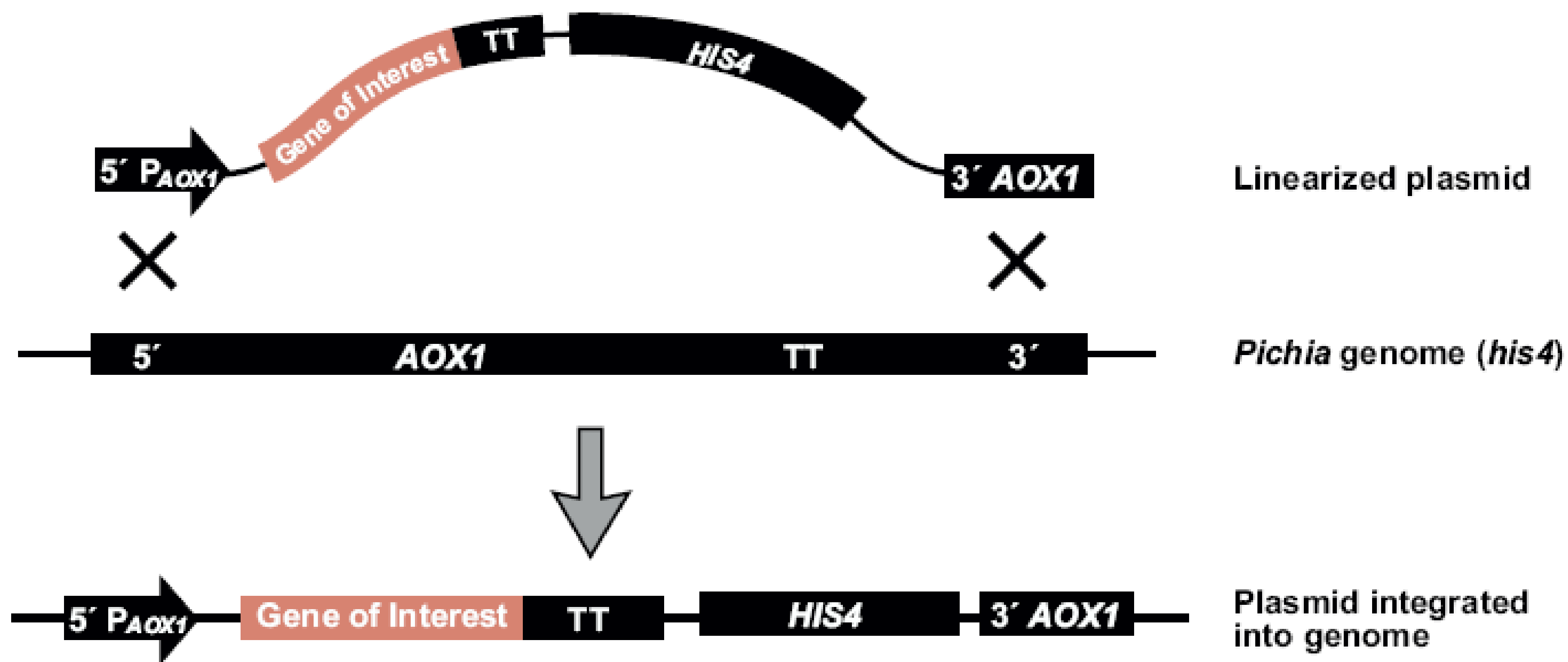
Vector for Intracellular Expression

http://tools.invitrogen.com/content/sfs/manuals/pich_man.pdf



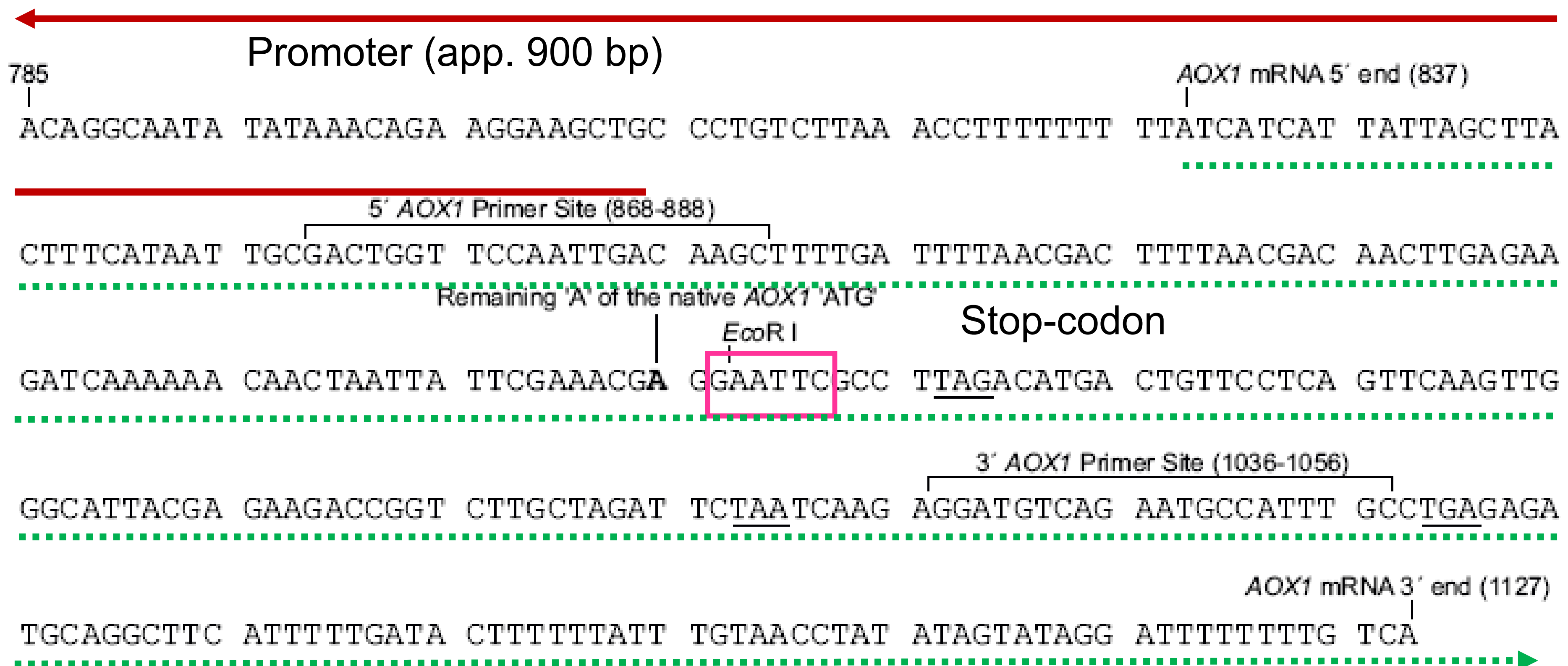
Comments for pHIL-D2:
8209 nucleotides

5' *AOX1* promoter fragment: bases 14-941
5' *AOX1* primer site: bases 868-888
*Eco*R I Site: bases 956-961
3' *AOX1* primer site: bases 1036-1056
3' *AOX1* transcription
termination (TT) fragment: bases 963-1295
HIS4 ORF: bases 4223-1689
3' *AOX1* fragment: bases 4578-5334
Ampicillin resistance gene: bases 5686-6546
f1 origin of replication: bases 7043-6588
pBR322 origin: bases 7138-7757


Gene of Interest
TT
HIS4
5´ PAOX1
3´ AOX1
Linearized plasmid
5´
AOX1
TT
3´
Pichia genome (his4)
5´ PAOX1
Gene of Interest
TT
HIS4
3´ AOX1
Plasmid integrated
into genome

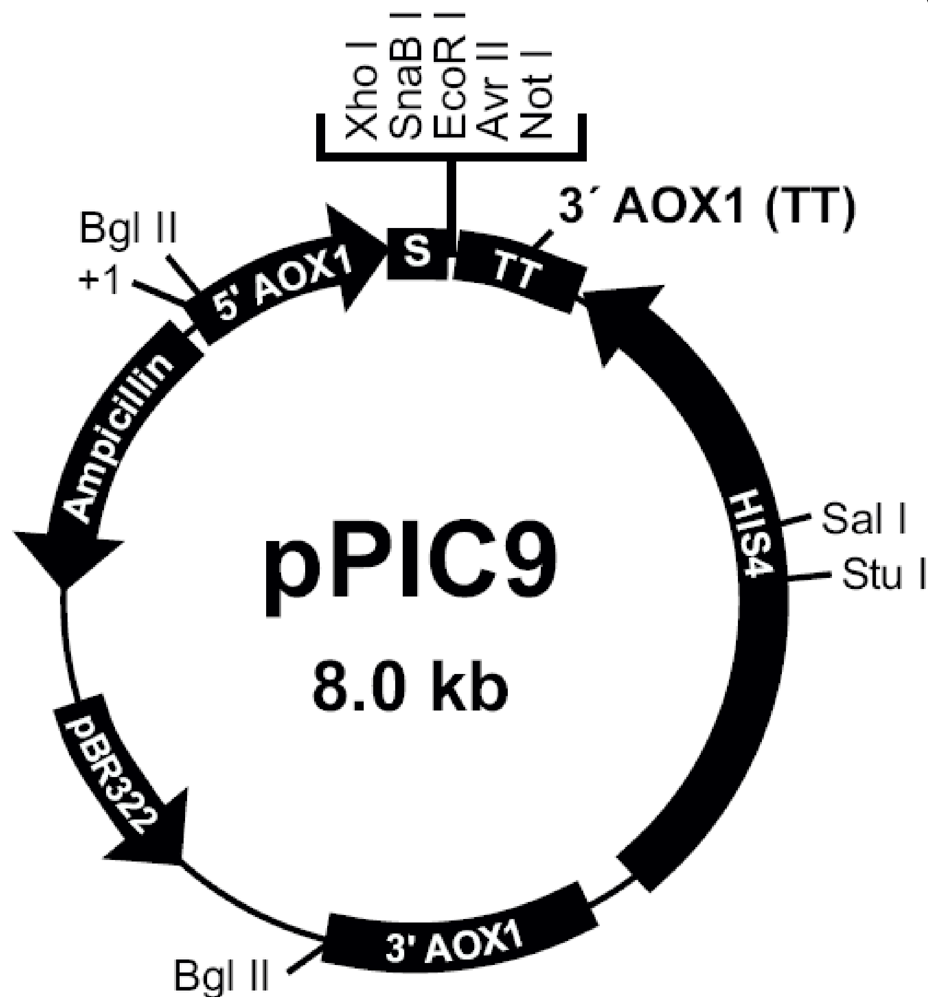
Vector for Intracellular Expression

pHILD2



- For pHIL-D2, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **ACC** **ATG** **G** is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990).
- Shorter, 5´ untranslated leaders reportedly work better in *AOX1* expression. In pHIL-D2, make the untranslated region as short as possible when cloning your gene.

Vector for Secretory Expression



Comments for pPIC9:
8023 nucleotides

5´ *AOX1* promoter fragment: bases 1-948
5´ *AOX1* primer site: bases 855-875
α-Factor secretion signal(s): bases 949-1215
α-Factor primer site: bases 1152-1172
Multiple Cloning Site: bases 1192-1241
3´ *AOX1* primer site: bases 1327-1347
3´ *AOX1* transcription
termination (TT): bases 1253-1586
HIS4 ORF: bases 4514-1980
3´ *AOX1* fragment: bases 4870-5626
pBR322 origin: bases 6708-6034
Ampicillin resistance gene: bases 7713-6853

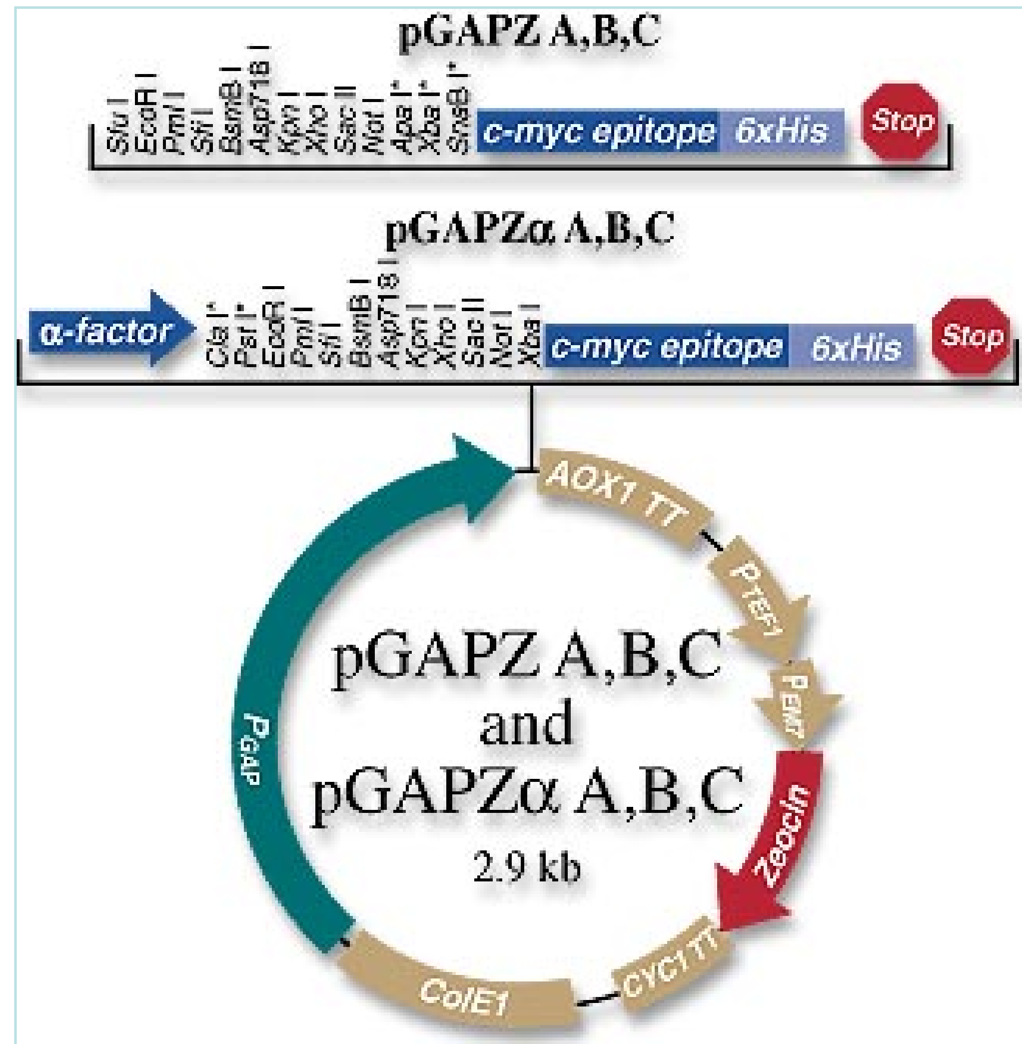
Vector for Secretory Expression

pPIC9

```
773                                                                     AOX1 mRNA 5´ end (824)
ACAGCAATAT ATAAACAGAA GGAAGCTGCC CTGTCTTAAA CCTTTTTTTT TATCATCATT ATTAGCTTAC

              5´ AOX1 Primer Site (855-875)
TTTCATAATT GCGACTGGTT CCAATTGACA AGCTTTTGAT TTTAACGACT TTTAACGACA ACTTGAGAAG

                                                α-Factor (949-1215)
ATCAAAAAAC AACTAATTAT TCGAAGGATC CAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCA
                                        Met Arg Phe Pro Ser Ile Phe Thr Ala
                                                  Signal sequence

GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT
Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp
                                                  Pro- sequence

GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT
Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp

TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA
Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe Ile

              α-Factor Primer Site (1152-1172)                 Xho I           Kex2
AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC GAG AAA AGA
Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu Glu Lys Arg

                   SnaB I  EcoR I    Avr II     Not I
GAG GCT GAA GCT TAC GTA GAA TTC CCT AGG GCG GCC GCG AAT TAA TTCGCCTTAG
Glu Ala Glu Ala Tyr Val Glu Phe Pro Arg Ala Ala Ala Asn ***
   Ste13
ACATGACTGT TCCTCAGTTC AAGTTGGGCA CTTACGAGAA GACCGGTCTT GCTAGATTCT AATCAAGAGG

 3´ AOX1 Primer Site (1327-1347)
ATGTCAGAAT GCCATTTGCC TGAGAGATGC AGGCTTCATT TTTGATACTT TTTTATTTGT AACCTATATA

                    AOX1 mRNA 3´ end (1418)
GTATAGGATT TTTTTTGTCA
```

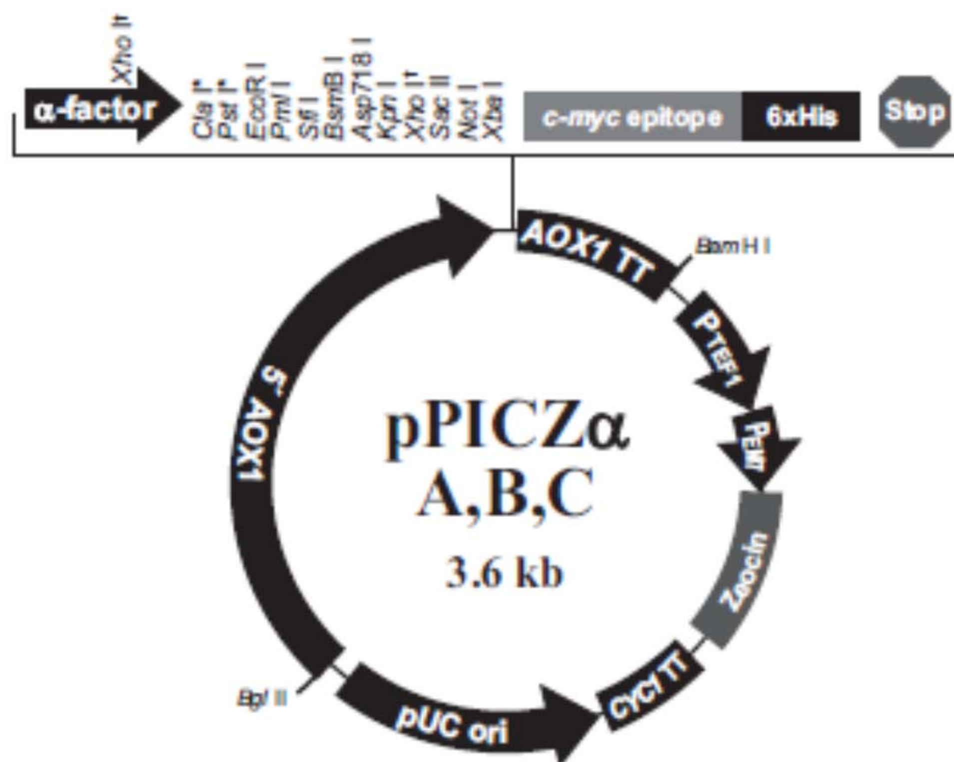
Resistance selection in *Pichia pastoris* , multiple integration and secretion

- P_{GAP}
- *AOX1* TT
- Zeo^R
- C-myc Epitope
- 6xHis
- alpha-factor
- ColE1 ori
- Multicopy Integration "in vivo"



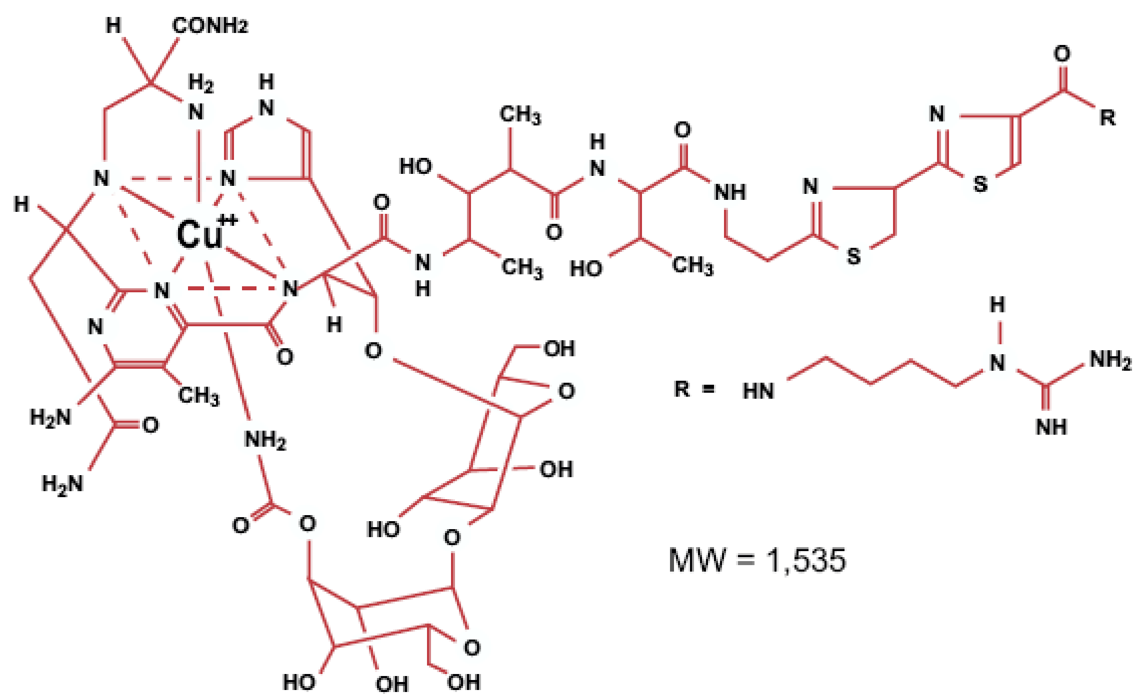
Comments for pPICZα A
3593 nucleotides

Feature	Benefit
5´ *AOX1*	A 942 bp fragment containing the *AOX1* promoter that allows methanol-inducible, high-level expression in *Pichia* Targets plasmid integration to the *AOX1* locus.
Native *Saccharomyces cerevisiae* α-factor secretion signal	Allows for efficient secretion of most proteins from *Pichia*
Multiple cloning site with 10 unique restriction sites	Allows insertion of your gene into the expression vector
C-terminal *myc* epitope tag	(Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) Permits detection of the fusion protein by the Anti-*myc* Antibody or Anti-*myc*-HRP Antibody (see page viii for ordering information) (Evan *et al.*, 1985)
C-terminal polyhistidine tag	Permits purification of your recombinant fusion protein on metal-chelating resin such as ProBond™ In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody and the Anti-His(C-term)-HRP Antibody (see page viii for ordering information) (Lindner *et al.*, 1997)
AOX1 Transcription Termination (TT)	Native transcription termination and polyadenylation signal from *AOX1* gene (~260 bp) that permits efficient 3´ mRNA processing, including polyadenylation, for increased mRNA stability
TEF1 promoter	Transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae* that drives expression of the *Sh ble* gene in *Pichia*, conferring Zeocin™ resistance (GenBank Acc. no. D12478, D01130)
EM7 (synthetic prokaryotic promoter)	Constitutive promoter that drives expression of the *Sh ble* gene in *E. coli*, conferring Zeocin™ resistance
Sh ble gene (*Streptoalloteichus hindustanus ble* gene)	Zeocin™ resistance gene
CYC1 transcription termination region	3´ end of the *Saccharomyces cerevisiae CYC1* gene that allows efficient 3´ mRNA processing of the *Sh ble* gene for increased stability (GenBank Acc. no. M34014)
pUC origin	Allows replication and maintenance of the plasmid in *E. coli*
Sac I, *Pme* I, *BstX* I	Unique restriction sites that permit linearization of the vectors at the *AOX1* locus for efficient integration into the *Pichia* genome



5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 941-1207
α-factor priming site: bases 1144-1164
Multiple cloning site: bases 1208-1276
c-myc epitope: bases 1275-1304
Polyhistidine (6xHis) tag: bases 1320-1337
3´ *AOX1* priming site: bases 1423-1443
AOX1 transcription termination region: bases 1341-1682
TEF1 promoter: bases 1683-2093
EM7 promoter: bases 2095-2162
Sh ble ORF: bases 2163-2537
CYC1 transcription termination region: bases 2538-2855
pUC origin: bases 2866-3539 (complementary strand)

Zeocin



P.pastoris vectors for intracellular expression

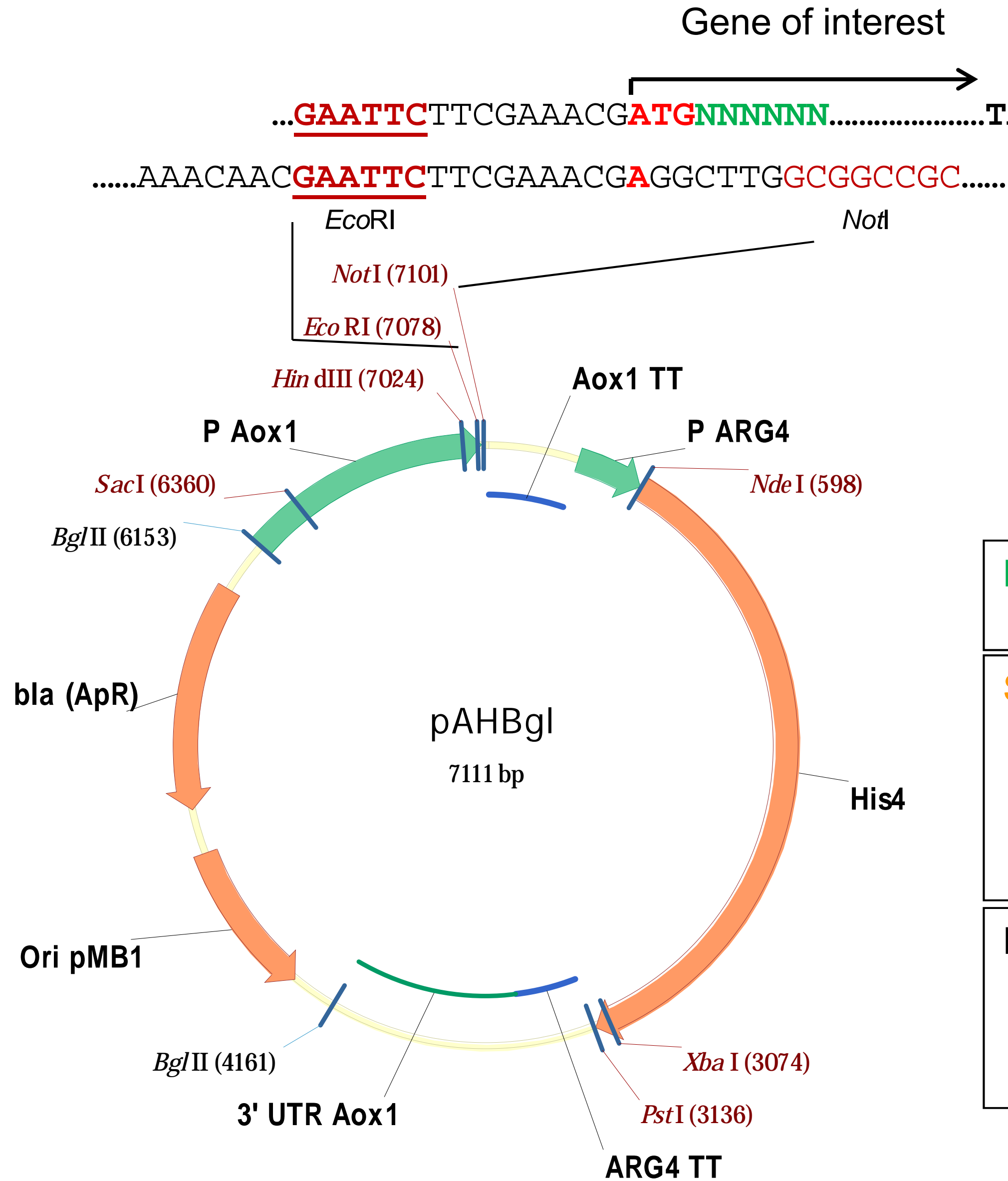
Gene of interest

NotI

...GAATTCTTCGAAACGATGNNNNNN....................TAAGCGGCCGC...

......AAACAACGAATTCTTCGAAACGAGGCTTGGCGGCCGC......

EcoRI NotI



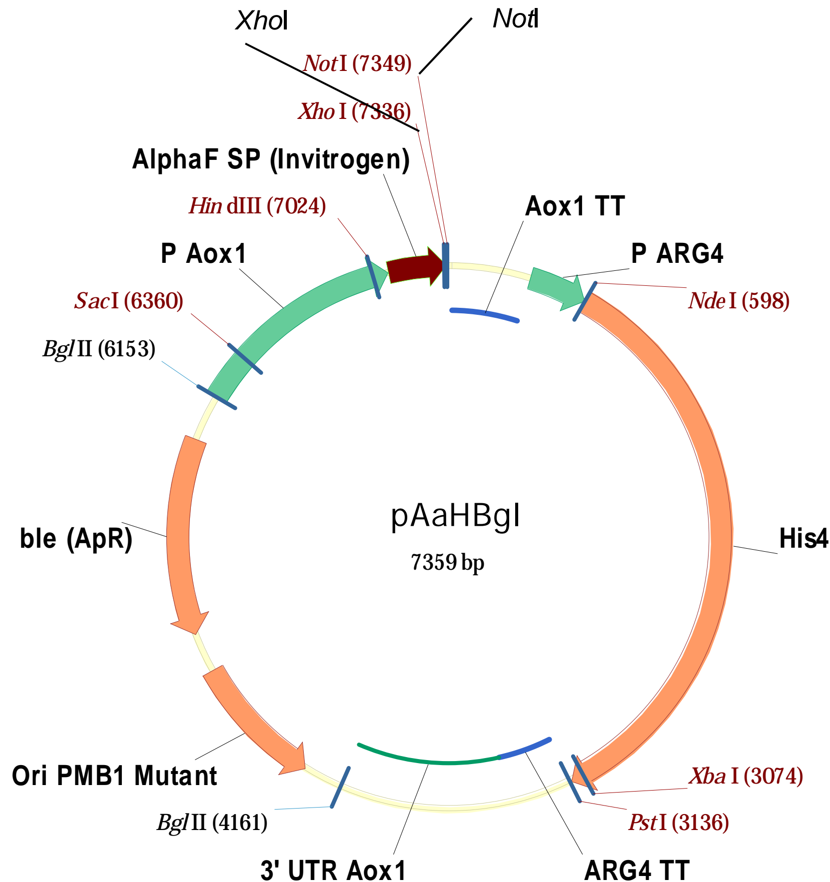
p A H Bgl

Promoter:
A → *AOX1*

Selection Marker:
H → *HIS4*
Z → Zeocin^R
A → *ARG4*
K → Kanamycin^R

Restriction site:
Bgl → *Bgl*II
Sph → *Sph*I
Swa → *Swa*I

P.pastoris vectors for secretory expression



p A a H Bgl

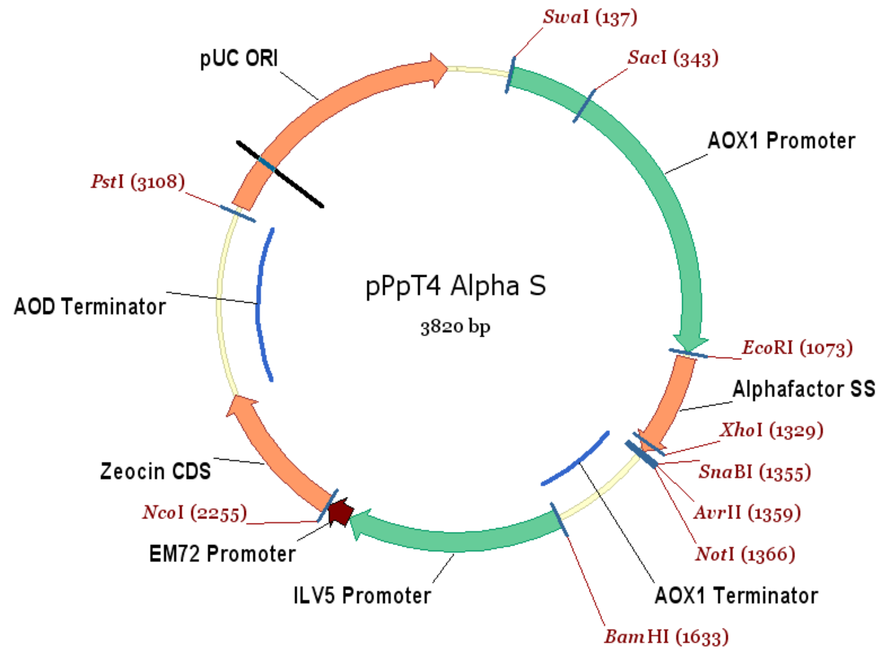
Promoter:
A → *AOX1*

Secretion Signal:
a → *alpha factor*

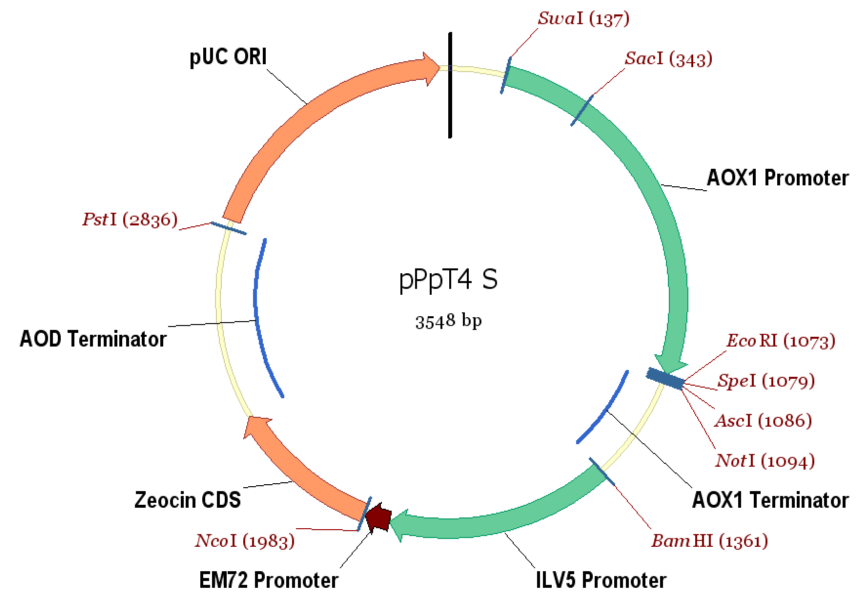
Selection Marker:
H → *HIS4*
Z → Zeocin^R
A → *ARG4*
K → Kanamycin^R

Restriction site:
Bgl → *Bgl*II
Sph → *Sph*I
Swa → *Swa*I

Vectors for multiple Integrations



secretory



intracellular

Protein expressed	Expression Level (mg/L)	Reference
Bacterial proteins		
Tetanus toxin fragment C	12,000	Clare, J.J. *et al.* (1991) *Bio/Technology* 9: 455–460
α-amylase	2,500	Paifer, E. *et al.* (1994) *Yeast* 10: 1415–1419
T2A peroxidase	2,470	Thomas, L. *et al.* (1998) *Can. J. Microbiol.* 44: 364–372
C. botulinum neurotoxin fragment	78	Smith, L.A. (1998) *Toxicon* 36: 1539–1548
Yeast proteins		
Catalase L	2,300	Calera, J.A. *et al.* (1997) *Infect. Immun.* 65: 4718–4724
Glucoamylase	400	Fierobe, H.-P. *et al.* (1997) *Protein Expr. Purif.* 9: 159–170
Lipase	60	Minning, S. *et al.* (1998) *J. Biotechnol.* 66: 147–156
Plant proteins		
Hydroxynitrile lyase	22,000	Hasslacher, M. *et al.* (1997) *Protein Expr. Purif.* 11: 61–71
Wheat lipid transfer protein	720	Klein, C. *et al.* (1998) *Protein Expr. Purif.* 13: 73–82
Aeroallergen	60	Huecas, S. *et al.* (1999) *Eur. J. Biochem.* 261: 539–546.
Invertebrate proteins		
Hirudin	1,500	Rosenfeld, S.A. *et al.* (1996) *Protein Expr. Purif.* 8: 476–482.
Spider dragline silk protein	663	Fahnestock, S.R. *et al.* (1997) *Appl. Micro. Biotechnol.* 47: 33–39
Honeybee olfactory protein	200	Danty, E. *et al.* (1999) *J. Neuroscience* 19: 7468-7475
Mammalian proteins		
Mouse gelatin	14,800	Werten, M.W. *et al.* (1999) *Yeast* 15: 1087-1096
Porcine carboxypeptidase B	200	Ventura, S. *et al.* (1999) *J. Biol. Chem.* 274: 19925-33
Human tumor necrosis factor	10,000	Sreekrishna, K. *et al.* (1989) *Biochemistry* 28: 4117-4125
Human IGF-1	600	Brierley, R.A. (1998) *Methods Mol. Biol.* 103: 149–177
Human CD38	455	Munshi, C.B. (1997) *Methods Enzymol.* 280: 318–330
15N-Interferon τ	10	Johnson, T.M. *et al.* (1999) *J. Interferon Cytokine Res.* 19: 631-636

Baculovirus Expression System

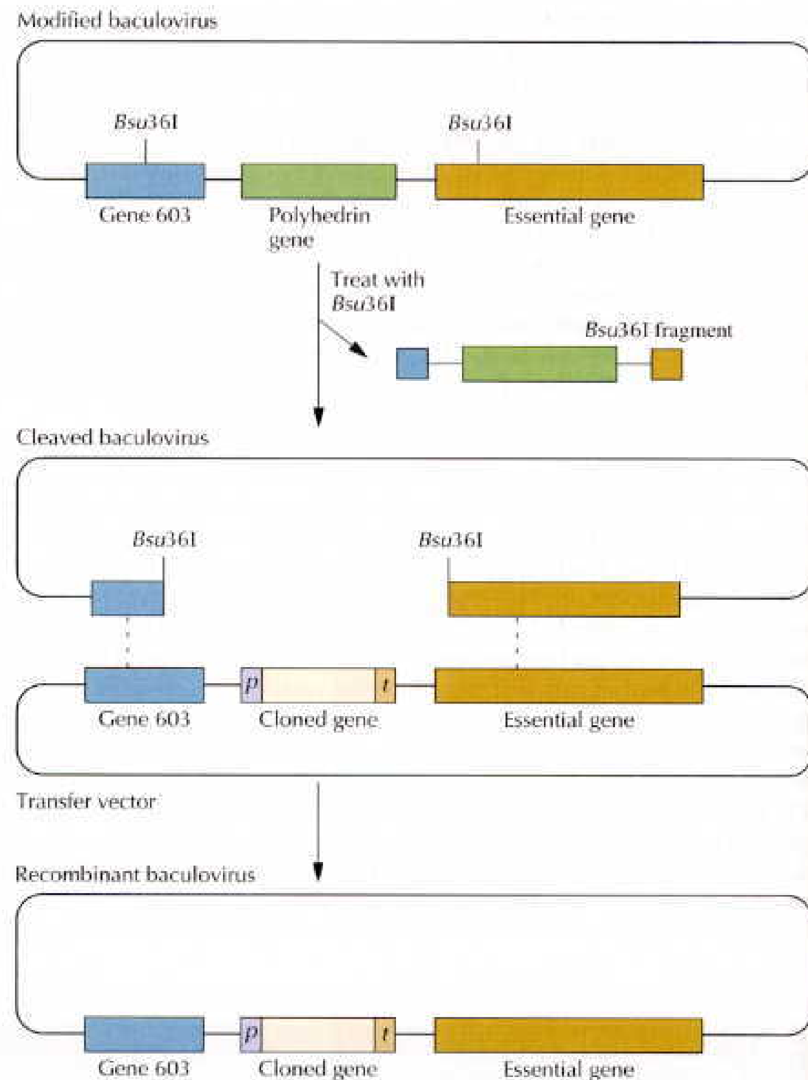
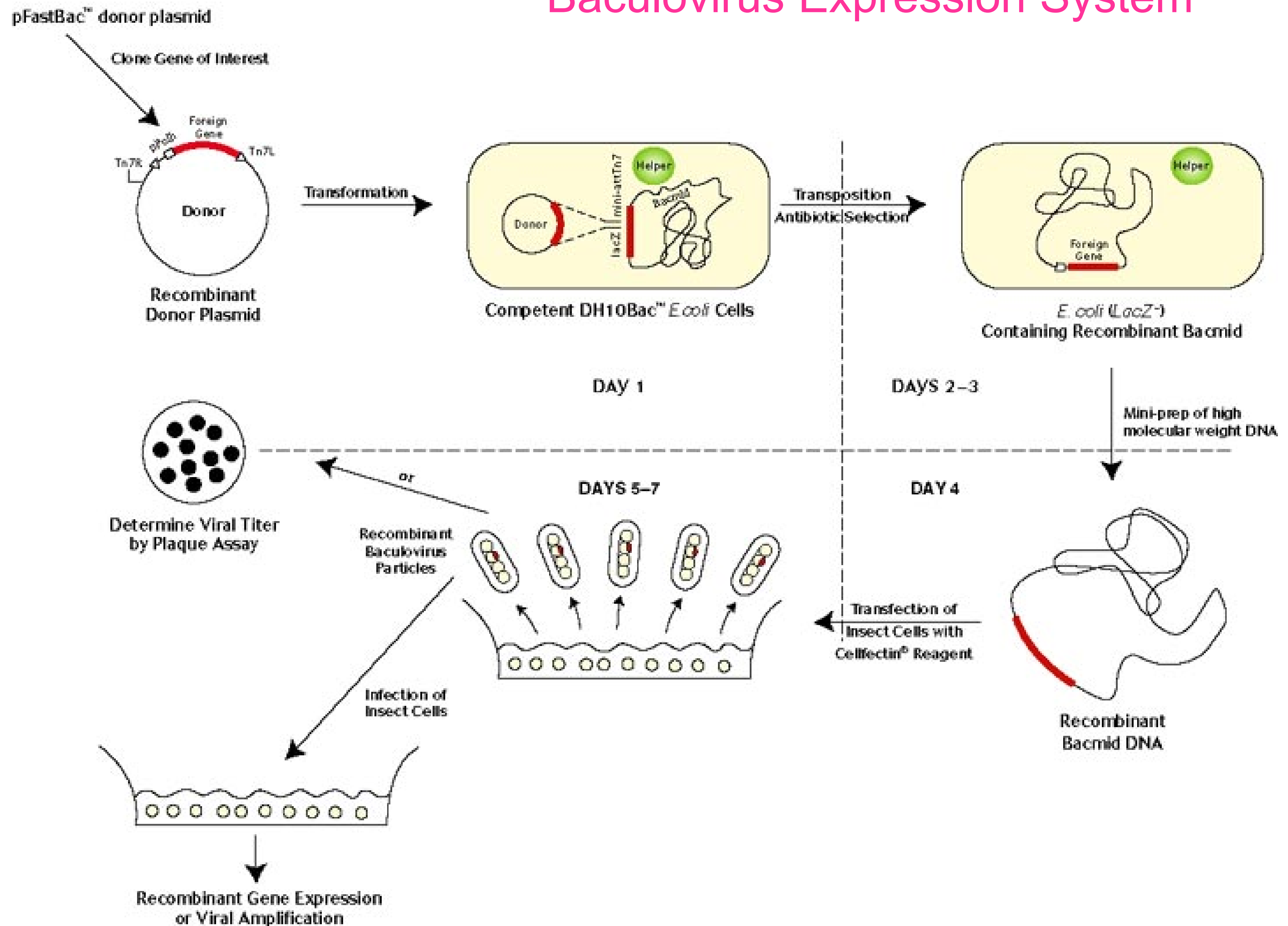
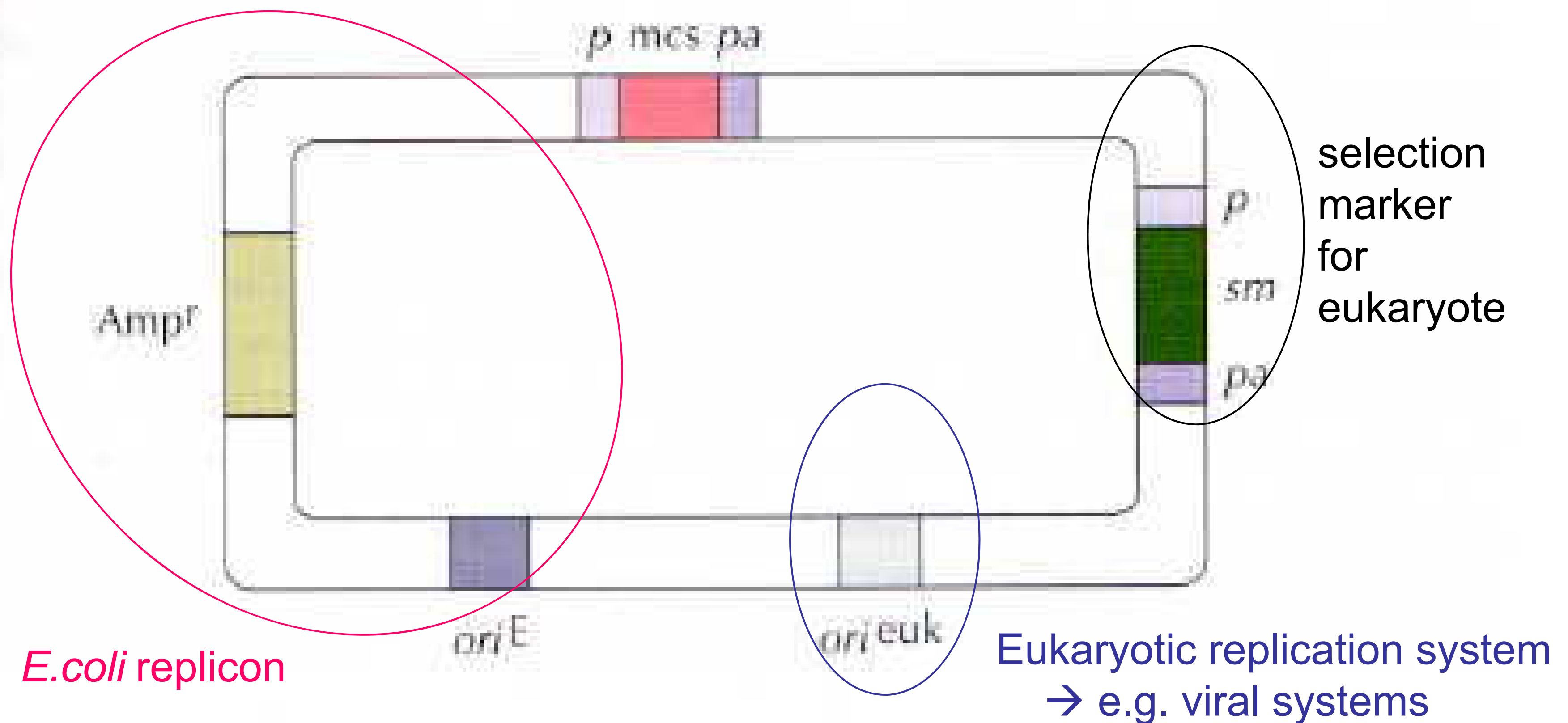


Figure 7.11 Production of recombinant baculovirus. Single *Bsu*36I sites are engineered into gene 603 and a gene (ORF1629) that is essential for baculovirus replication in insect cells (essential gene) of the AcMNPV genome. These genes flank the polyhedrin gene of the AcMNPV genome. After a baculovirus with two engineered *Bsu*36I sites is treated with *Bsu*36I, the segment between the *Bsu*36I sites is deleted. After transfection of an insect cell carrying a *Bsu*36I-treated baculovirus with a transfer vector that has a cloned gene under the control of the promoter (*p*) and terminator (*t*) elements of the polyhedrin gene and the complete sequence of both gene 603 and the essential gene, a double crossover event (dashed lines) generates a recombinant baculovirus with a functional essential gene. This system produces up to 99% recombinant baculoviruses.

Baculovirus Expression System

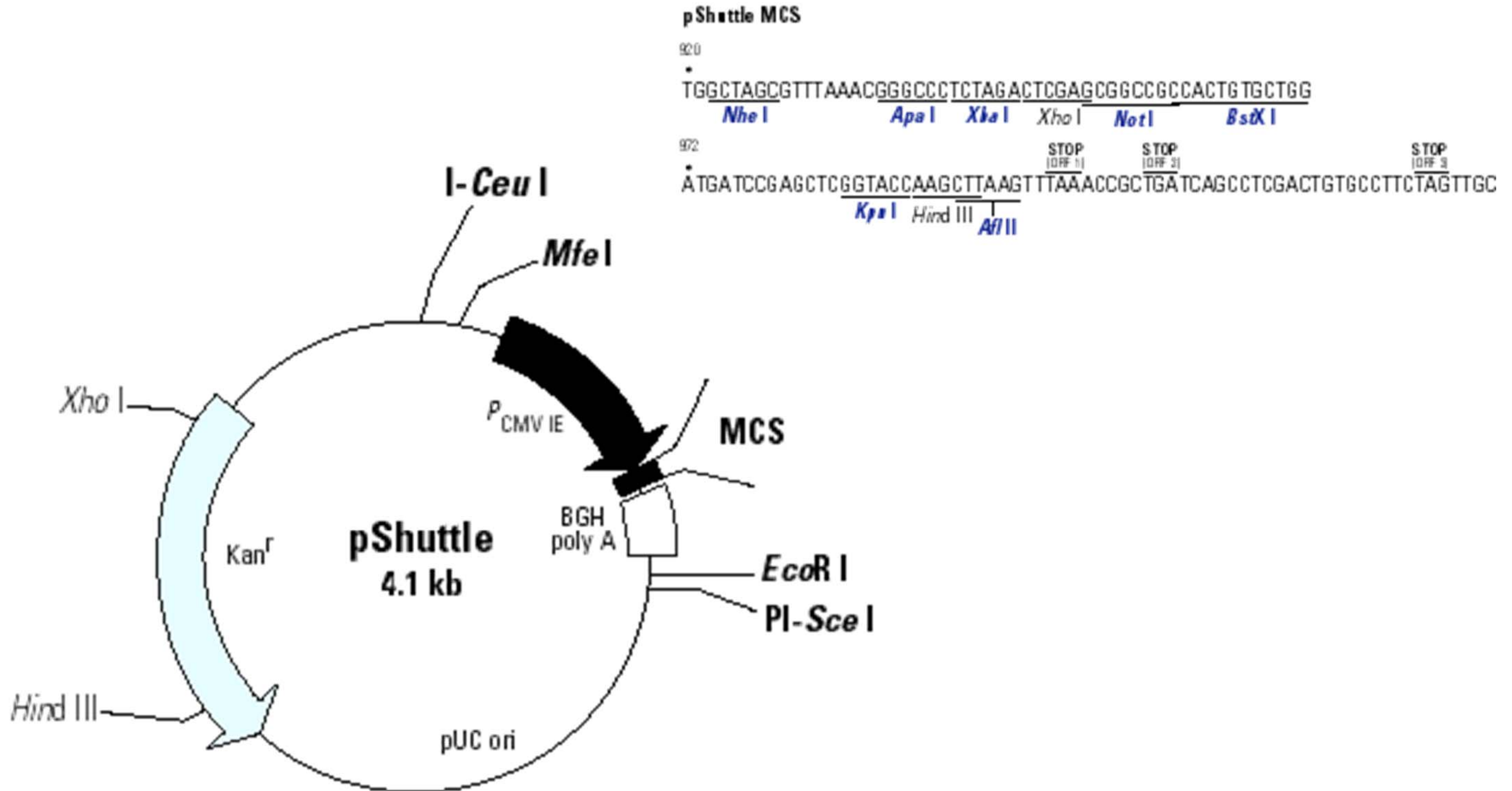


Mammalian Expression System



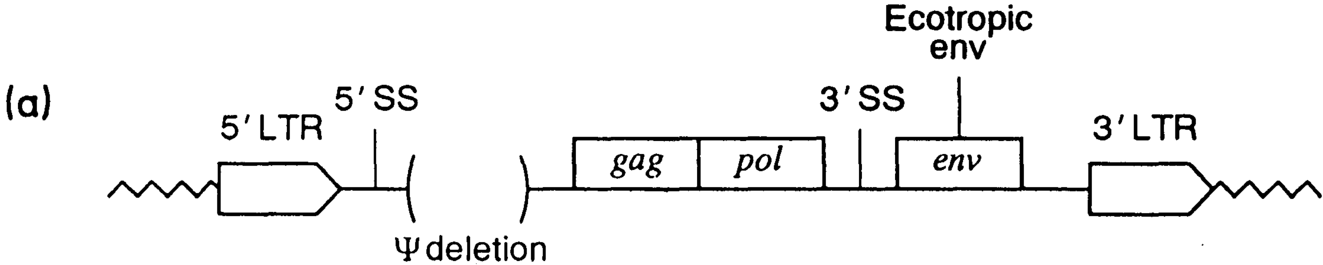
Mammalian Expression System

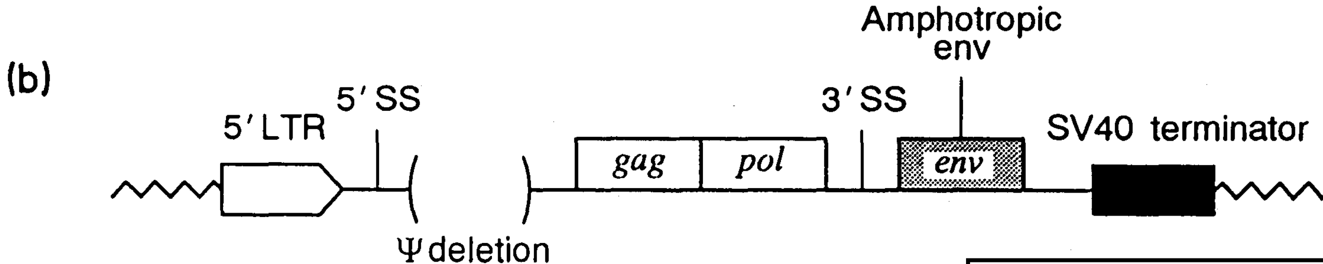
Simple Plasmid for ectopic integration

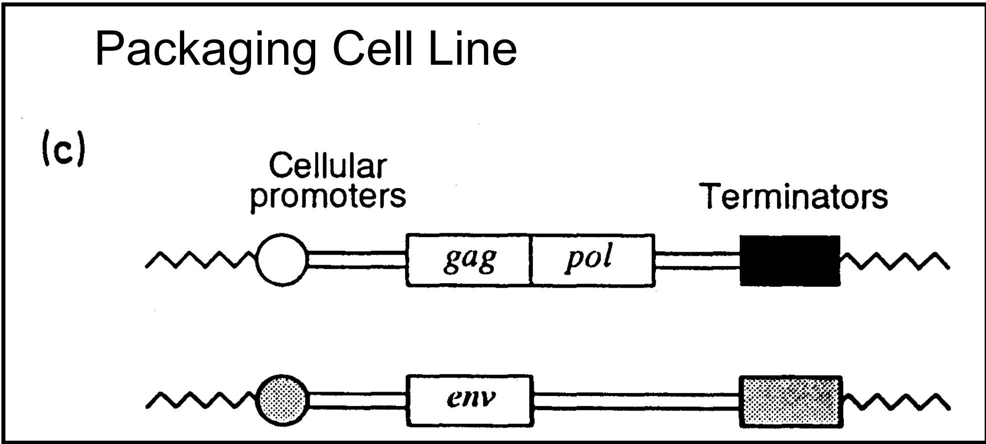


6.11.14

Retroviral Expression system







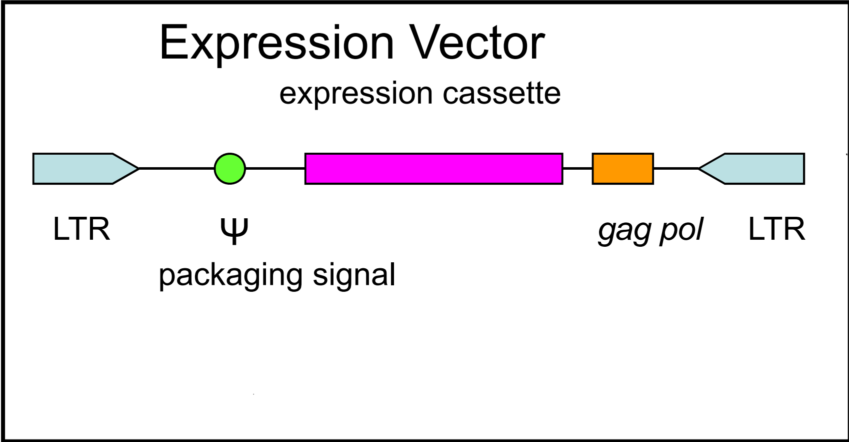


Fig. 30. Recombinant retroviral genomes in packaging cell lines. The factors required to rescue defective

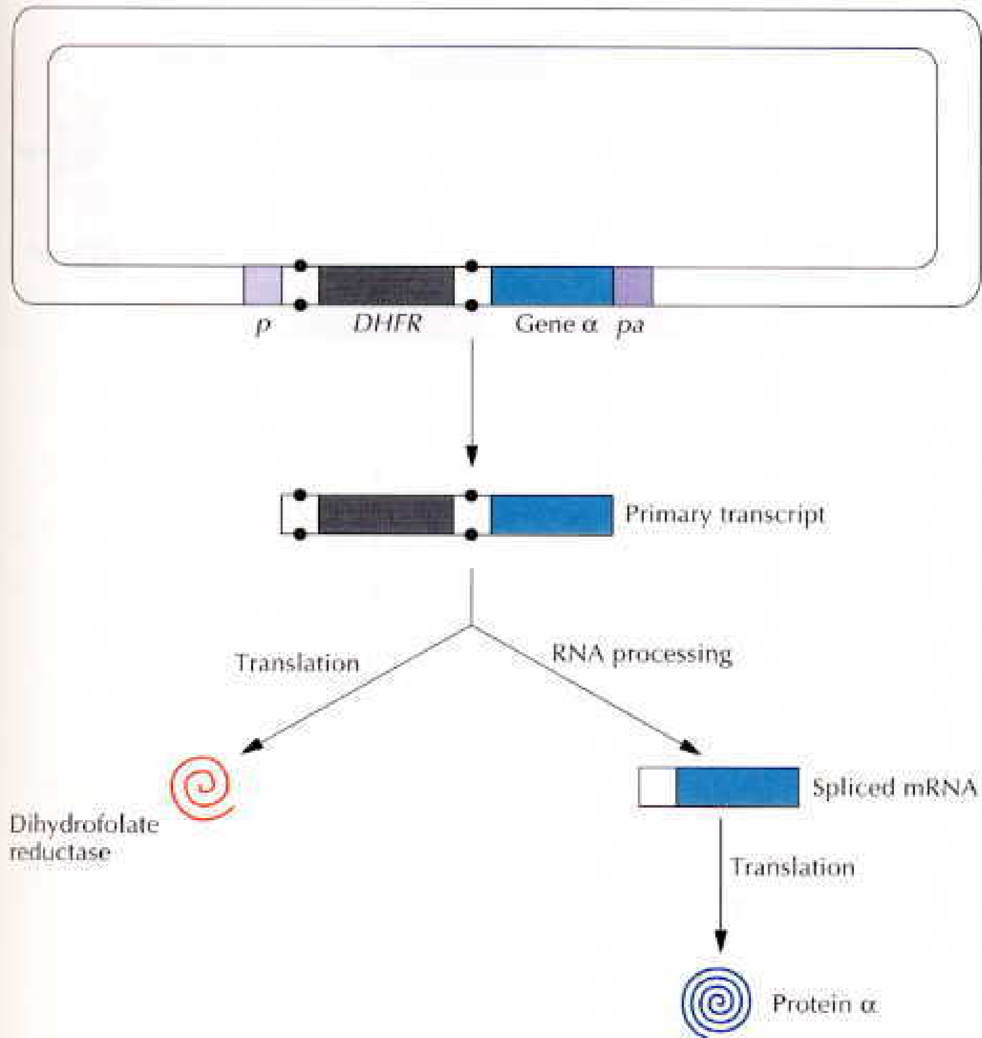
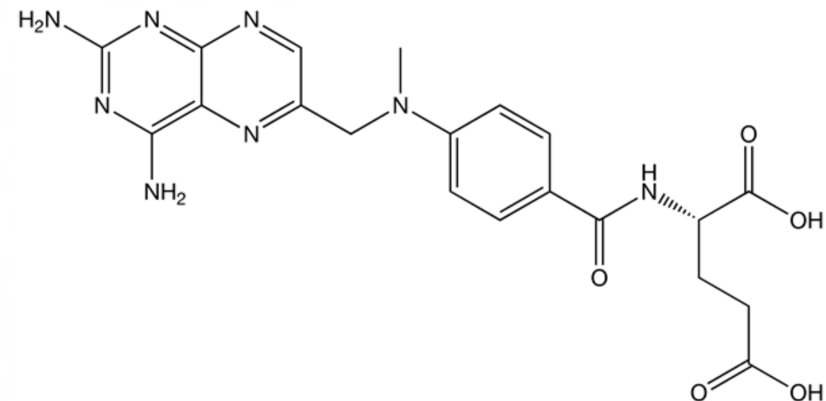
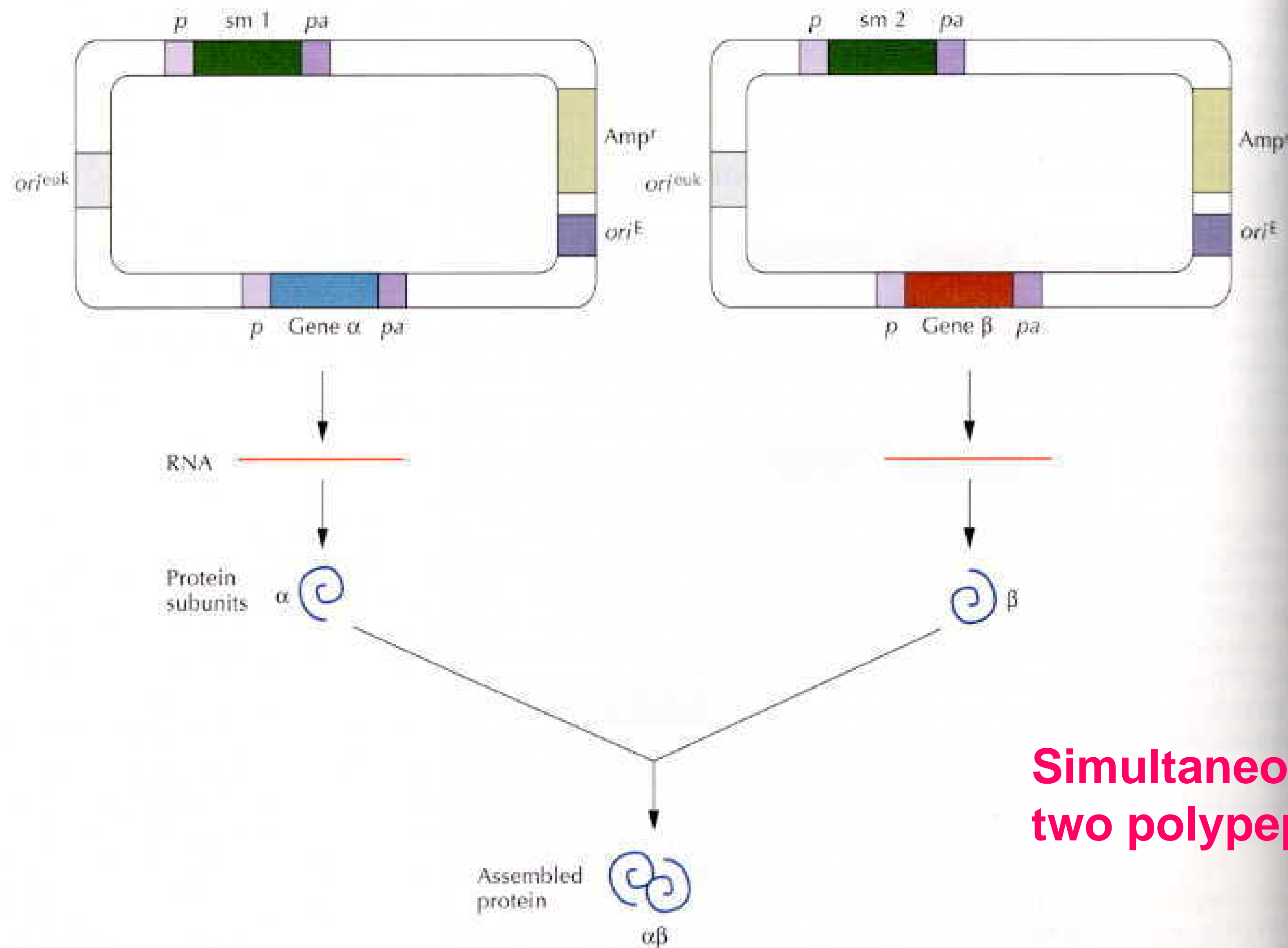


Figure 7.14 Coordinated expression of DHFR and a recombinant protein. A *DHFR* gene is cloned between intron donor and acceptor splice sites (dots) and upstream from a cloned gene (gene α). Both the *DHFR* and cloned genes are under the control of eukaryotic promoter (*p*) and polyadenylation (*pa*) sequences. Dihydrofolate reductase and the heterologous protein (protein α) are translated from the unspliced (primary) and processed (spliced) transcripts, respectively.

Expression strategies

DHFR:
Selection for high expression with methotrexate





Simultaneous expression of two polypeptides

Figure 7.15 Two-vector expression system. The cloned genes (gene α and gene β) encode subunits of a protein dimer ($\alpha\beta$). After cotransfection, both subunits (α and β) are synthesized and assembled into a functional protein dimer. Both vectors carry origins of replication for *E. coli* (ori^E) and mammalian cells (ori^{euk}), a marker gene (Amp^r) for selecting transformed *E. coli*; and eukaryotic promoter (*p*) and polyadenylation (*pa*) sequences that control a selectable marker gene (sm) and each of the cloned genes (gene α and gene β).

Simultaneous expression of two polypeptides

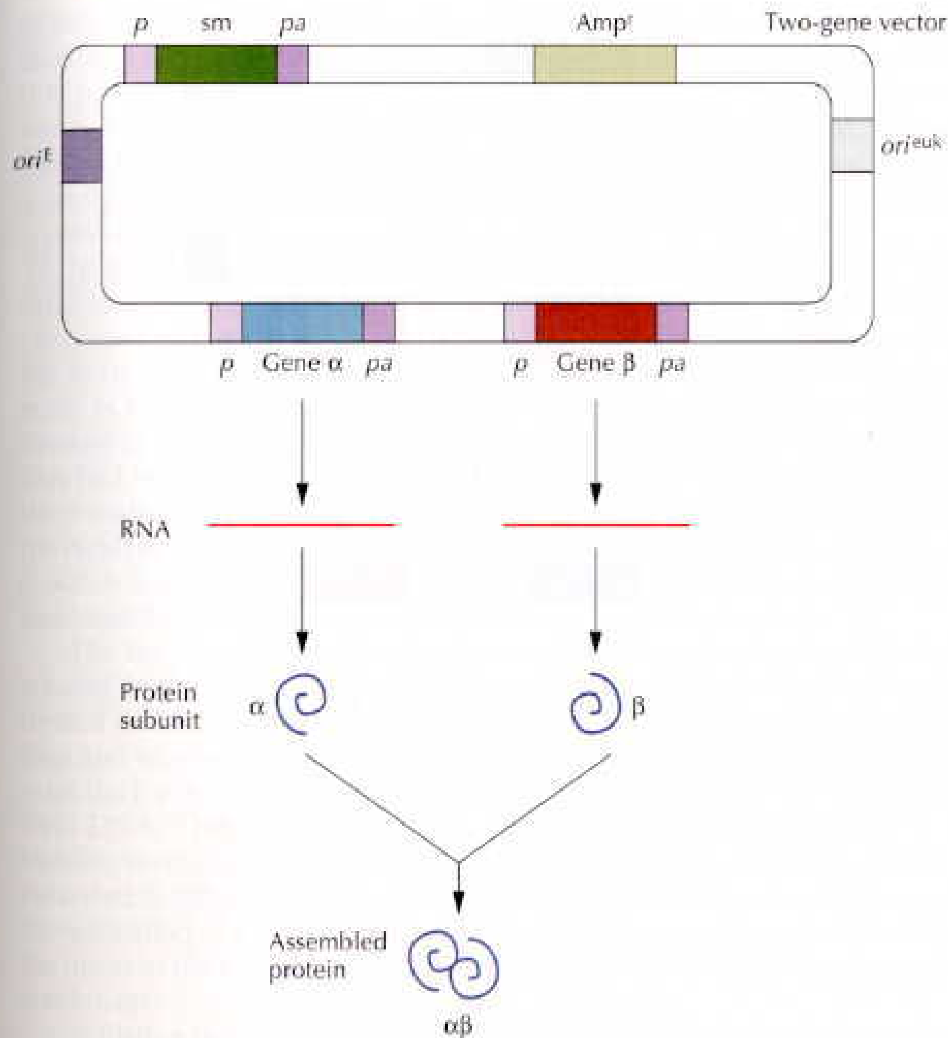


Figure 7.16 Two-gene expression vector. The cloned genes (gene α and gene β) encode subunits of a protein dimer ($\alpha\beta$). Each cloned gene is inserted into a vector as part of its own transcription unit under the control of a eukaryotic promoter (*p*) and polyadenylation sequence (*pa*). Each subunit is translated from a separate mRNA, and a functional protein dimer ($\alpha\beta$) is assembled. Each vector carries origins of replication for *E. coli* (ori^E) and mammalian cells (ori^{euk}), a marker gene (Amp^r) for selecting transformed *E. coli*, and a selectable marker gene (sm) that is under the control of a eukaryotic promoter (*p*) and sequence polyadenylation (*pa*).

Simultaneous expression of two polypeptides

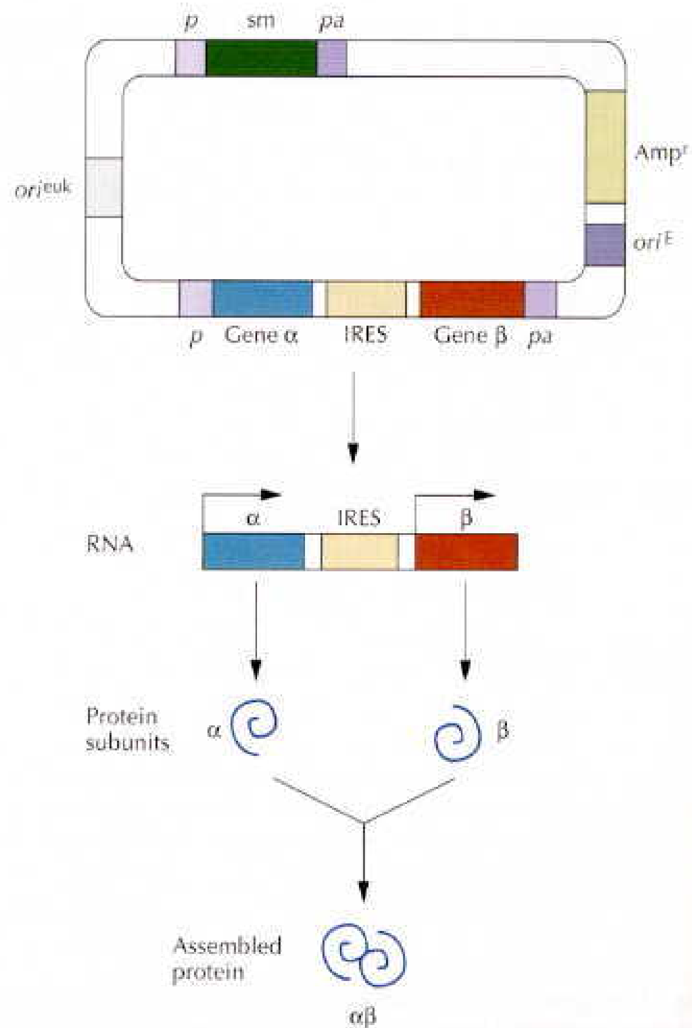


Figure 7.17 Dicistronic expression vector. The cloned genes (gene α and gene β) encode subunits of a protein dimer ($\alpha\beta$). Each cloned gene is inserted into a vector, and they are separated by a DNA sequence that after transcription, as part of an mRNA, acts as an IRES. The two cloned genes are under the control of a eukaryotic promoter (*p*) and sequence polyadenylation (*pa*). Translation of the mRNA occurs from the 5′ end and internally (right-angled arrows). Both subunits (α and β) are synthesized and assembled into a functional protein dimer. The vector carries origins of replication for *E. coli* (ori^E) and mammalian cells (ori^{euk}), a selectable marker (Amp^r) for selecting transformed *E. coli*, and a selectable marker gene (sm) that is under the control of a eukaryotic promoter (*p*) and polyadenylation sequence (*pa*).

IRES:

Internal Ribosome Entry Site