Biotechnology of drugs– Expression of recombinant proteins in eukaryotic cells

Doc. RNDr. Jan Hošek, Ph.D.
hosekj@pharm.muni.cz

Department of Molecular Pharmacy
FaF MU

Table 1.3. Characteristics of various recombinant protein expression systems.

Expression system	Most common application	Advantages	Challenges
Mammalian 	• Functional assays • Structural analysis • Antibody production • Expression of complex proteins • Protein interactions • Virus production	• Highest-level protein processing • Can produce proteins either transiently, or by stable expression • Robust optimized transient systems for rapid, ultrahigh-yield protein production	• Gram-per-liter yields only possible in suspension cultures • More demanding culture conditions
Insect 	• Functional assays • Structural analysis • Expression of intracellular proteins • Expression of protein complexes • Virus production	• Similar to mammalian protein processing • Can be used in static or suspension culture	• More demanding culture conditions than prokaryotic systems • Production of recombinant baculovirus vectors is time consuming
Yeast 	• Structural analysis • Antibody generation • Functional analysis • Protein interactions	• Eukaryotic protein processing • Scalable up to fermentation (grams per liter) • Simple media requirements	• Fermentation required for very high yields • Growth conditions may require optimization
Bacterial 	• Structural analysis • Antibody generation • Functional assays • Protein interactions	• Scalable • Low cost • Simple culture conditions	• Protein solubility • May require protein-specific optimization • May be difficult to express some mammalian proteins
Algal 	• Studying photosynthesis, plant biology, lipid metabolism • Genetic engineering • Biofuel production	• Genetic modification and expression systems for photosynthetic microalgae • Superb experimental control for biofuel, nutraceuticals, and specialty chemical production • Optimized system for robust selection and expression	• Nascent technology • Less developed compared to other host platforms
Cell-free 	• Toxic proteins • Incorporation of unnatural label or amino acids • Functional assays • Protein interactions • Translational inhibitor screening	• Open system; able to add unnatural components • Fast expression • Simple format	• Scaling above multimilligram quantities may not be costly

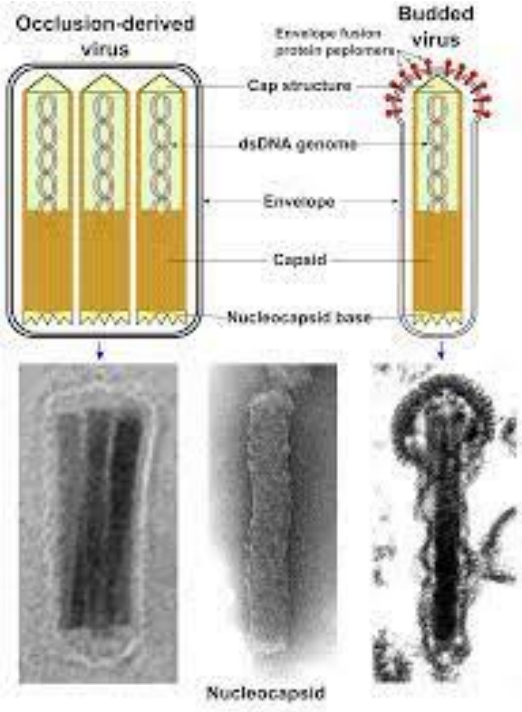
Aims of transfection of animal cells

1. Gene transfer into animal cells
   - searching for genes, learning their function and regulation and studying the phenotype (e.g. differentiation processes and their disorders)
   - the study of protein interactions
   - **formation and purification of foreign proteins**

2. Preparation of transgenic animals
   - the study of the functioning of genes within the whole organism
   - models for the study of genetic diseases
   - preparation of animals with better useful properties
   - searching for options for gene therapy
   - **creation of foreign proteins (gene pharming)**

Insect cell expression systems

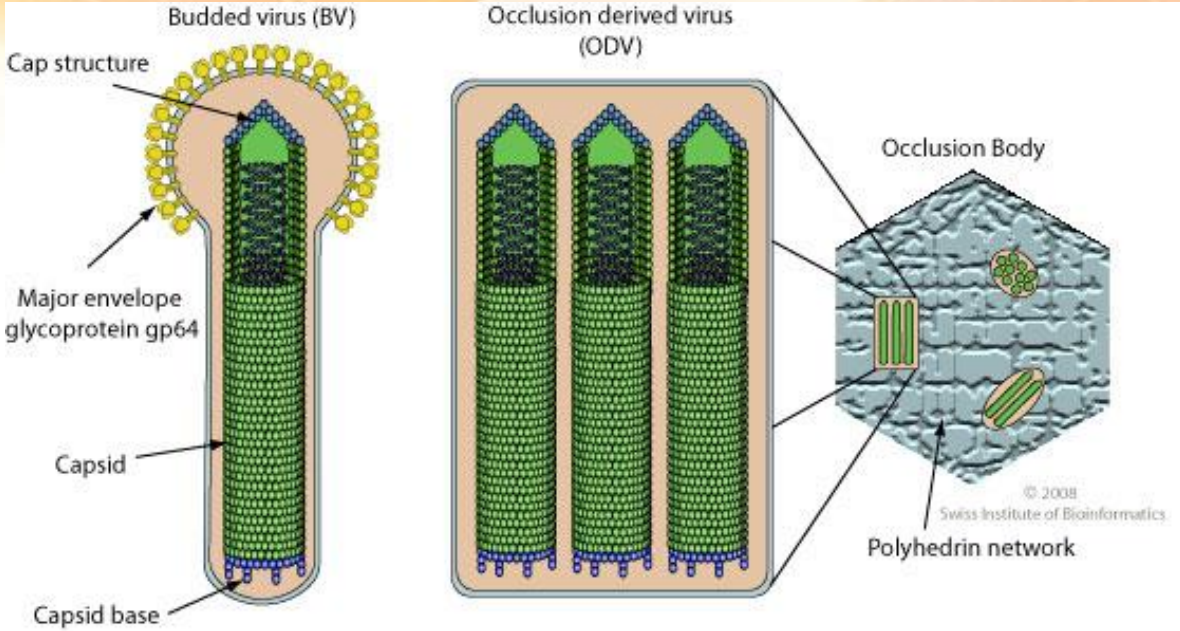
- Host cells derived from
  - *Spodoptera frugiperda* → Sf9 cells – **transient expression**
  - *Trichoplusia ni* → High Five™ cells – **transient expression**
  - *Drosophila melanogaster* → S2 cells – **stable expression**
  - They can grow adherently or in suspension



- Baculovirus vector

Baculoviruses

- Large rod-shaped **dsDNA** viruses infecting insects
- The most common member is *Autographa californica* multiple nuclear polyhedrosis virus (**AcMNPV**) parasitizing members of the order lepidoptera (Spodoptera frugiperda and Trichoplusia) → **a wide range of hosts**



- Viral particles are enveloped by a protective matrix consisting of the protein **polyhedrin** - it allows survival in the external environment and efficient spread between cells, in *in vitro* conditions are not required

- The **pPolh promoter** allows expression of polyhedrin or **recombinant protein** - up to 50% of the total protein at the end of the baculovirus cycle

Baculovirus expression vectors I.

- Gene of interest under pPolh promoter
- GOI on plasmid → homologous recombination to AcMNPV DNA
- Low success rate of recombination (approx. 0.1%)
- More complicated search for recombinant (polyhedrin-negative) viral plaques
- Use of linearized (non-replicating) AcMNPV DNA → recombination success rate 10-20%, BakPAC system up to 95%

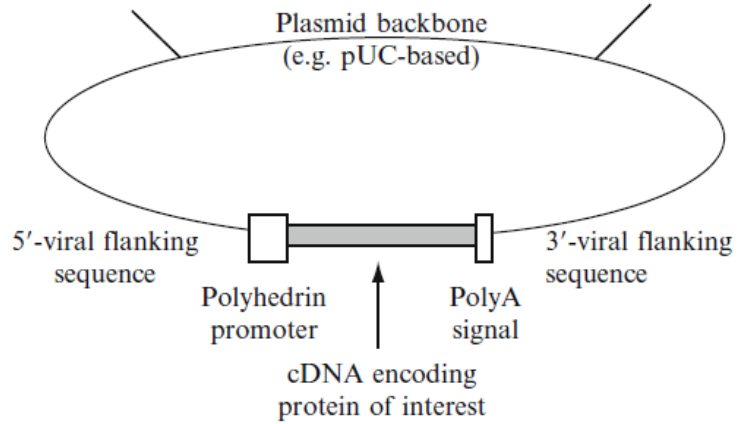


Figure 14.1 A simple baculovirus transfer plasmid.

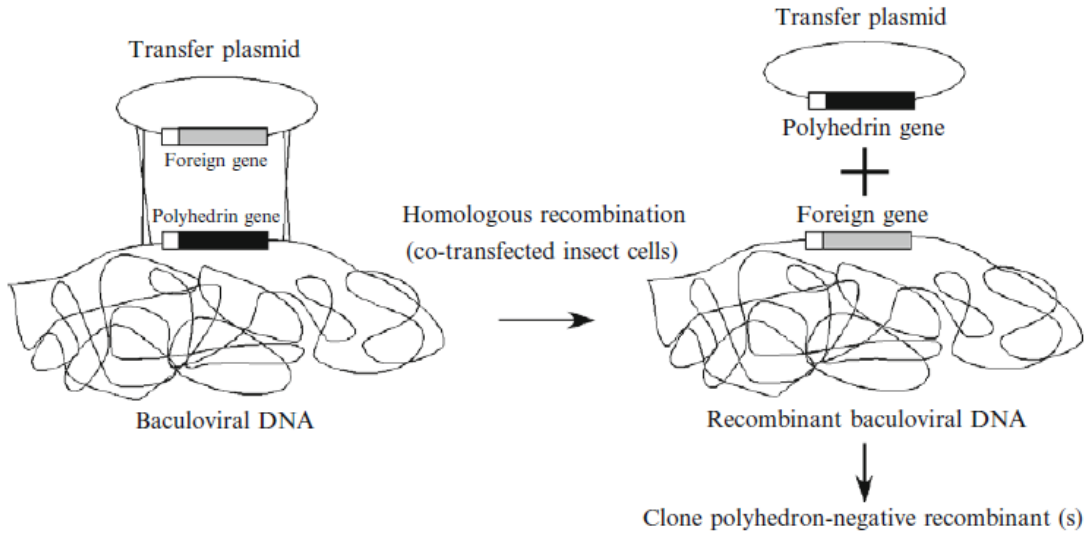


Figure 14.2 Producing a baculovirus expression vector by homologous recombination.

DOI: 10.1016/S0076-6879(09)63014-7

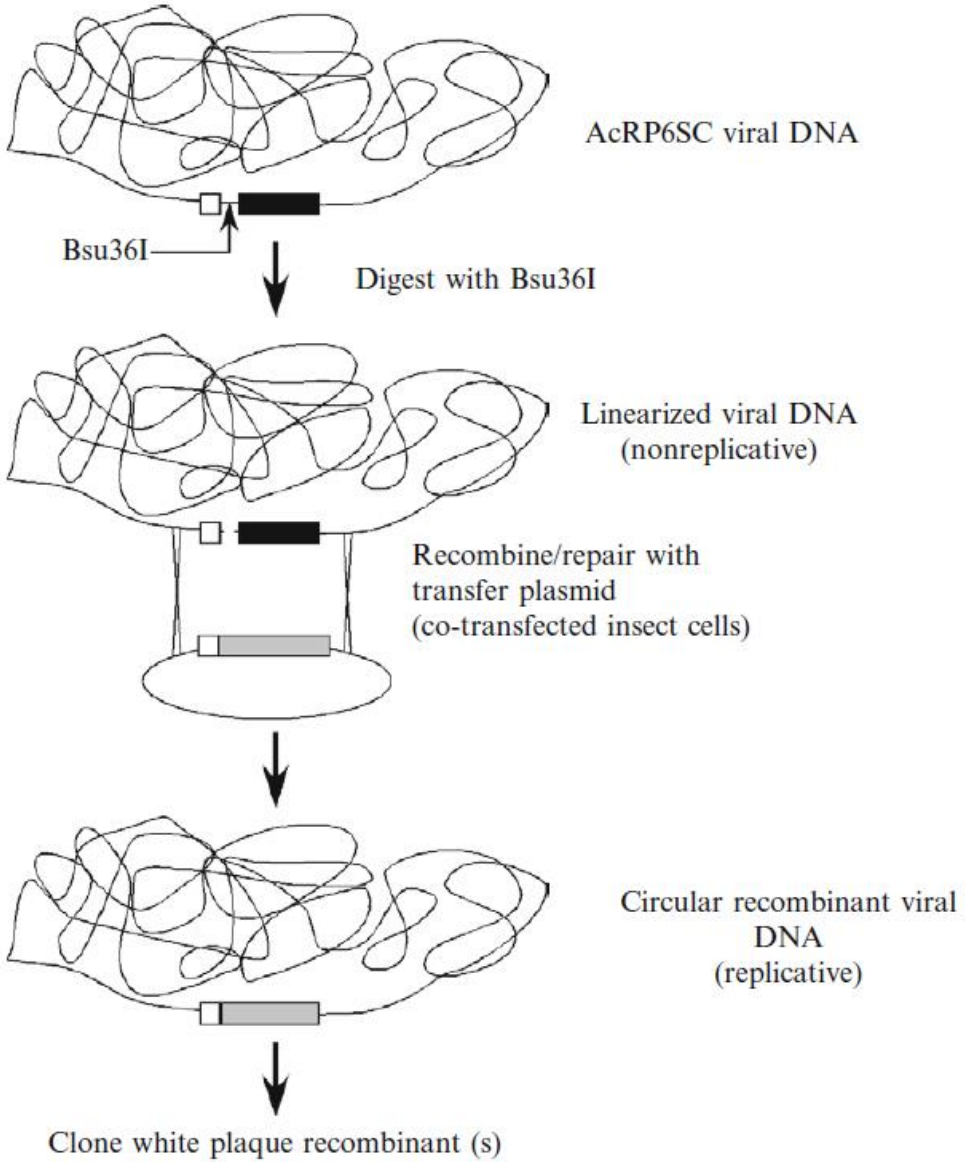


Figure 14.3 Producing a baculovirus expression vector by homologous recombination with a linearized parental viral genome.

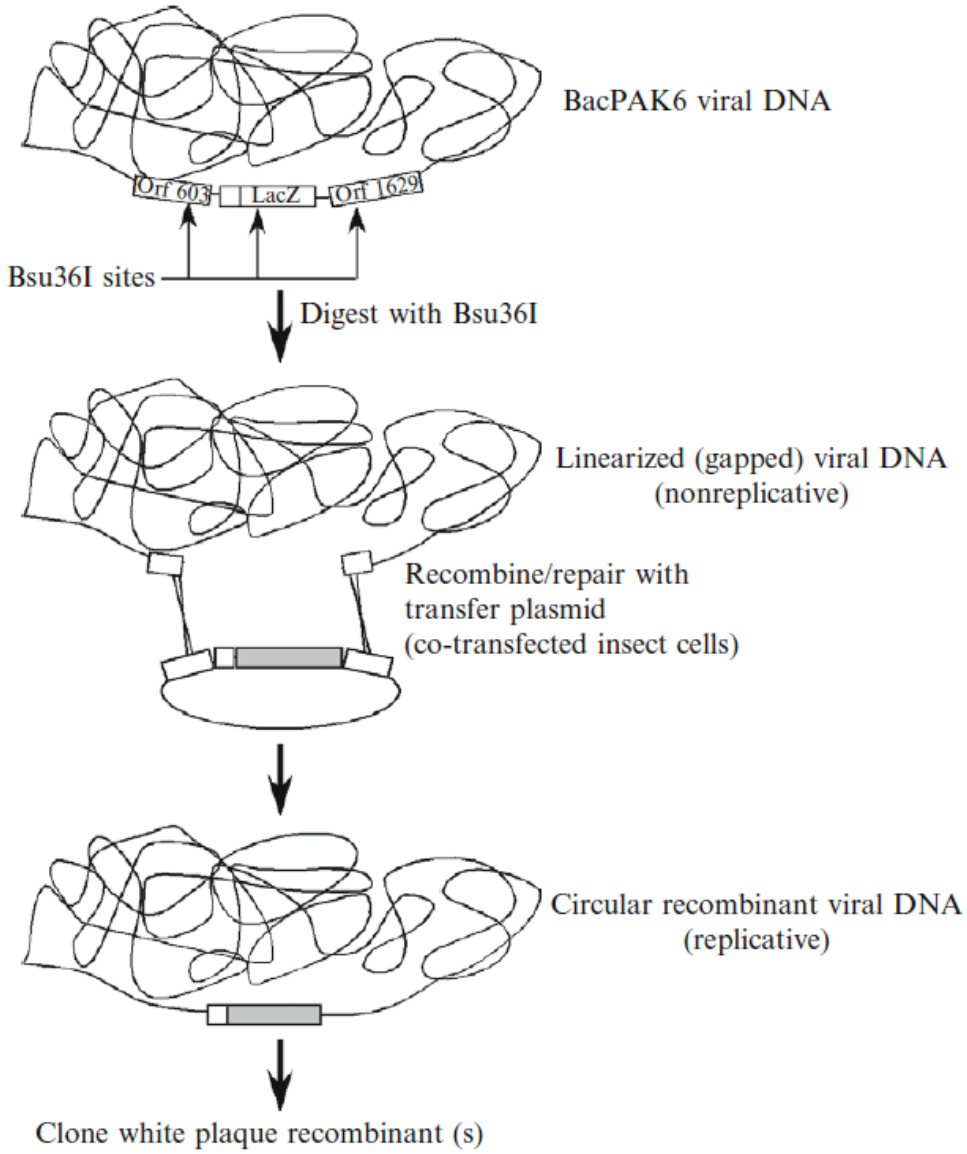


Figure 14.4 Producing a baculovirus expression vector by homologous recombination with a linearized/gapped parental viral genome.

DOI: 10.1016/S0076-6879(09)63014-7

Baculovirus expression vectors II.

- Use of **transposition** instead of homologous recombination
- Transposition takes place in *E. coli*, which carries a "helper" plasmid encoding the transposase
- Recombination success rate 100%

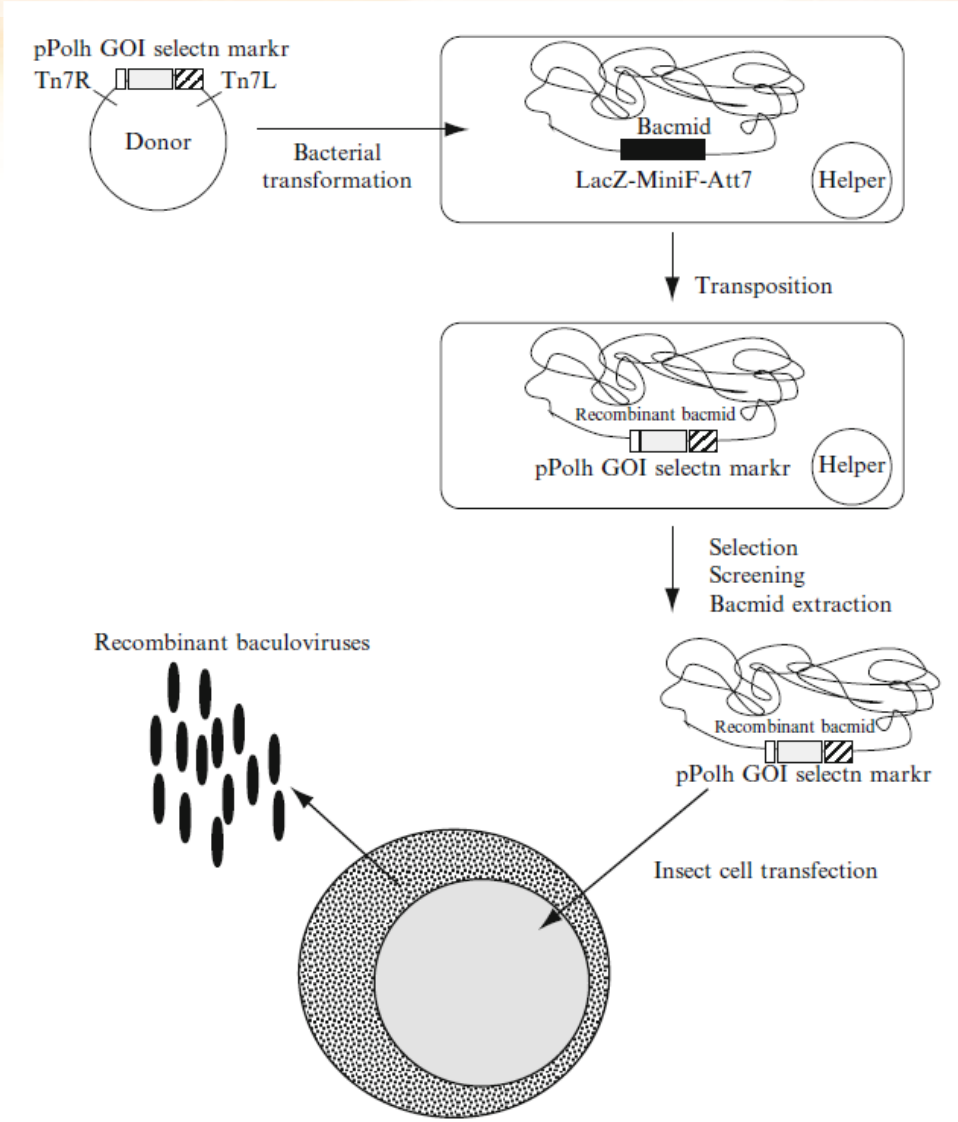
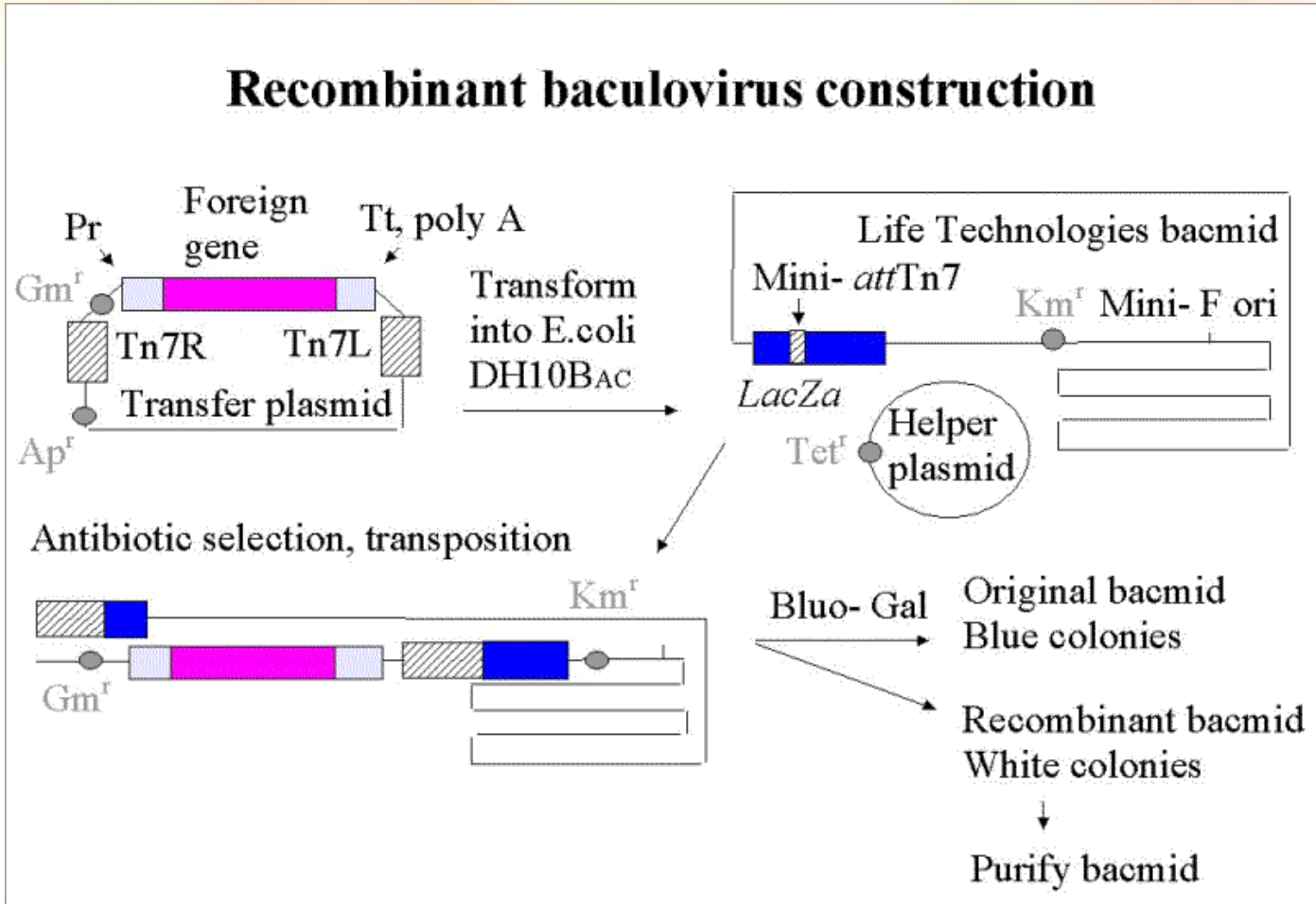


Figure 14.5 Producing a baculovirus expression vector by transposition between a bacmid and a transfer plasmid.

DOI: 10.1016/S0076-6879(09)63014-7

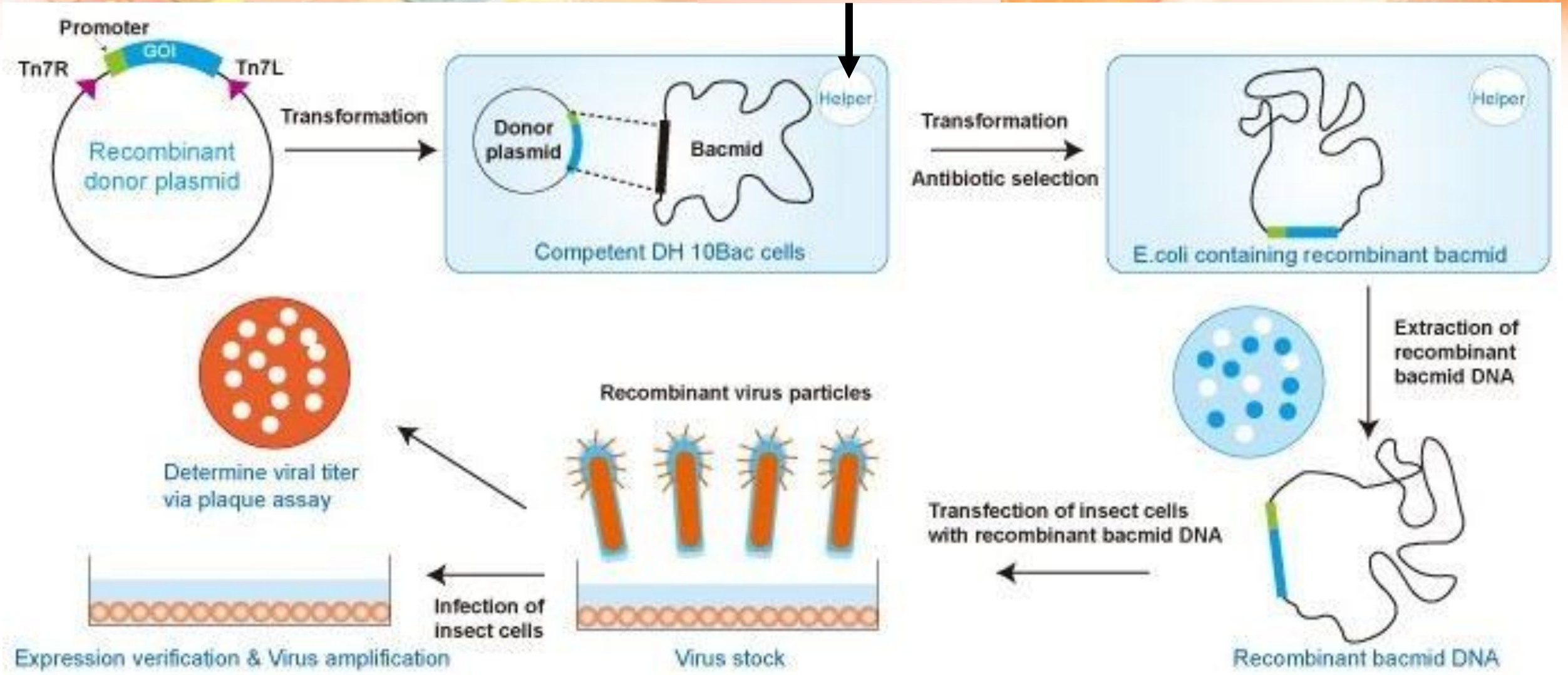
Bacmid

- **binary vector that can replicate in *Escherichia coli* and insect cells (contains complete baculovirus genome (no PH), low-copy origin of replication from F plasmid and att site for T7 transposon)**
- **carries resistance to kanamycin**
- **carries the *lacZ* gene, so colonies transformed by it grow blue in the presence of IPTG**
- **the recombinant gene is introduced into it by transposition from another vector, the transposition is directed to *lacZ*, the resulting colonies grow white**



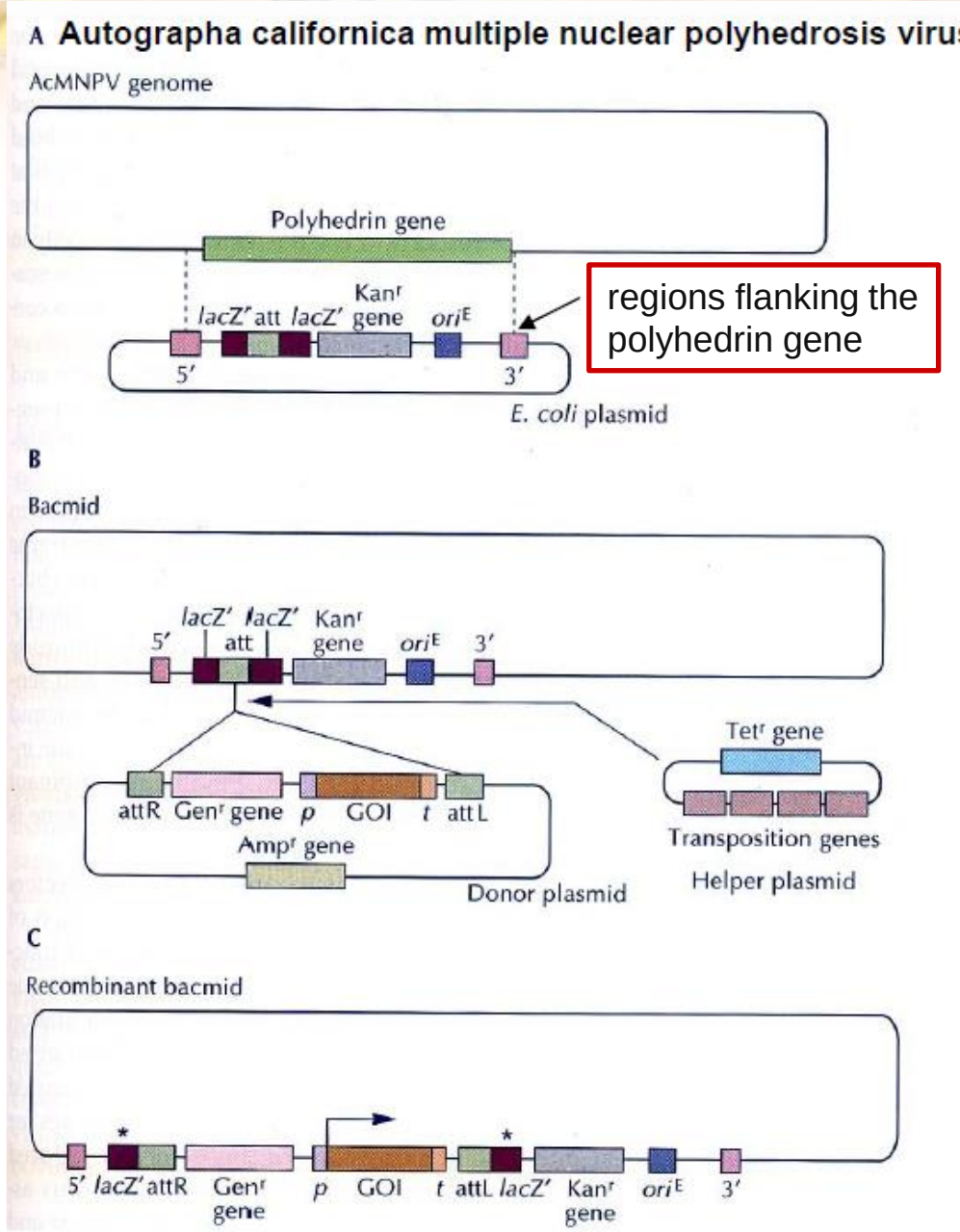
http://strubiol.icr.ac.uk/extra/baculovirus/introduction.html

transposase



https://www.creativebiomart.net/baculovirus-insect-cell-expression-systems.htm

Construction of recombinant Bacmid



regions flanking the polyhedrin gene

by incorporating the plasmid into the baculovirus genome (2x CO) a shuttle vector is created (*E. coli* + insect cells)

the transposition function of the helper plasmid will enable the transposition of the stretch of the donor plasmid containing the GOI, which is under the control of the baculovirus promoter and terminator (***p*** and ***t***)

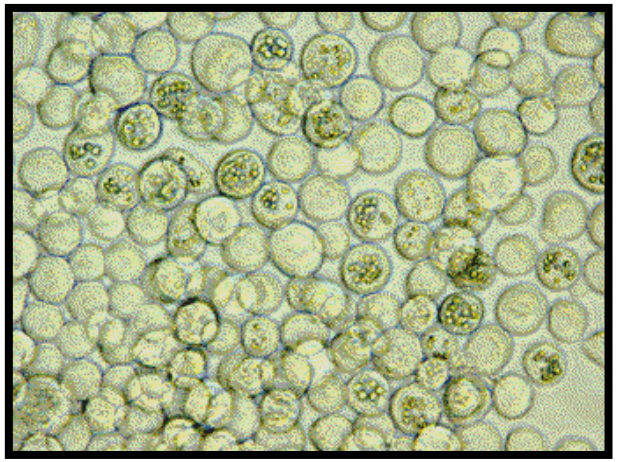
the recombinant bacmid has a disrupted lacZ' gene; *E. coli* cells containing recombinant bacmid are unable to form functional β-galactosidase (white colonies)

Basic features of the insect system I.

- suitable for the production of high levels of recombinant proteins (up to 1000 mg/mL)
- correctly post-translationally modified (folding, formation of S-S bridges, oligomerization, glycosylation, acylation, proteolytic cleavage)
- biologically active and functional recombinant proteins
- the recombinant gene is inserted into regions of the viral genome that are not required for virus replication

Basic features of the insect system II.

- recombinant baculovirus **loses one of the dispensable genes** (polh, v-cath, chiA ...), which is replaced by a recombinant gene
- the recombinant protein is **expressed in insect cell cultures** or insect larvae (not so common)



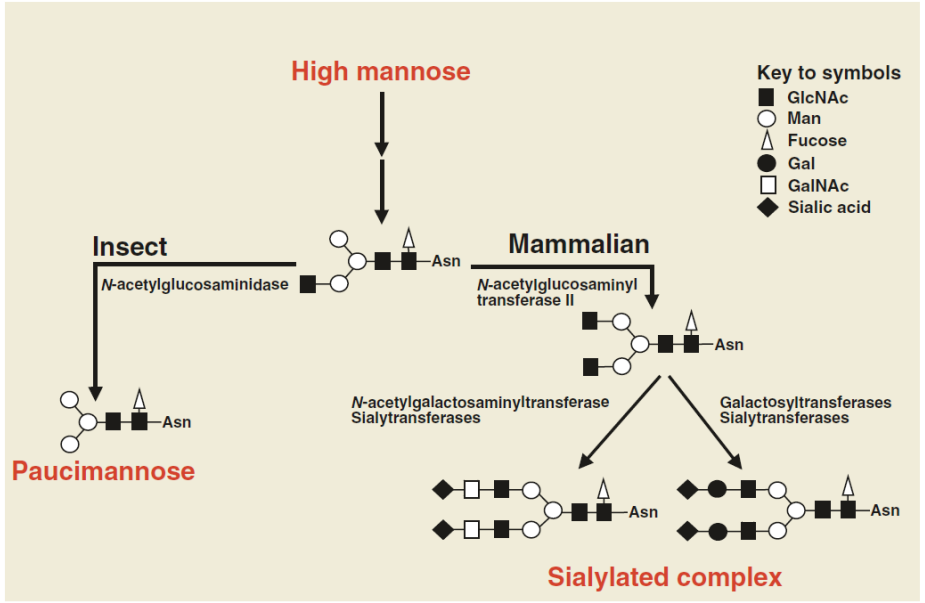
http://www.ent.iastate.edu/dept/faculty/bonningb/files/images/zebracaterpillar.jpg

Advantages of the insect express system

- **High levels of gene expression, especially for intracellular proteins**
- **Recombinant proteins are mostly soluble, post-translationally modified and easy to isolate, the content of parental proteins is low, expression takes place in the late stages of infection**
- **Insect cells grow well in suspension cultures - easy transfer to bioreactors**
- **Heterooligomeric proteins can be expressed using simultaneous infection with two or more viral vectors or a vector with multiple expression cassettes**
- **Baculoviruses have a limited spectrum of invertebrate hosts - safe technology**

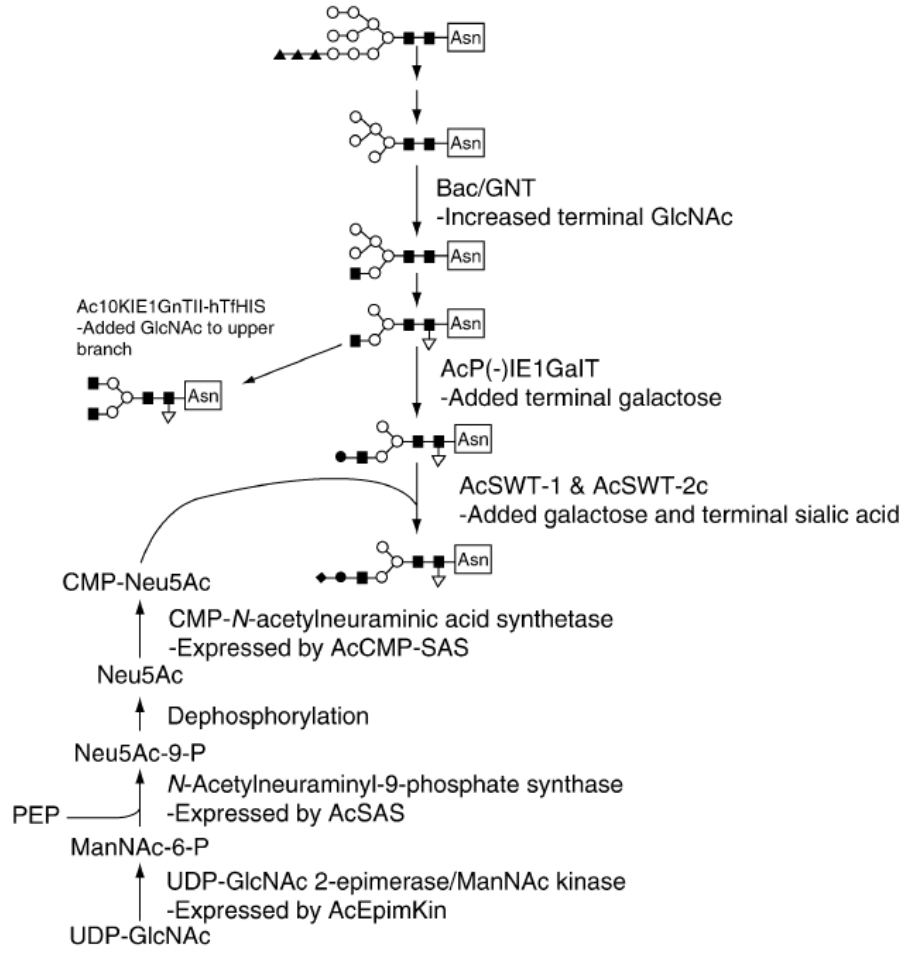
Glycosylation of proteins in insect cells

- Insect cells have different glycosylation compared to mammalian cells
  - **Reduced biological activity**
  - **Immunogenicity / allergic reaction**
- Use of insect cells transfected with mammalian glycosyltransferases



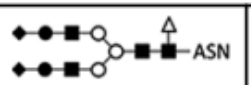
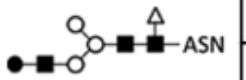
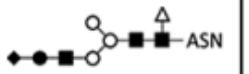
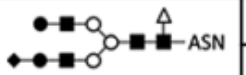
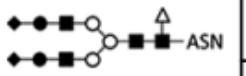
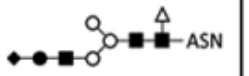
doi:10.1038/nbt1095

Figure 2 Overview of processing pathways and major *N*-glycans produced by insect and mammalian cell systems. The processing pathways in both systems yield a common intermediate. The major insect-cell end product (paucimannose) is produced by further trimming of this intermediate (left-hand branch), whereas the major mammalian-cell end products (including sialylated complex) are produced by elongation of this intermediate (right-hand branch).

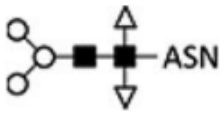


DOI: 10.1016/S0065-3527(06)68005-6

FIG 2. Structures of the *N*-glycans produced in insect cells infected with recombinant baculoviruses expressing mammalian glycosyltransferases. The pathway for synthesis of the sialylation donor substrate CMP-sialic acid (CMP-Neu5Ac) also is shown, and the names of the recombinant baculoviruses encoding mammalian enzymes that participate in this pathway are indicated. The standard monosaccharide symbols used in this figure are defined in the key shown in Fig. 1.

	Line #	Cell line	Glycotransferases					Sialic Acid Biosynthesis				Glycosylation Complexity	
			GalT	ST6Gal	ST3GalI	GlcNAc-TI	GlcNAc-TII	Synthase	Synthetase	Transporter	Epimerase	Key to Symbols: ● Galactose ■ GlcNAc △ α-1,6-core fucose ◆ Sialic Acid ○ Mannose ▽ α-1,3-core fucose	
Mammalian cell lines	1	Wild type											Biantennary sialylation
Sf9 cell line	2	Wild type											Paucimannose glycosylation[61]
	3	Wild type				B							Increased GlcNAe addition[77]
	4	Wild type	B										Improved galactosylation[115]
	5	Sfp4GalT[116]	I										First glycoengineered cell line
	6	Sfp4GalT/ST6[117]	I	I									Partial sialylation
	7	SfSWT-1[42]	I	I	I	I	I						First biantennary complexity
	8	SfSWT-3[118]	I	I	I	I	I	I	I				Improved sialylation
	9	SfSWT-6[119]	I	I	I		I	I	I	I			1% terminal sialylation
	10	SfSWT-21[120]	I	I			I	I	I	I	I		Terminal sialylation without Ac_4ManAc precursor
	11	SflElSWT[64]	I	I	I	I	I	I	I	I	I		13% terminal sialylation
	12	S09KSWT[64]	I	I	I	I	I	I	I	I	I		40% terminal sialylation
S2 cell line	13	S2/GalT-ST[121,122]	I	I									Partial sialylation

doi:10.1021/acs.iecr.8b0098

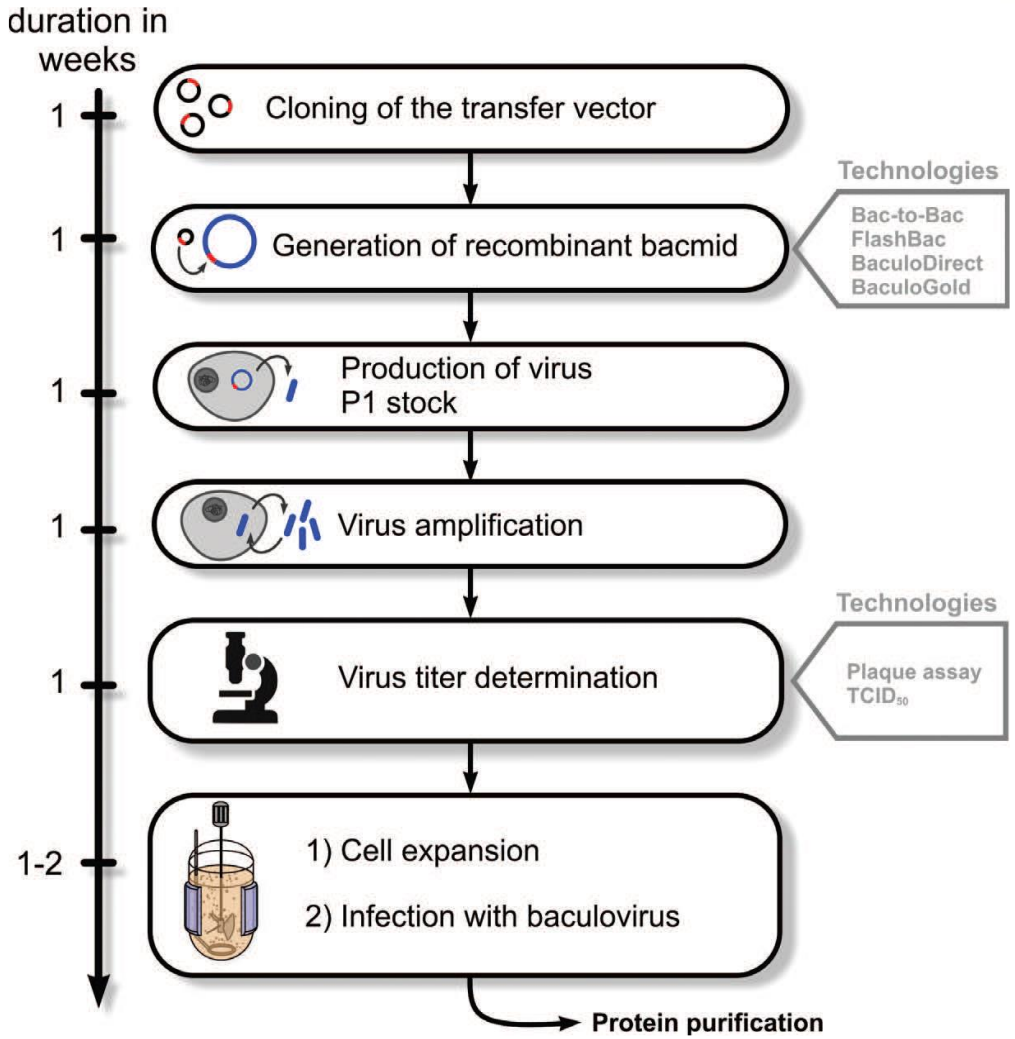
	Line #	Cell line	Glycotransferases					Sialic Acid Biosynthesis				Glycosylation Complexity	
			GalT	ST6Gal	ST3GalI	GlcNAc-TI	GlcNAc-TII	Synthase	Synthetase	Transporter	Epimerase	Key to Symbols: ● Galactose ■ GlcNAc △ α-1,6-core fucose ◆ Sialic Acid ○ Mannose ▽ α-1,3-core fucose	
High-Five	14	Wild type with α-1,3-core fucose											Allergenic glycan[76]
	15	Wild type	B				B						MultiBac single-infection[82]

[a] GalT, β-1;4-galactosyltransferase; ST6Gal, α−2,6-sialyltransferase; ST3Gal, α−2,3-sialyltransferase; GlcNAc-TI, N-acetylglucosaminyltransferase I; GlcNAc-TII, N-acetylglucosaminyltransferase II); ASN, Asparagine; B, expression from BEVS; I, expression from integrated genes in stable cell lines.

doi:10.1021/acs.iecr.8b0098

Transient expression in insect cells

- Mainly Sf9 cells are used



http://dx.doi.org/10.5772/67849

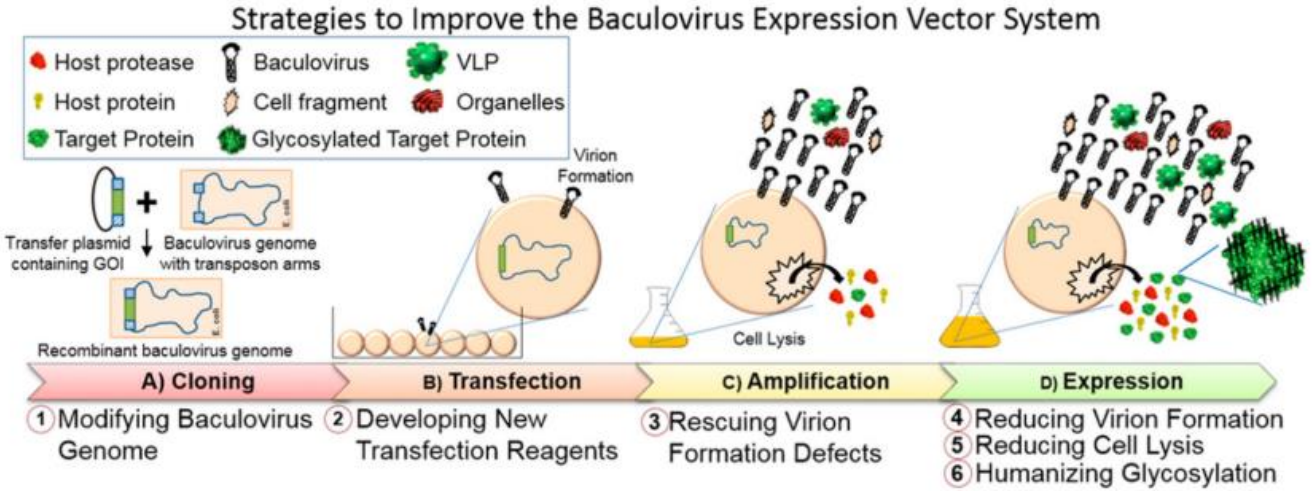


Figure 2.
Overview of the BEVS and strategies to address issues associated with product contamination and glycosylation. BEVS includes four major steps: (A) Cloning the gene of interest (GOI) into a transfer plasmid and transposing into the baculovirus genome in a specialized *E. coli* strain (DH10Bac). (B) Transfection of the purified recombinant baculovirus genome in insect cells to form recombinant baculovirus progeny (Note: direct homologous recombination in insect cells could also be used but is not shown for simplicity). (C) Amplification to generate high-titer viral stocks. (D) Expression of the target protein. The six strategies listed have been applied at one of the four steps.

doi:10.1021/acs.iecr.8b0098

Use of transient expression

Application	Product name	Company	Stage	References
For human use				
Cervical cancer	CERVARIX®	GSK	Approved	[27]
Prostate cancer	PROVENGE®	Dendreon	Approved	[28]
Influenza	FluBlok®	Protein Sciences	Approved	[29, 30]
Influenza	A/H5N1 Virus-like particle	Novavax	Phase I (NCT01596725)	[31]
For veterinary use				
Procrine circovirus 2 (PCV2)	Porcilis® PCV	Merck	Approved	[32]
PCV2	CircoFLEX®	Boehringer Ingelheim	Approved	[33]
Swine fever	Porcilis Pesti®	Merck	Approved	[34]

Table 1. Selected human and veterinary vaccines produced using BEVS.

http://dx.doi.org/10.5772/67849

BEVS-derived products licensed for commercial use

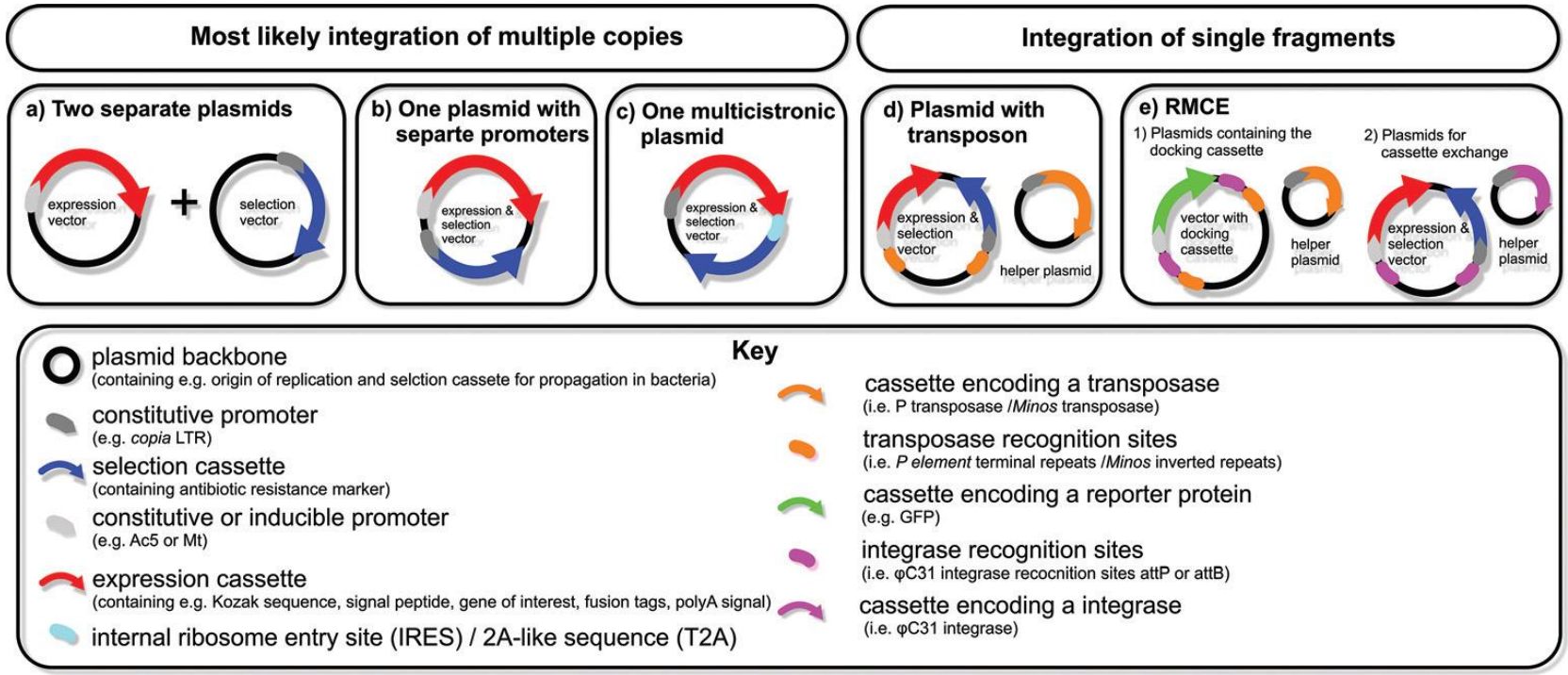
Product/Indication	Manufacturer	Product Type
Veterinary Vaccines (pigs)		
Classical swine fever		
– Porcilis® Pesti	MSD Animal Health	subunit
– BAYOVAC CSF E2®/Advasure[a)]	Bayer AG/Pfizer Animal Health	subunit
Porcine circovirus type 2		
– Circumvent® PCV	Merck Animal Health	VLP
– Ingelvac CircoFLEX®	Boehringer Ingelheim Vetmedica	VLP
– Porcilis® PCV	MSD Animal Health	VLP
Human Vaccines		
Human papillomavirus		
– Cervarix®	GlaxoSmithKline	VLP
Influenza		
– Flublok®	Protein Sciences Corporation	subunit
Human Therapeutics		
Prostate cancer		
– Provenge®	Dendreon	immunotherapy
Lipoprotein lipase deficiency		
– Glybera®	uniQure	rAAV-based gene therapy

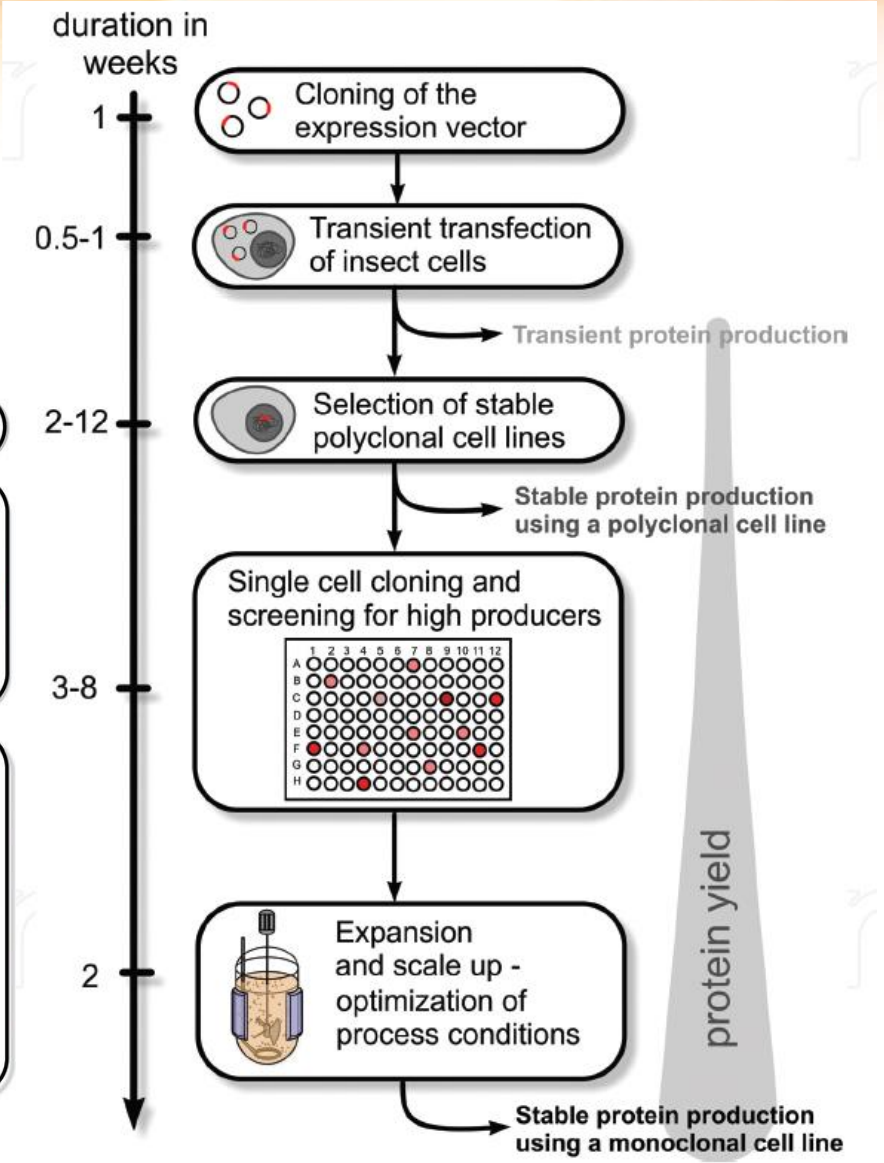
doi: 10.1002/biot.201400438

[a)] Discontinued

Stable expression in insect cells

- Mainly S2 cells → recombinant rS2 cells
- The gene is integrated into the nuclear DNA
- Stable expression, high batch homogeneity
- The possibility of using perfusion cultivation systems





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Use of stable expression

Proteins in clinical development		
Recombinant placental malaria vaccine	Phase I	[76, 89, 90]
West Nile virus vaccine	Phase I	[76, 91]
HER-2 protein AutoVac™ (breast cancer)	Phase II	[76]
Proteins for research and process development		
HIV-1 VLP and soluble HIV gp120	VLP	[92]
Arabidopsis thaliana sterol glycosyltransferase	Enzyme	[93]
Psalmotoxin 1	Small peptide toxin	[83]
M2 muscarinic and glucagon receptor	G-protein-coupled receptor	[94]
Atlantic salmon serum C-type lectin	Lectin	[95]
Monoclonal antibody against H5N1 influenza hemagglutinin	Antibody	[81]
Enhanced green fluorescent protein (eGFP)	Fluorescent marker protein	[96]

http://dx.doi.org/10.5772/67849

Vectors for transfer into mammalian cells

1. **Plasmid vectors** - the plasmid does not replicate, it is rarely incorporated into the genome of the cell
   - prokaryotic plasmid + eukaryotic transcription unit + selection marker
   - they are used to select transfected cells during co-transfection and to monitor transient gene expression
2. **Viral vectors** - shuttle vectors, replicating in host cells
   - part of the bacterial vector + sequence of eukaryotic viruses + selection marker
   - vectors derived from SV40, bovine papillomavirus, EBV, retroviruses, baculoviruses, vaccinia virus, adenoviruses, etc.
   - they are used to monitor stable or transient gene expression and to obtain recombinant proteins in large quantities

General structure of a mammalian expression vector

- Promoter
- Enhancer of transcription
- polyA sequence
- Selection marker
- Replication origin

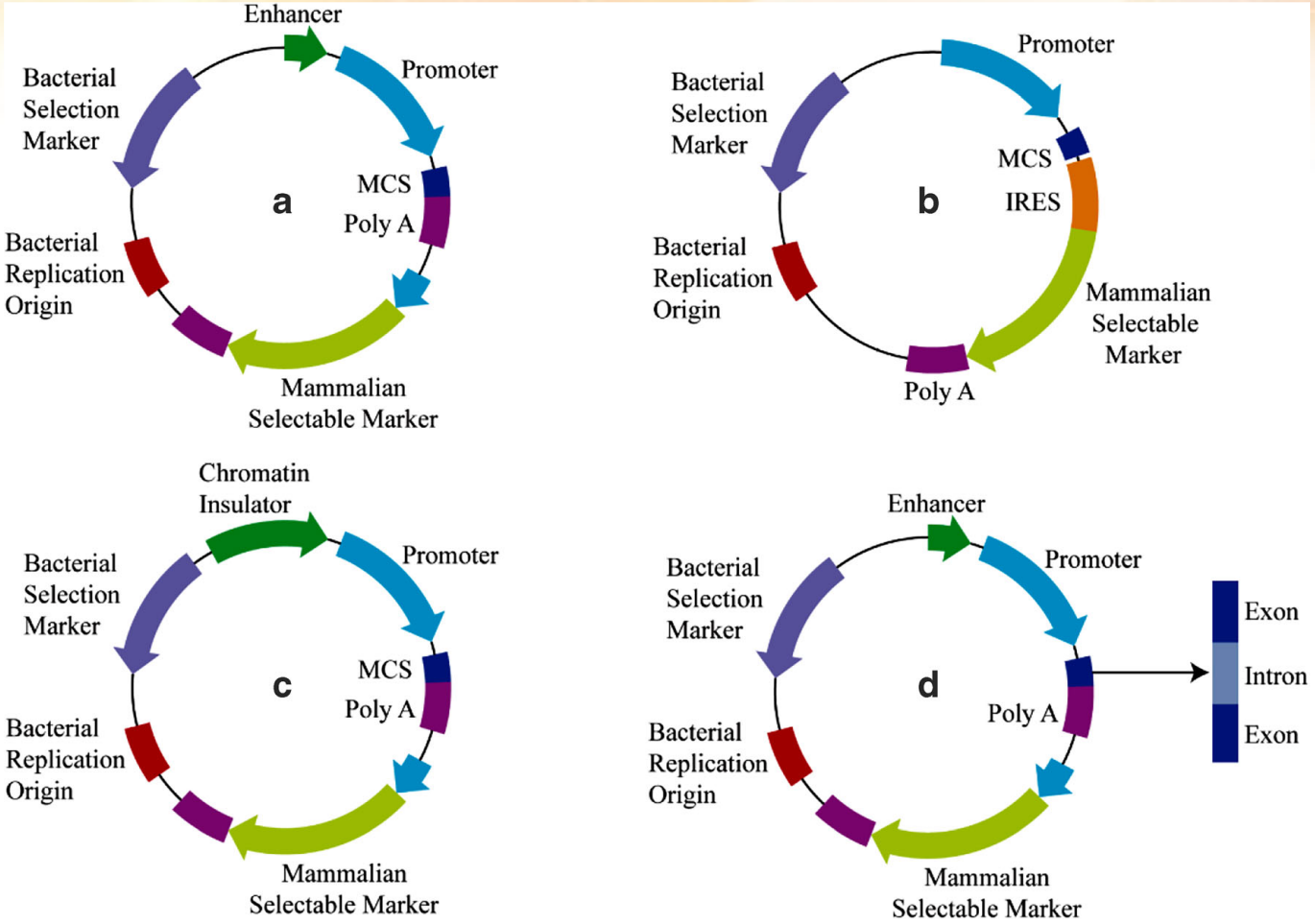


Fig. 1 Schematic of mammalian expression vector components (revised from). **a** Expression vector which contains promoter (with or without an enhancer element) and a 3′ polyadenylation sequence, and GOI sequences are inserted into a multiple cloning site (MCS). The mammalian and bacterial selectable marker is regulated by a separate set of regulatory elements. **b** Bistronic vectors contain a single cassette for expression of a GOI and the MCS and a mammalian selectable marker, separated by the IRES and under control of an upstream promoter and 3′ polyA. **c** Expression vectors containing DNA opening elements (e.g., MARs or UCOEs), the element is typically placed upstream—and possibly also downstream—of the polyA. **d** Expression vectors containing one or more introns are frequently inserted into the coding sequence for a gene of interest

https://doi.org/10.1007/s00253-020-10640-w

Mammalian vector promoters

- The most common promoters for expression in mammalian cells:
  - human cytomegalovirus enhancer/promoter (hCMV)
  - Simian virus 40 early promoter (SV40E)
  - CMV enhancer/chicken β-actin promoter (CAG)
  - human elongation factor-1α (hEF-1α)
  - Elongation factor-1α of Chinese hamster (CHEF-1α)
- Artificially created SCP1 (super core promoter 1) promoter → 3x faster than CMV promoter

Elements		Size (bps)	Source	Vector example
Promoter	hCMV	589	*Homo sapiens* cytomegalvirus	pRc, pCI, pAdCMV5, pcDNA3.1, pBudCE4.1
	mCMV	522	Murinecytomegalovirus	GS vector
	SV40	351	Simian virus	pGL2, PSF-SV40
	RSV	229	Rous sarcoma virus	pRSV, pRC-RSV
	PGK	555	Mouse phosphoglycerate kinase 1	pDRIVE5-SEAP-mPGK
	hEF1α	1335	*Homo sapiens* elongation factor 1α	pDRIVE5-GFP-1
	CHEF1α	1660	CHO elongation factor 1a gene	pSF-CHEF1-Fluc
	CAG	1662	CMV enhancer/ß-actin promoter	pCAGG

https://doi.org/10.1007/s00253-020-10640-w

Viral constitutive promoters

- High level of GOI expression
- Viral promoters are often only active during a specific phase of the cell cycle (e.g. CMV is active during S phase)
- High production of the target protein inhibits cell growth and can also cause apoptosis
- Viral promoters are susceptible to epigenetic silencing

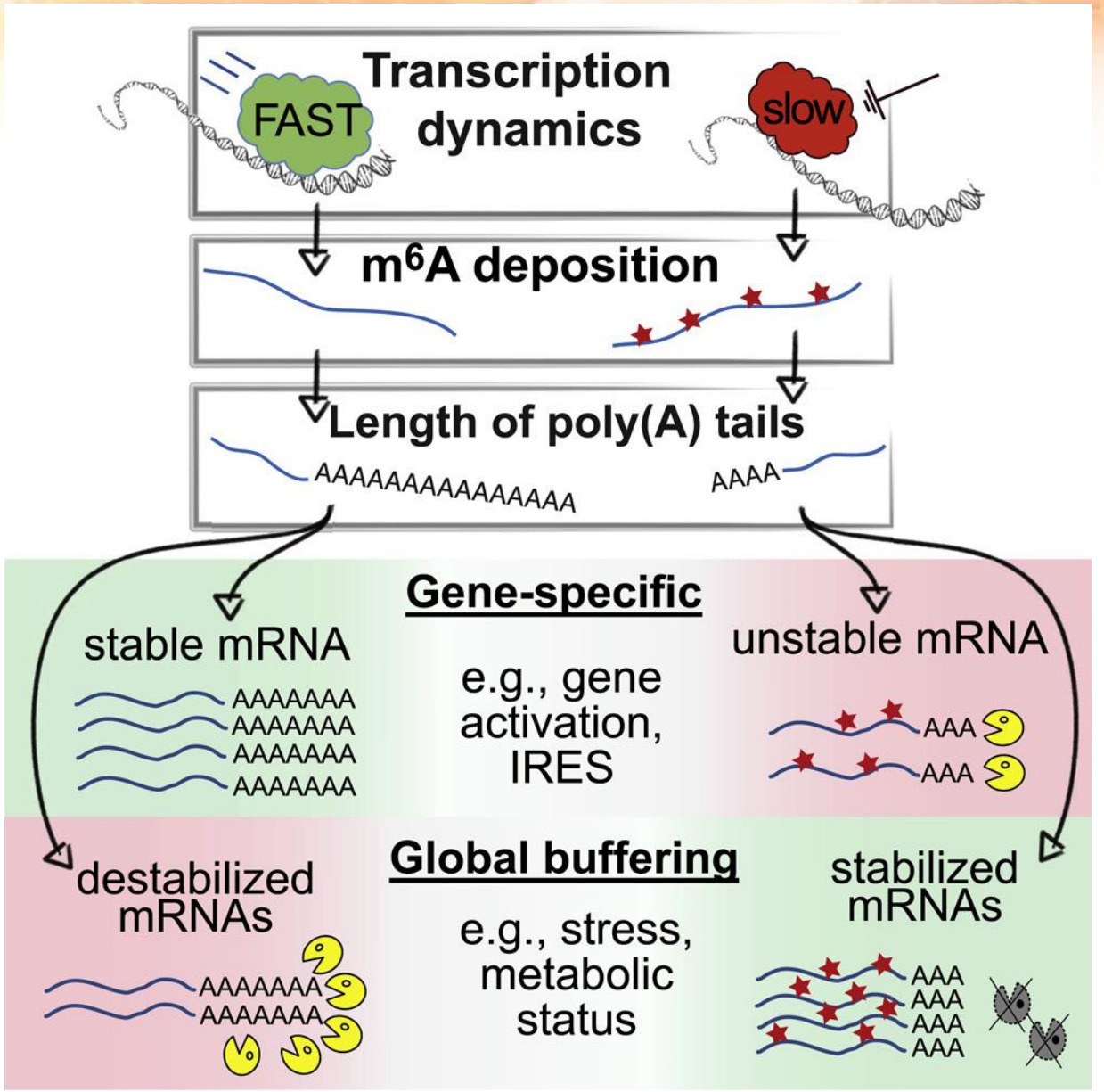
Inducible promoters

- They are not cell cycle dependent
- Induction of protein expression at the peak of the exponential phase of growth will ensure maximum yields

- IPTG-inducible *lac* operon
- Tetracycline resistance repressor operons Tn10 (tetracycline resistance repressor operons) – Tet-Off/Tet-On

Other regulatory sites

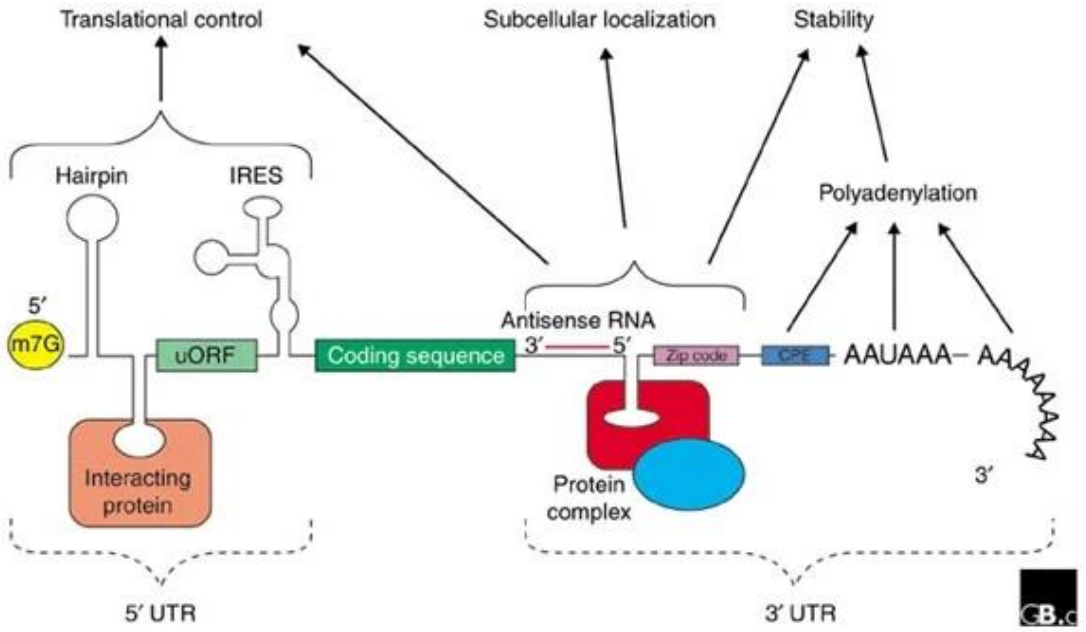
- **PolyA sequence**
  - in 3‘-UTR of terminator
  - polyA sequence from the SV40 virus – increased resistance to nucleases



https://doi.org/10.1016/j.molcel.2020.03.022

Other regulatory sites

- **Introns**
  - inserting an intron or untranslated exon before the start codon increases the efficiency of mRNA transport from the nucleus to the cytoplasm and prolongs its half-life



The generic structure of a eukaryotic mRNA, illustrating some post-transcriptional regulatory elements that affect gene expression. Abbreviations (from 5' to 3'): UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal.

https://genomebiology.biomedcentral.com/articles/10.1186/gb-2002-3-3-reviews0004

Other regulatory sites

- **IRES**
  - internal ribosome entry site
  - it is inserted between the reading frames of bi/poly-cistronic mRNAs
  - ensures the expression of multiple genes from a single promoter
  - translation of the gene down-stream the IRES is lower than up-stream the IRES

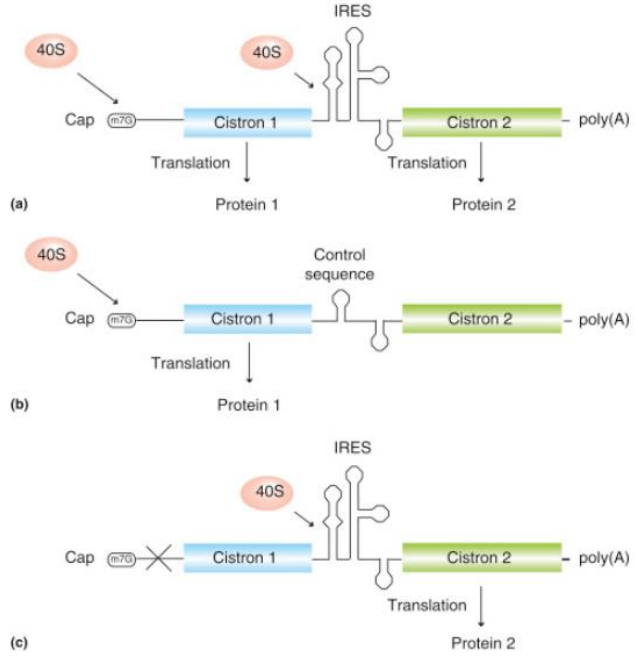


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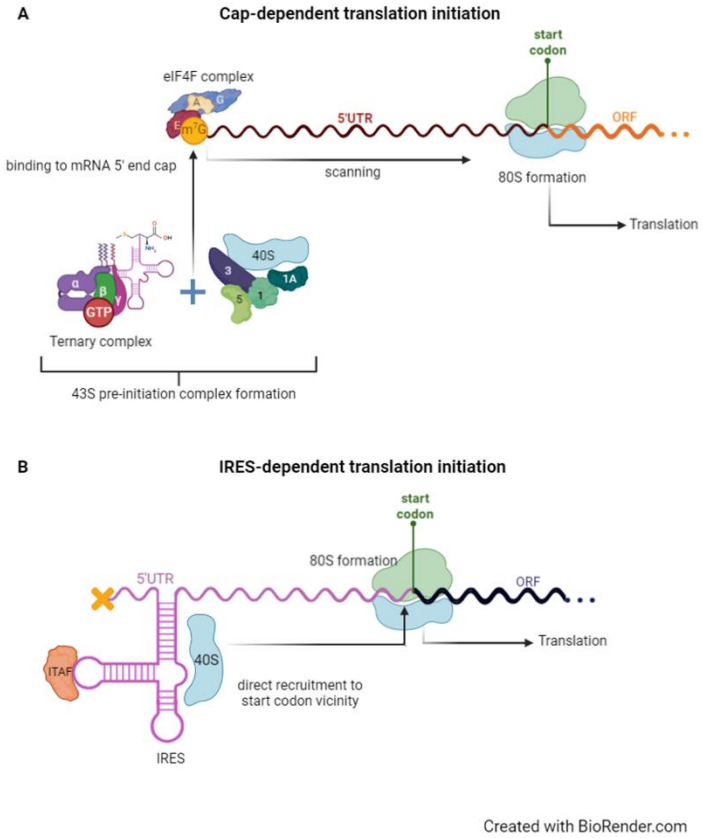
Figure 1. Schematic representation of the bicistronic construct assay. (a and b) In mRNAs transcribed from bicistronic constructs, the first cistron is translated by a cap-dependent scanning mechanism while translation of the second cistron depends on the presence of IRES in the intercistronic spacer. (c) Ribosome recruitment by IRES is independent of the first cistron expression. A 5′-UTR sequence is qualified as IRES if it significantly increases the Cistron-2/Cistron-1 expression ratio after insertion in the construct. For simplicity, the diagram does not show the 60S ribosomal subunits, eIFs and ITAFs, which participate in these processes.

https://doi.org/10.1016/B978-0-12-394447-4.30042-6



https://doi.org/10.3390/biomedicines10081865

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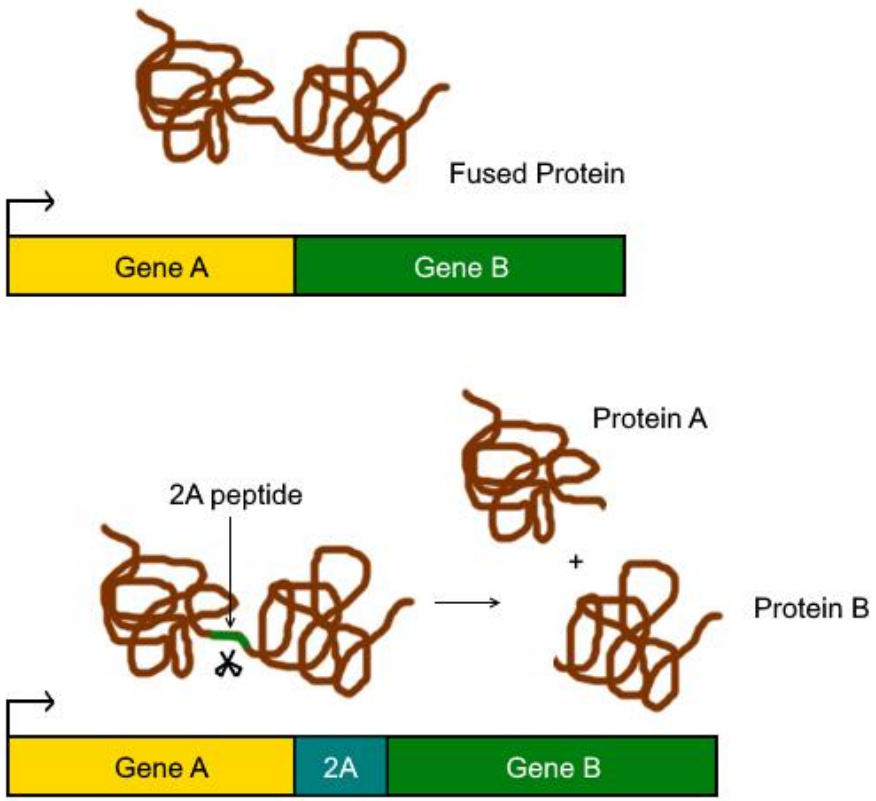
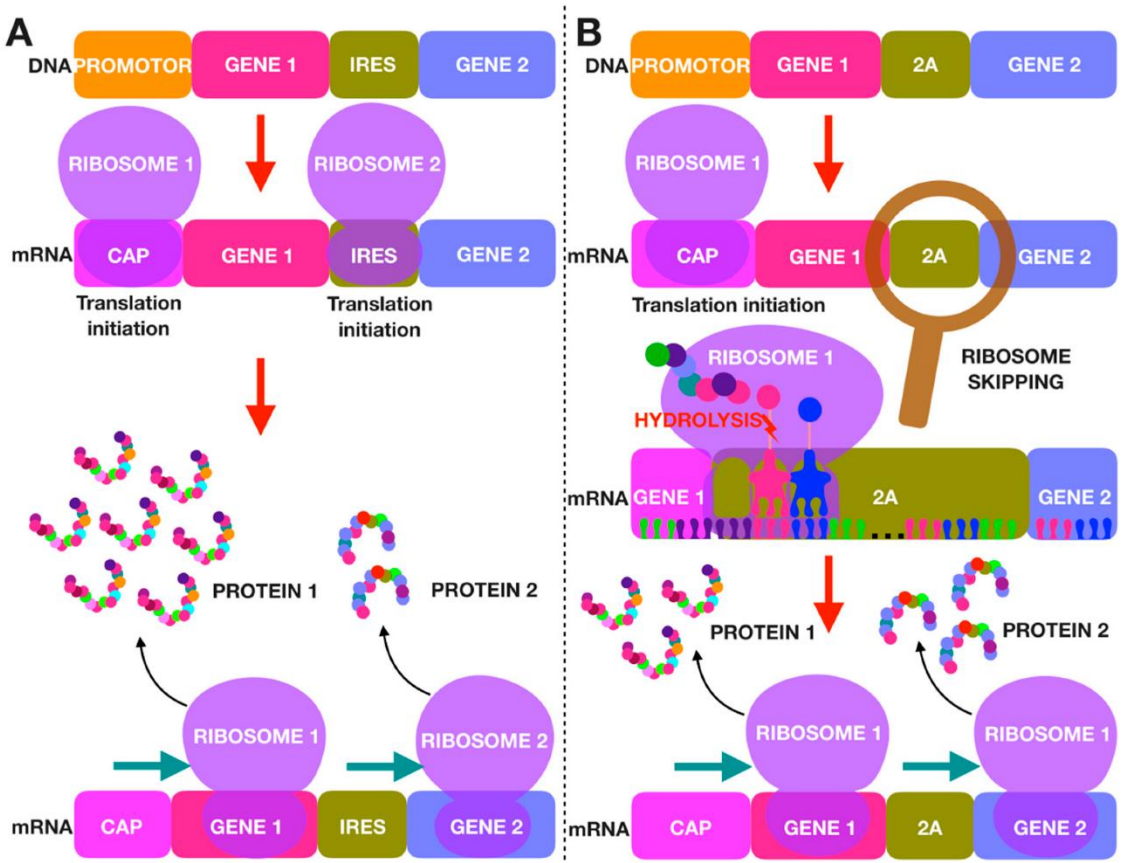
Other regulatory sites

- **Furin-2A sequence**
  - small self-cleaving peptides
  - they are inserted between the two ORFs instead of the IRES
  - will ensure equal expression of both genes



https://commons.wikimedia.org/wiki/File:2A_peptide_Working_Mechanism.jpg



https://doi.org/10.3390/pharmaceutics11110580

Selective markers

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

Table 1 Common used elements of expression vector in mammalian cells

Elements		Size (bps)	Source	Vector example	Reference(s)
Promoter	hCMV	589	*Homo sapiens* cytomegalvirus	pRc, pCI, pAdCMV5, pcDNA3.1, pBudCE4.1	Ho and Yang (2014)
	mCMV	522	Murinecytomegalovirus	GS vector	Xia et al. (2006)
	SV40	351	Simian virus	pGL2, PSF-SV40	Wang et al. (2016)
	RSV	229	Rous sarcoma virus	pRSV, pRC-RSV	Wang et al. (2016)
	PGK	555	Mouse phosphoglycerate kinase 1	pDRIVE5-SEAP-mPGK	Wang et al. (2016)
	hEF1α	1335	*Homo sapiens* elongation factor 1α	pDRIVE5-GFP-1	Wang et al. (2017); Veith et al. (2016)
	CHEF1α	1660	CHO elongation factor 1a gene	pSF-CHEF1-Fluc	Wang et al. (2016)
	CAG	1662	CMV enhancer/ß-actin promoter	pCAGG	Wang et al. (2016)
PolyA	HGH	624	*Homo sapiens* growth hormone	pCMV5	Ostedgaard et al. (2005)
	SV40 late	222	Simian virus 40	pCHO1.0, pYL1	Liu et al. (2017)
	Synthetic polyA (SPA)	49	Rabbit β-globin	pAAV-CW3SA-EGFP	Choi et al. (2014)
	bGH	250	Bovine growth hormone	pcDNA3.1, pTS1012	Berman et al. (2018); Naddafi et al. (2018)
	Mutation BGH	164	Bovine growth hormone mutation	pVAX1GFP-BGH-M1	Azzoni et al. (2007)
	HSV TK	19	Herpes simplex virus	pTLD39	Leidy-Davis et al. (2018)
Intron	hCMV intron	814	*Homo sapiens* cytomegalovirus major immediate-early protein gene	pGA1	Chapman et al. (1991); Ross et al. (2000)
	Intron hEF1 promoter	942	*Homo sapiens* elongation factor EF-1-alpha gene	pTT5, pCK25	Kemmer et al. (2010)
	Chimeric intron	133	Synthetic intron	pPAL	Alcolea et al. (2018)
	Modified SV40 intron	99	Synthetic intron	pCMH148, AF286077	Wang et al. (1995)
	β-Globin intron	572	Rabbit beta-globin intron II	pSG5, pMVAX1(c)	Du et al. (2014)
IRES	EMCV WT	505	Encephalomyocarditis virus	Tricistronic vectors	Ho et al. (2012)
	EMCV ATT	505	Mutated EMCV IRES with attenuated translation effificiency	Tricistronic vectors	Ho et al. (2012)
	FMDV	458	Foot and mouth disease virus	CHEF-F2A	Ebadat et al. (2017)
	HCV	368	Hepatitis C virus		Lafuente et al. (2002)
	HRV	622	*Homo sapiens* rhino virus		Stoneley et al. (1998)
	PV	635	Poliovirus		Johansen et al. (2000)
	AQP-4	292	Aquaporin 4		Baird et al. (2007)
	Apaf-1	581	Apoptotic protease-activating factor1		Holcik et al. (2003)
	BIP	222	Immunoglobulin heavy chain binding protein		Nevins et al. (2003)

Table 1 (continued)

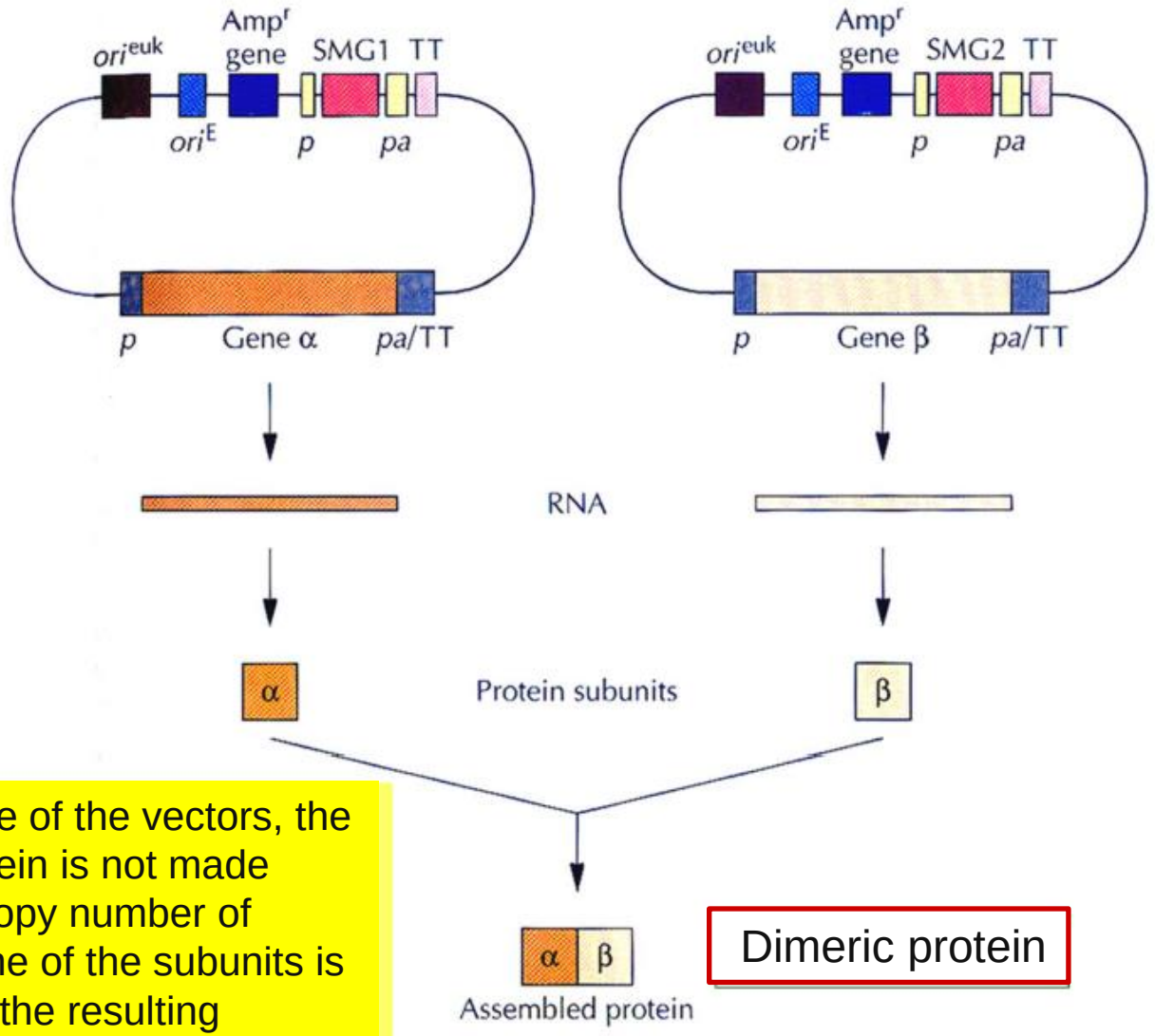
Elements		Size (bps)	Source	Vector example	Reference(s)
	CAT-1	274	Cationic amino acid (arginine/lysine) transporter 1		Fernandez et al. (2001)
	c-myc	352	c-myc proto-oncogene		Fernandez et al. (2001)
	NRF	653	NF-kappaB repressing factor		Oumard et al. (2000)
	Rbm3	721	Cold stress–induced mRNA		Chappell et al. (2001)
	VCIP	571	Vascular endothelial growth factor (VEGF) and type 1 collagen inducible protein		Blais et al. (2006)
Furin-2A	FMDV 2A(P2A)	22	Porcine teschovirus-1 2A	pLeo695	Scheller et al. (2018)
	T2A	21	Thosea asigna virus 2A	pCS4+	Kim et al. (2011)
	E2A	23	Equine rhinitis A virus	pCS4+	Kim et al. (2011)
	F2A	25	Foot-and-mouth disease virus	pCS4+	Kim et al. (2011)
Selection marker	DHFR	564	CHO cell	pCHO1.0	Invitrogen
	GS	1119	CHO cell	pEE12.4	Lonza biologics
	NeoR(G418)	804	Neomycin resistance gene from *E. coli*	pLeo695	Scheller et al. (2018)
	BSR	399	Blasticidin resistance gene from *Bacillus cereus*	pSG601	Goldfless et al. (2014)
	HygR	1026	Aminoglycoside phosphotransferase from *E. coli*	pNDC_SUB4	GenBank: LN866853.1
	Zeocin	375	*Streptomyces* CL990	pBudCE4.1	Thermo Fisher Scientific

Formation of multimeric proteins

- Preparation options :
  1. cloning the genes for the subunits separately and then joining them *in vitro* – low efficiency
  2. gene cloning in two vectors in one cell - protein assembly *in cellulo* - high efficiency
- Possible complications:
  - Loss of one of the vectors, the active protein is not made
  - Different copy number of vectors, one of the subunits is in excess, the resulting product is not enough

- Solution: **Cloning genes in a bicistronic expression vector**

Two-vector expression system in a single cell

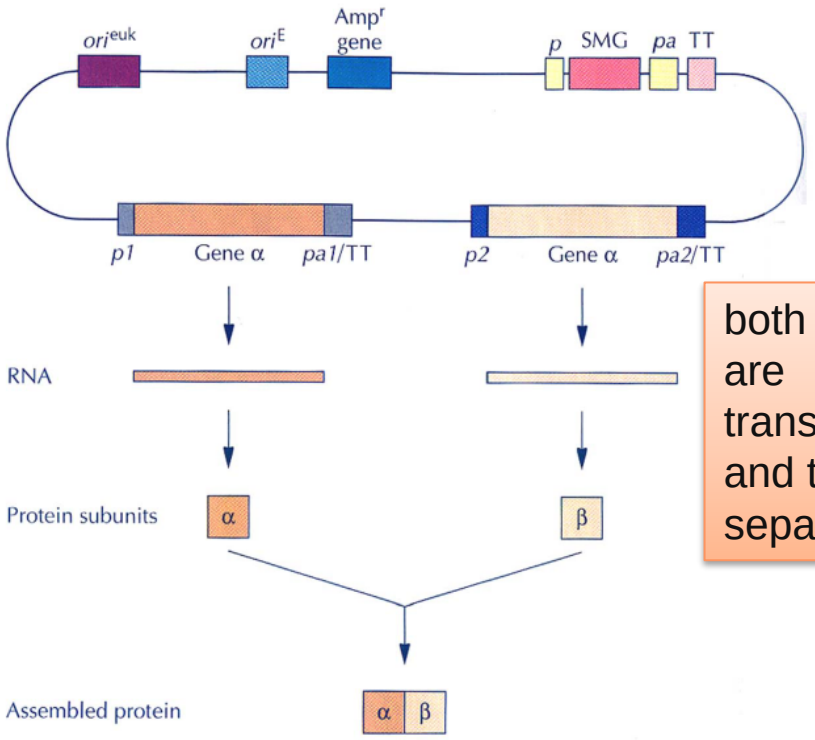


- Loss of one of the vectors, the active protein is not made
- Different copy number of vectors, one of the subunits is in excess, the resulting product is not enough

Dimeric protein

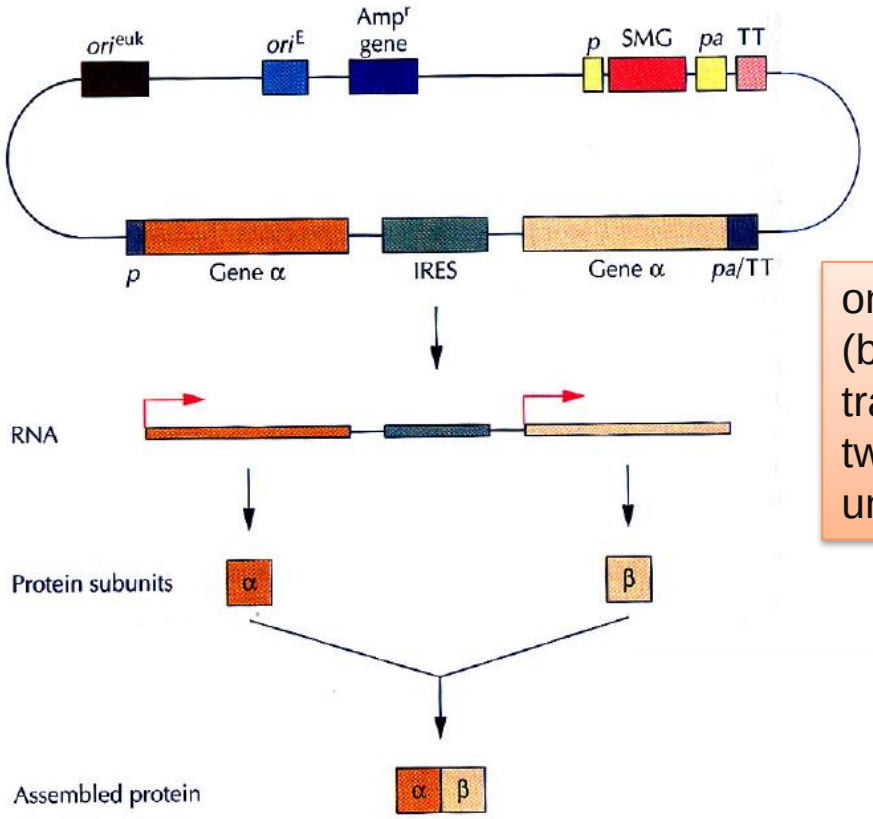
Two genes on one vector

Expression vector with two cloned genes encoding heterodimer subunits



both genes are transcribed and translated separately

Bicistronic expression vector for cloning genes encoding heterodimer subunits



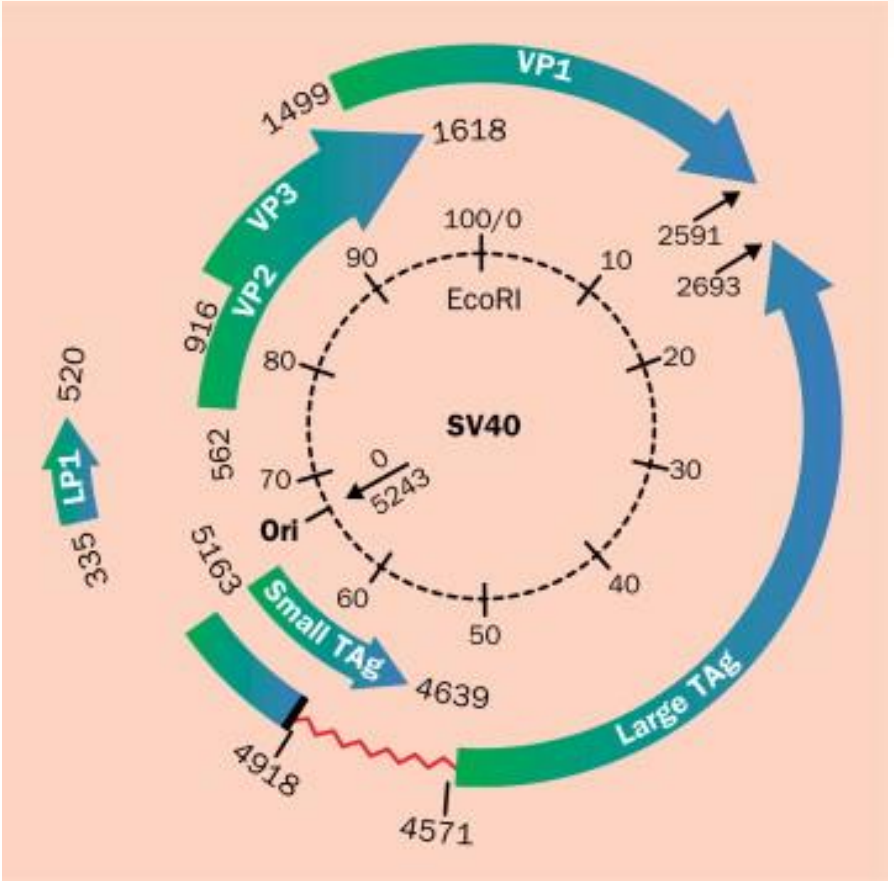
one transcript (bicistronic) translated into two protein units

Retroviral vectors

- They infect a wide range of animal species and various types of human cells
- Infection leads to the integration of the viral genome into the genome of the host cell - the place of integration is arbitrary (preferably in transcriptionally active chromatin)
- Retrovirus infection does not result in cell death, it often leads to constant production of new virions
- A disadvantage is the ability of retroviral vectors to activate the transcription of genes adjacent to their insertion sites

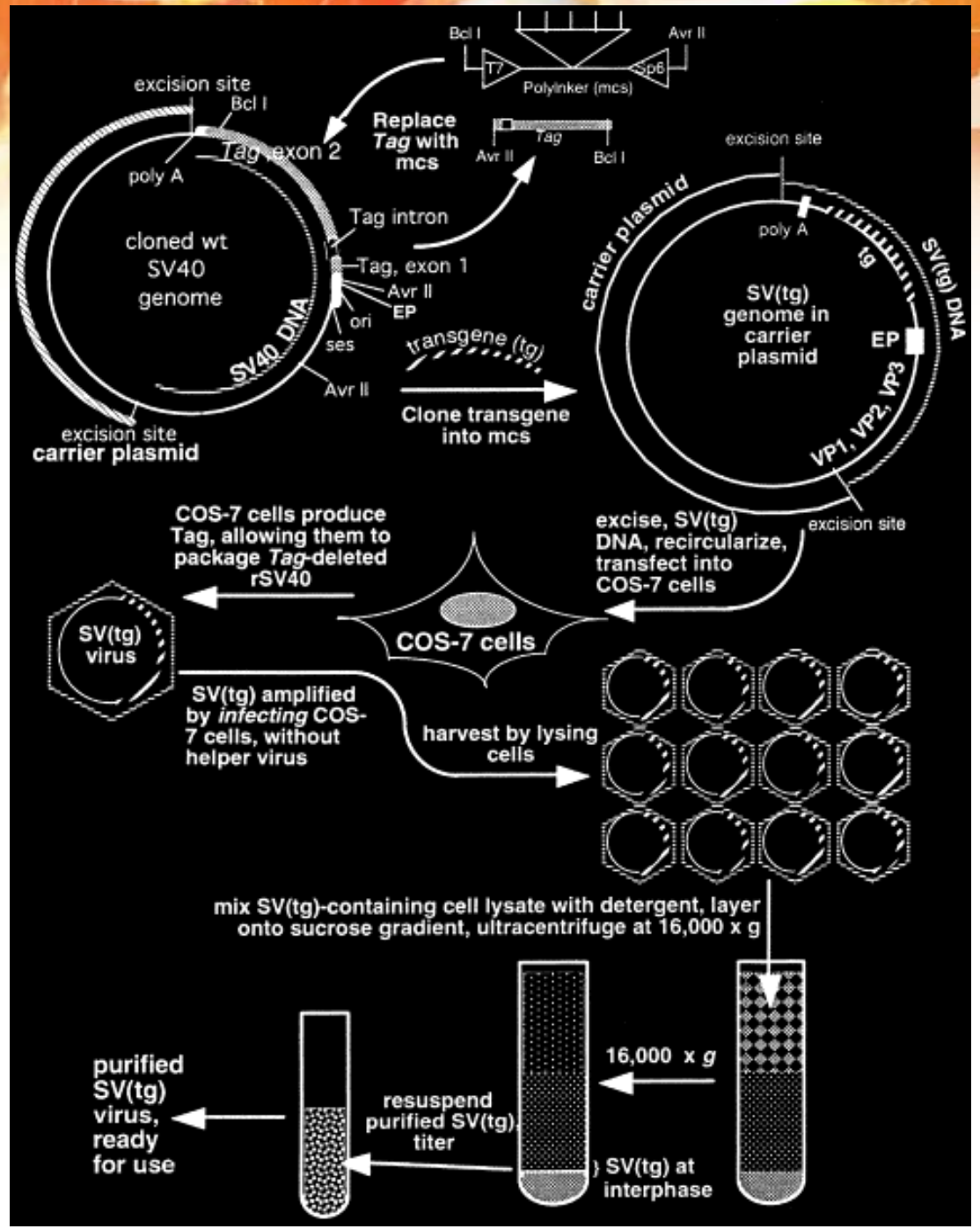
Vectors derived from SV40 virus

- Simian virus 40 – polyomavirus attacking monkeys and humans
- genome = 5.2 kb circular dsDNA
- life cycle:
  - in permissive cells (monkeys) – lytic cycle
  - in non-permissive cells (mouse, hamster) – integration into the genome, cell transformation
- Construction of vectors:
  - replacement of the early or late region with foreign genes, complementation of missing functions with a helper virus or helper cells (**COS**)



COS cells

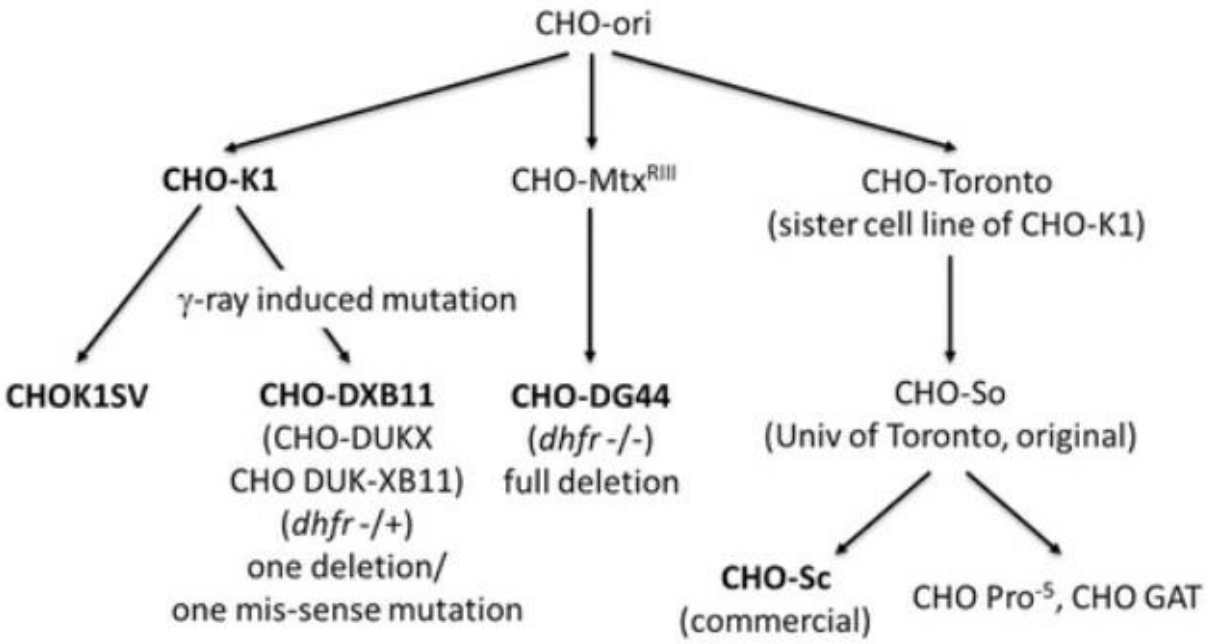
- Fibroblast-like cells derived from monkey kidney tissue
- COS = **C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic material
- Defective SV40 virus integrated into the genome - cannot replicate independently, but makes all proteins
- Replication of recombinant SV40 viruses or plasmids with **oriSV40**
- mainly transient expression



https://doi.org/10.1016/S0167-7306(03)38005-6

Mammalian cells used

- Most commonly used CHO cells (60-70% of recombinant biopharmaceuticals on the market)
  - **mainly stable expression**
  - **grow in suspension**
  - **protein production up to 10 g/L**
- Sp2/0 and NS0 mouse myeloma, YB2/0 rat myeloma and baby hamster kidney (BHK) cells
- Human lines PER C6 and CAP
  - **ideal post-translational modification → low immunogenicity of therapeutic proteins**



doi: 10.1007/978-3-319-52287-6_29

Table 1 Biotherapeutics ranking in the top 50 pharmaceuticals of 2014 based on global sales

Rank	Trade name	Proper name	Manufacturer	Age	Sales ($USB)	Technology	Application	Expression host
1	Humira	adalimumab	AbbVie & Eisai	12	12,890	Antibody, human IgG1/k	TNFa Inhibitor	CHO
3	Enbrel	etanercept	Amgen & Phizer & Takeda	16	8915	Recombinant Protein	TNFa Inhibitor	CHO
4	Remicade	infliximab	JNJ & Merck & Mitsubishi	16	8807	Antibody, chimeric IgG1/k	TNFa Inhibitor	Sp2/0
5	Lantus	insulin glargine	Sanofi	15	8428	Recombinant Protein	Insulin analogue	*E. coli*
6	Rituxan	rituximab	Roche	17	7547	Antibody, chimeric IgG1/k	Anti-CD20	CHO
8	Avastin	bevacizumab	Roche	10	7018	Antibody, humanized IgG1/k	Anti-VEGF	CHO
9	Herceptin	trastuzumab	Roche	16	6863	Antibody, humanized IgG1/k	Anti-HER2	CHO
16	Neulasta	pegfilgrastim	Amgen & Kyowa Hakko	16	4599	Recombinant Protein	G-CSF	E. coli
18	Lucentis	ranibizumab	Novartis & Roche	8	4301	Antibody, humanized IgG1/k Fab	Anti-VEGF	E. coli
20	Prevnar 13	pneumococcal vaccine	Pfizer & Daewoong	4	4297	Bacterial Vaccine	Pneumococcal vaccine	Pneumococcal strains
26	Epogen	epoetin alfa	Amgen & JNJ & Kyowa	25	3292	Recombinant Protein	Erythropoietin	CHO
27	NovoRapid	Insulin aspart	Novo Nordisk	16	3109	Recombinant protein	Insulin analogue	*S. cerevisiae*
29	Avonex	Interferon beta-1a	Biogen	18	3013	Recombinant Protein	Interferon beta	CHO
30	Eylea	aflibercept	Regeneron & Bayer & Santen	3	2972	Recombinant Protein	VEGFr kinase inhibitor	CHO
34	Humalog	Insulin lispro	Eli Lilly	19	2785	Recombinant Protein	Insulin analogue	E. coli
38	Levemir	Insulin detemir	Nov Nordisk	11	2533	Recombinant Protein	Insulin analogue	S. cerevisiae
40	Botox	onabotulinumtoxinA	Allergan & GlaxoSmithKline	12	2496	Bacterial Protein	Botulinum toxin	C. botulinum
42	Aranesp	darbepoetin alfa	Amgen & Kyowa Hakko	13	2454	Recombinant Protein	Erythropoietin	CHO
43	Rebif	Interferon beta-1a	Merck KGaA	17	2444	Recombinant Protein	Interferon beta	CHO
44	Prolia	denosumab	Amgen & Daiichi Sankyo	4	2411	Antibody, human IgG2/k	Anti-RANKL	CHO
45	Victoza	liraglutide	Novo Nordisk	6	2393	Recombinant Protein	Glucagon-like peptide 1 agonist	S. cerevisiae

doi: 10.1007/978-3-319-52287-6_29