VIROLOGIA

Engineering Viral Genomes: Viral vectors

Engineering Viral Genomes:
Rationale for the development of vectors

1. They contain strong promoter elements.

2. They replicate at high copy numbers (increased transient expression of the transgene).

3. Some of them have a replication cycle that include the integration of a dsDNA copy of viral genome into the cell genome (stable integration of the transgene)

Engineering Viral Genomes:
Rationale for the development of vectors

4. Some of them have large genomes containing several non-essential genes that may be deleted without losing infectivity (introduction of the transgene).

5. Some of them are tissue-specific and may present a latent phase of their replicative cycle (gene therapy).

6. Some of them have genomes with ori region and trans acting protein (episomal vectors)

Engineering Viral Genomes: purposes

- Expression at high levels of properly folded and post-translational modified recombinant proteins in eukaryotic cells

- Vaccine vectors

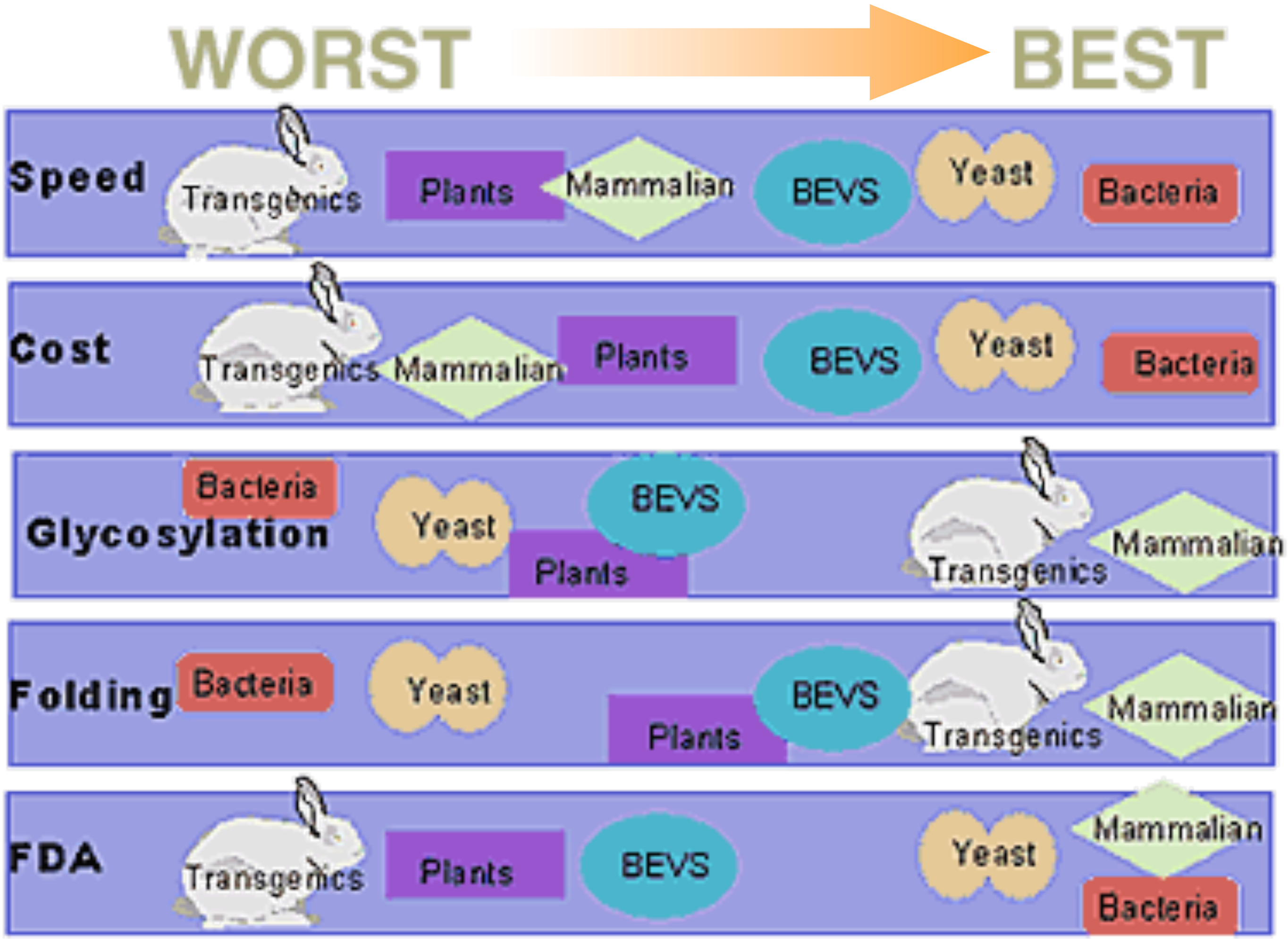
- Therapeutic purposes- Gene therapy

Comparison of protein production in various expression systems

Expression System	Baculovirus	E.coli	Yeast	Mammalian cells
Folding	++	+	+	+++
Glycosylation	++	–	+	+++
Phosphorylation	+++	–	+	+++
Speed	++	+++	++	+
Expression level	+++	+++	+	+

+++ excellent, ++ good, + poor, – none

Comparison of protein production in various expression systems



Engineering Viral Genomes:
design requirements

1) The inclusion of appropriate promoter elements.

2) The maintenance of genome size within the packaging limit of the virus.

3) The elimination of the capacity of the virus to cause disease

Engineering Viral Genomes: persistence

- Transient expression

    Adeno, Vaccinia, RNA viruses, Baculovirus

- Permanent expression

    -stable integration (integrative vectors) (Retrovirus, Lentivirus)

    -episomal vectors (AAV)

Viral vectors

Virus	Insert size	Integration	Duration of expression	Advantages	Disadvantages
Adeno-associated virus	~4.5–9 (?) kb	Low efficiency	Long	Nonpathogenic, episomal, infects nondividing cells	Immunogenic, toxicity
Adenovirus	2–38 kb	No	Short	Efficient gene delivery	Transient, immunogenic
Alphavirus	~5 kb	No	Short	Broad host range, high-level expression	Virulence
Herpes simplex virus	~30 kb	No	Long in central nervous system, short elsewhere	Neurotropic, large capacity	Virulence, persistence in neurons
Influenza virus	Unknown	No	Short	Strong immune response	Virulence
Lentivirus	7–18 kb	Yes	Long	Stable integration; infects nondividing and terminally differentiated mammalian cells	Insertional mutagenesis
Poliovirus	~300 bp for helper-free virus; ~3 kb for defective virus	No	Short	Excellent mucosal immunity	Limited capacity, reversion to neurovirulence
Retrovirus	1–7.5 kb	Yes	Shorter than formerly believed	Stable integration	May rearrange genome, insertional mutagenesis, require cell division
Rhabdovirus	Unknown	No	Short	High-level expression, rapid cell killing	Virulence, highly cytopathic
Vaccinia virus	At least ~25 kb, probably ~75–100 kb	No	Short	Wide host range, ease of isolation, large capacity, high-level expression	Transient, immunogenic

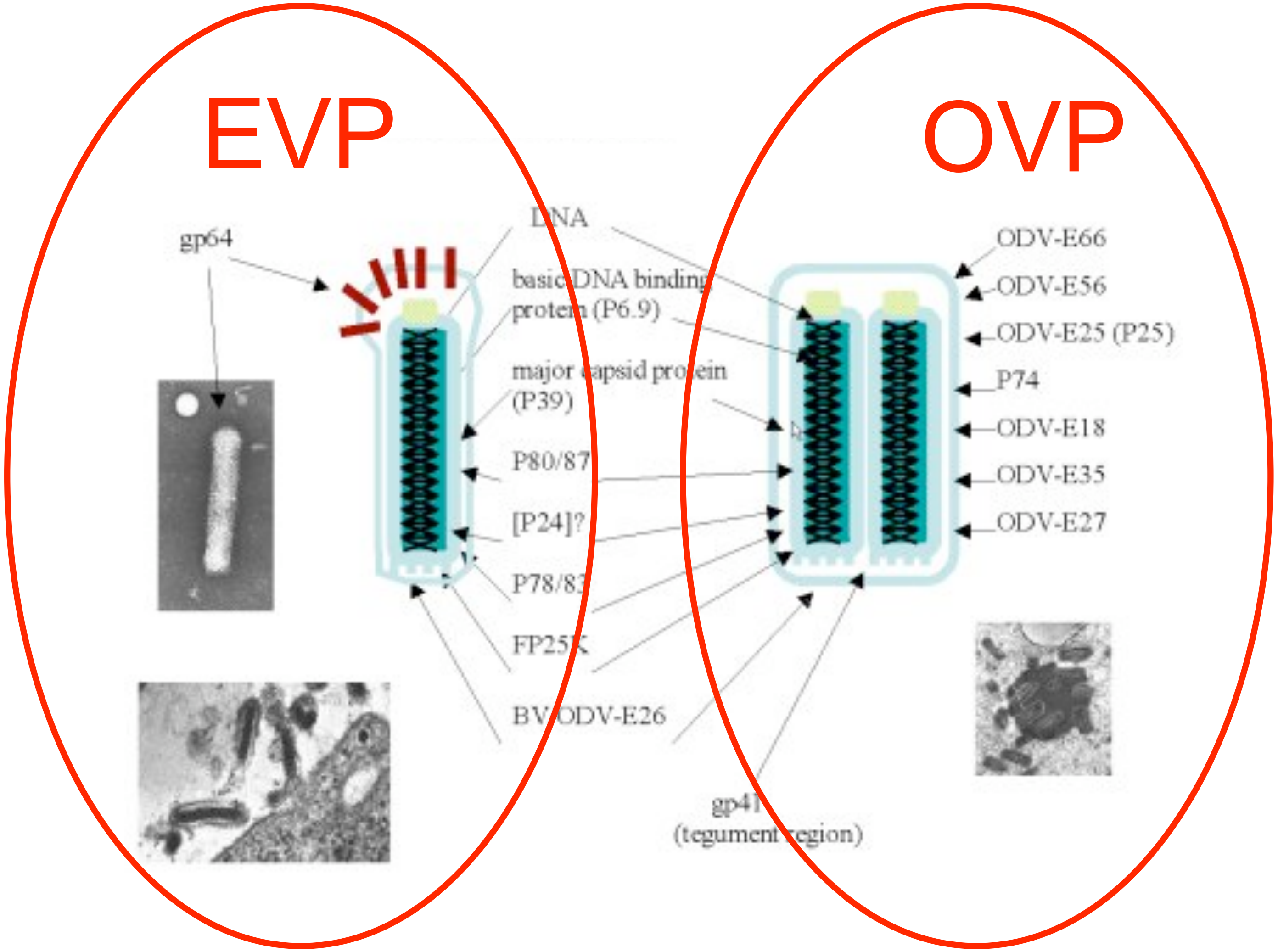
Application	Virus family	Virus species
Protein production	Adenovirus	Adenovirus type 5
	Retrovirus	Human immunodeficiency virus type 1
	Baculovirus	*Autographa californica* nucleopolyhedrovirus
	Togavirus	Semliki Forest virus, Sindbis virus
	Adenovirus	Adenovirus types 2 and 5
Recombinant vaccines	Coronavirus	Transmissible gastroenteritis virus, mouse hepatitis virus, infectious bronchitis virus
	Flavivirus	Japanese encephalitis virus, yellow fever virus
	Herpesvirus	Bovine herpesvirus
	Orthomyxovirus	Influenza A and B viruses
	Paramyxovirus	Sendai virus, simian virus 5
	Parvovirus	Adeno-associated virus
	Picornavirus	Poliovirus
	Poxvirus	Vaccinia virus, canarypox virus, fowlpox virus
	Rhabdovirus	Rabies virus, vesicular stomatitis virus
	Togavirus	Semliki Forest virus, Sindbis virus, western equine encephalitis virus, Venezuelan equine encephalitis virus
Gene replacement therapy	Adenovirus	Adenovirus types 2 and 5
	Baculovirus	*Autographa californica* nucleopolyhedrosis virus
	Flavivirus	Kunjin virus, tick-borne encephalitis virus
	Herpesvirus	Cytomegalovirus
	Retrovirus	Human immunodeficiency virus type 1, simian immunodeficiency virus type 1, murine leukemia virus
	Paramyxovirus	Sendai virus, human parainfluenza virus
	Parvovirus	Adeno-associated virus, B19 virus
	Polyomavirus	Simian virus 40
	Togavirus	Semliki Forest virus, Sindbis virus
Antitumor agents	Adenovirus	Adenovirus type 5
	Herpesvirus	Herpes simplex virus
	Paramyxovirus	Newcastle disease virus, simian virus 5
	Parvovirus	Minute virus of mice, H-1 parvovirus, LuIII virus
	Poxvirus	Vaccinia virus, avian poxviruses
	Reovirus	Reovirus
	Rhabdovirus	Vesicular stomatitis virus

Engineering Viral Genomes:

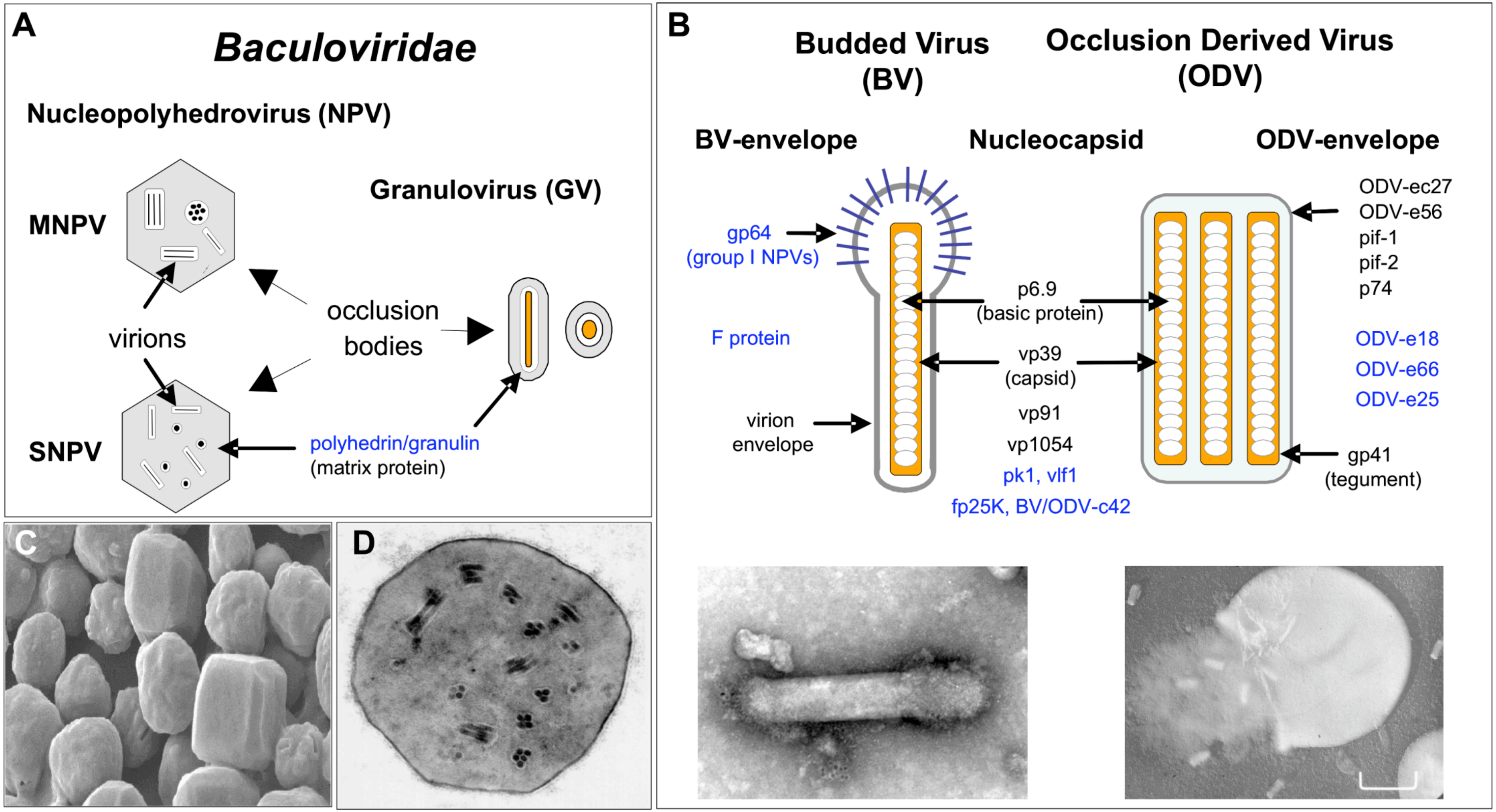
- Baculovirus
- Vaccinia virus
- Adenovirus
- Alphavirus
- AAVs
- Retrovirus
- Lentivirus
- VSV

Engineering Viral Genomes:
Baculovirus vectors

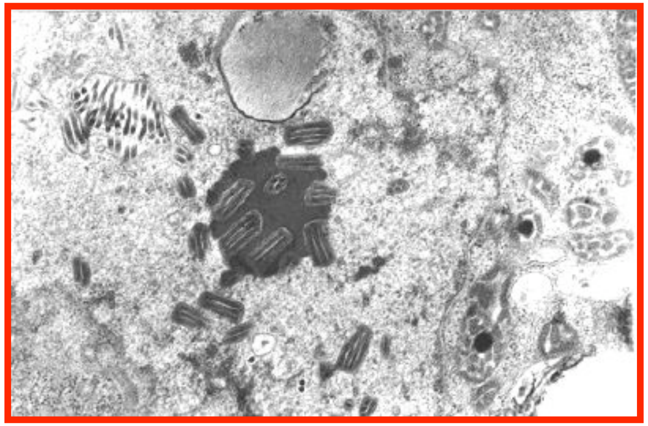
Baculovirus structures produced by infected cells

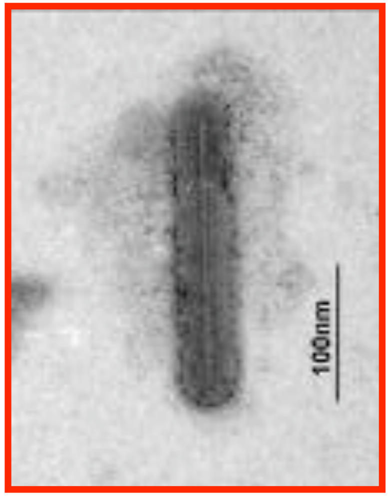


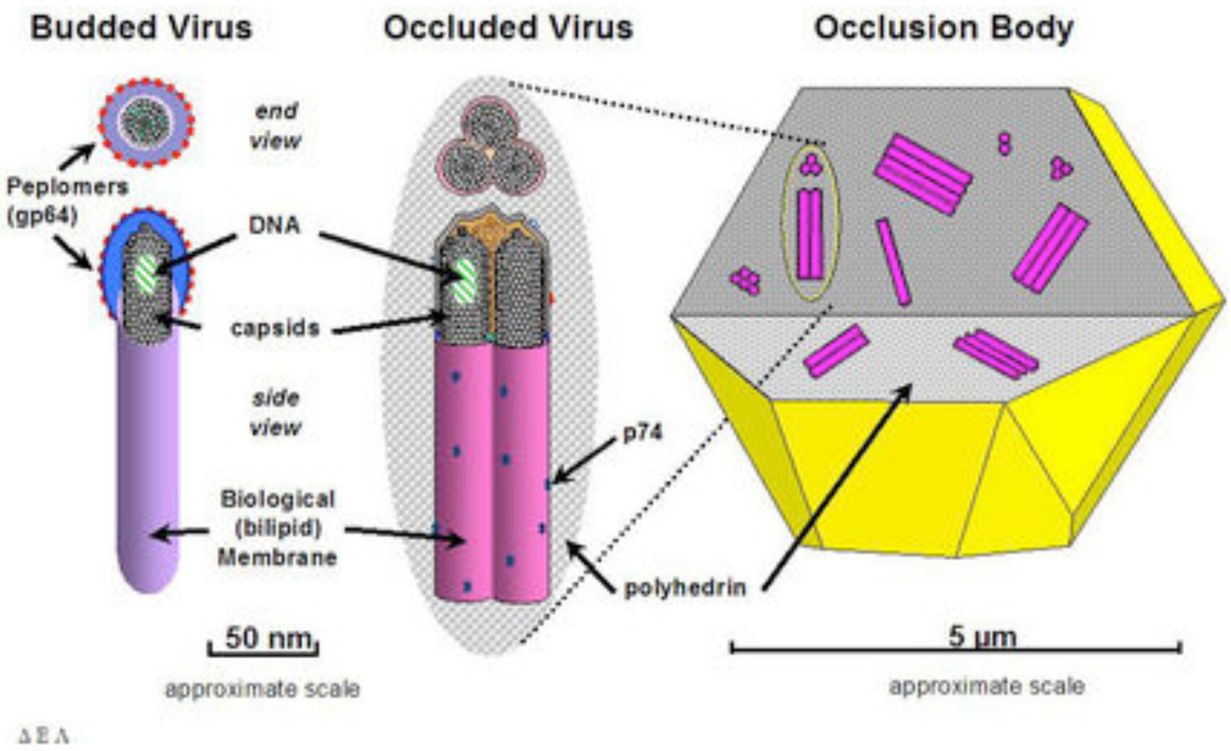
Baculovirus particles



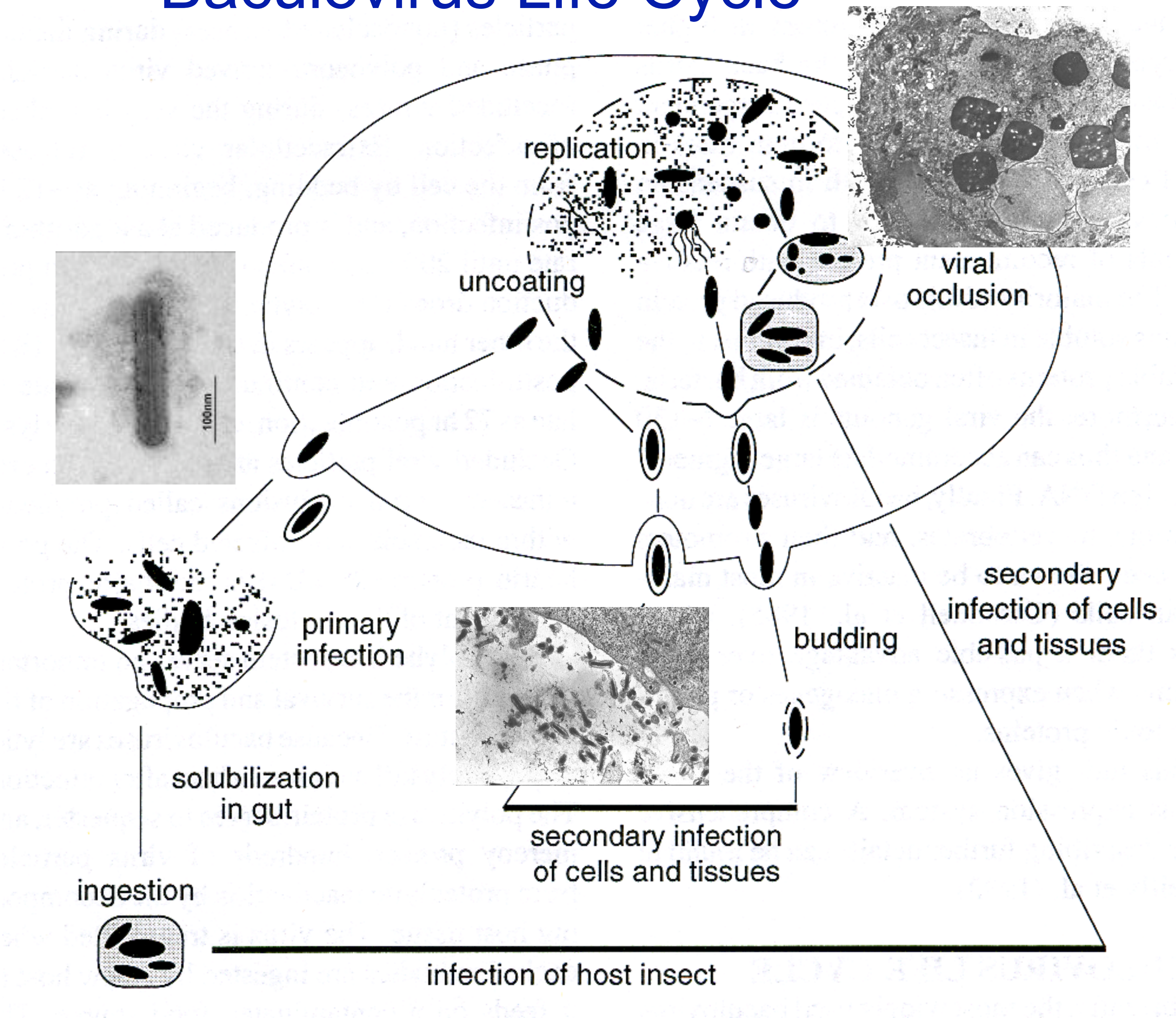
Baculovirus structures produced by infected cells







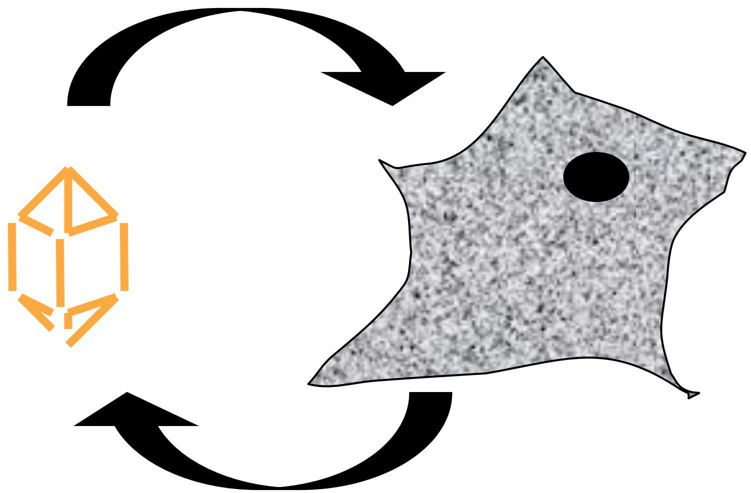
Baculovirus Life Cycle



Baculovirus Life Cycle

Infection - “IE, E, and L phases”

Baculovirus virion



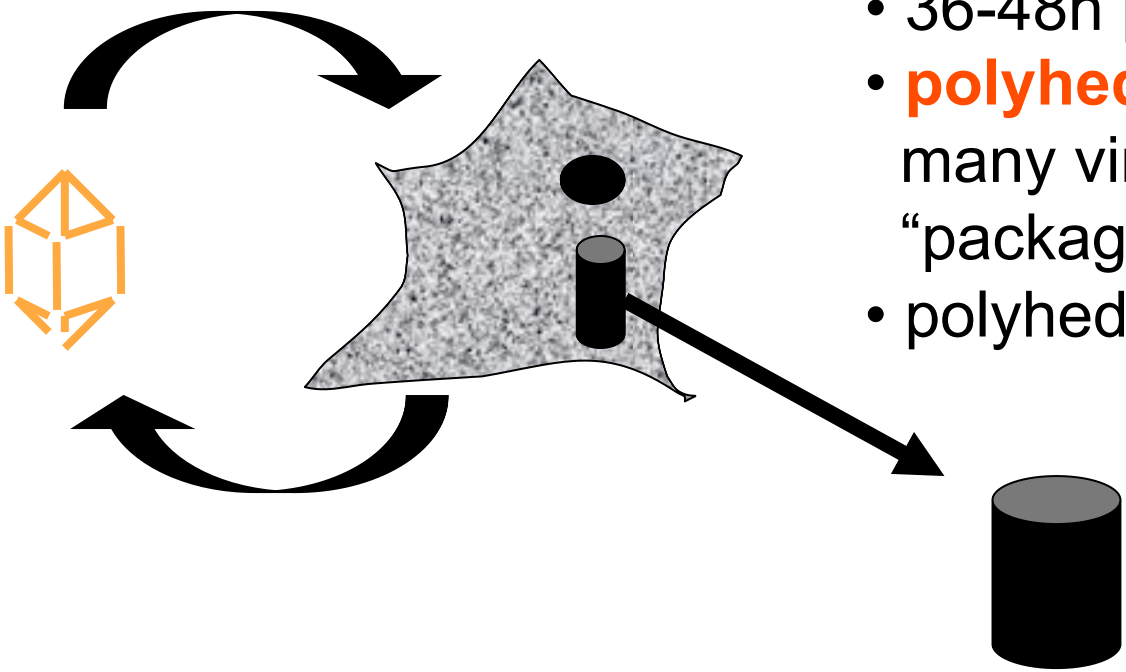
Insect cell

EVP Production

Baculovirus Life Cycle

Polyhedrin Formation - “Very Late”

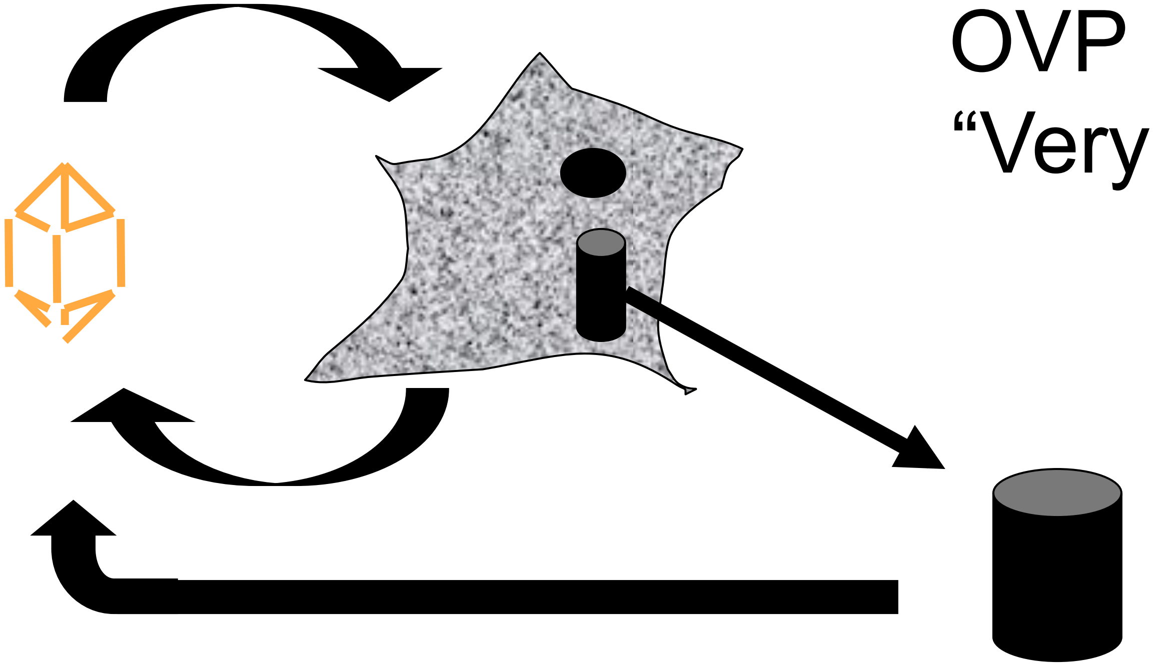
- 36-48h post infection
- **polyhedrin protein** traps many virions into polyhedra “package”
- polyhedra is stable



polyhedra
“package”

Baculovirus Life Cycle

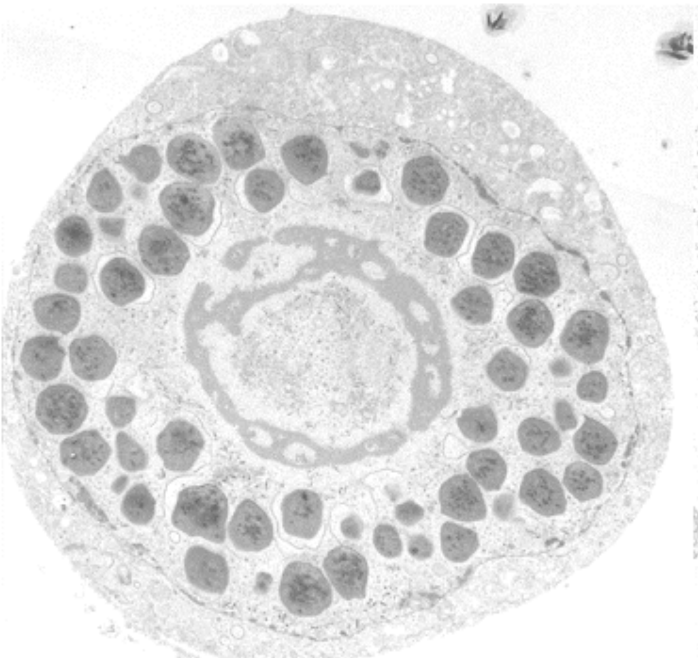
OVP production -
“Very Late” phase

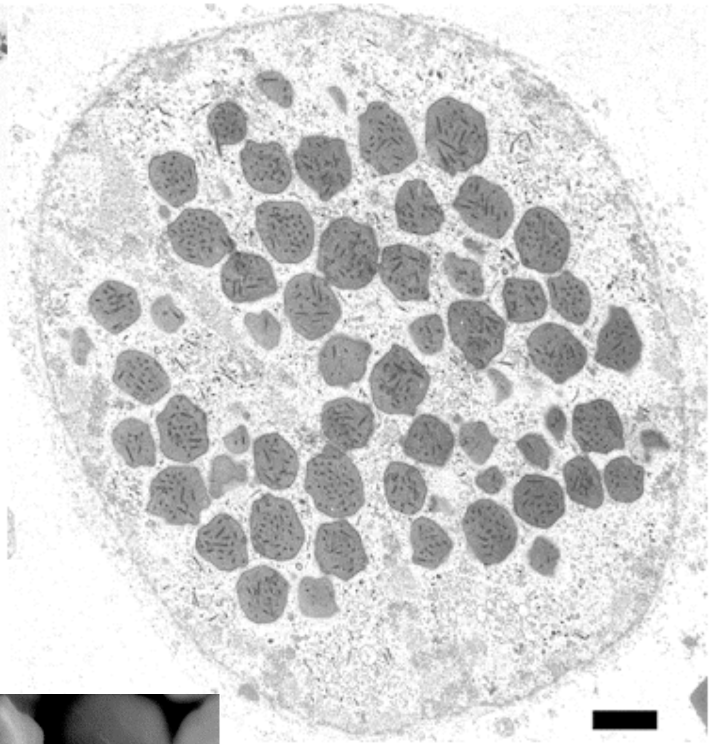


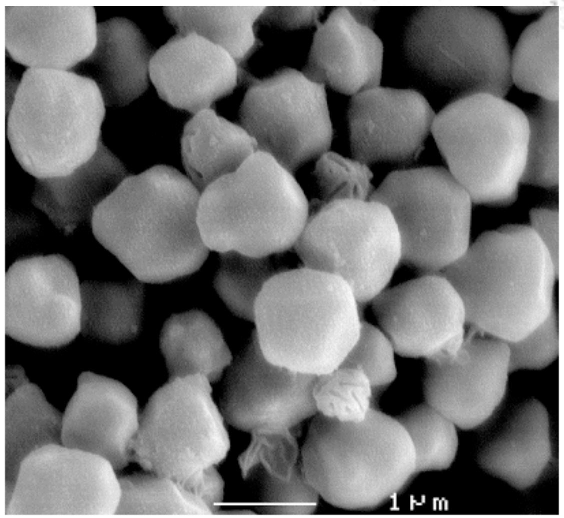
polyhedron

If ingested by insect host,
polyhedrin protein is broken down.
Virions will be released - infection starts again.

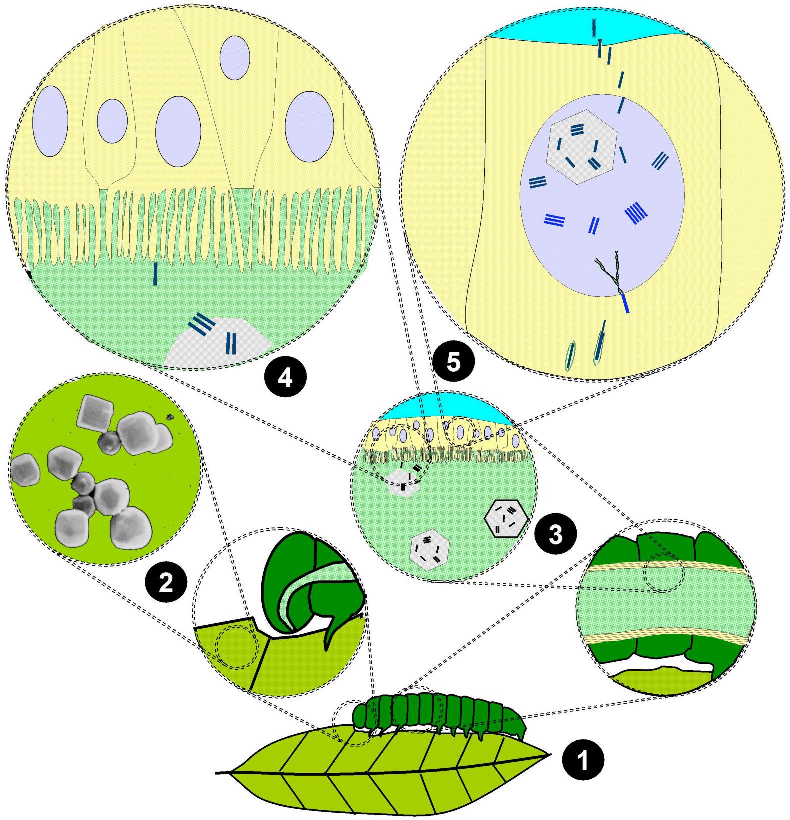
At the end, infected cells may harbour up to 200-300 occlusion bodies in the nucleus, which will be released as the cell lyses





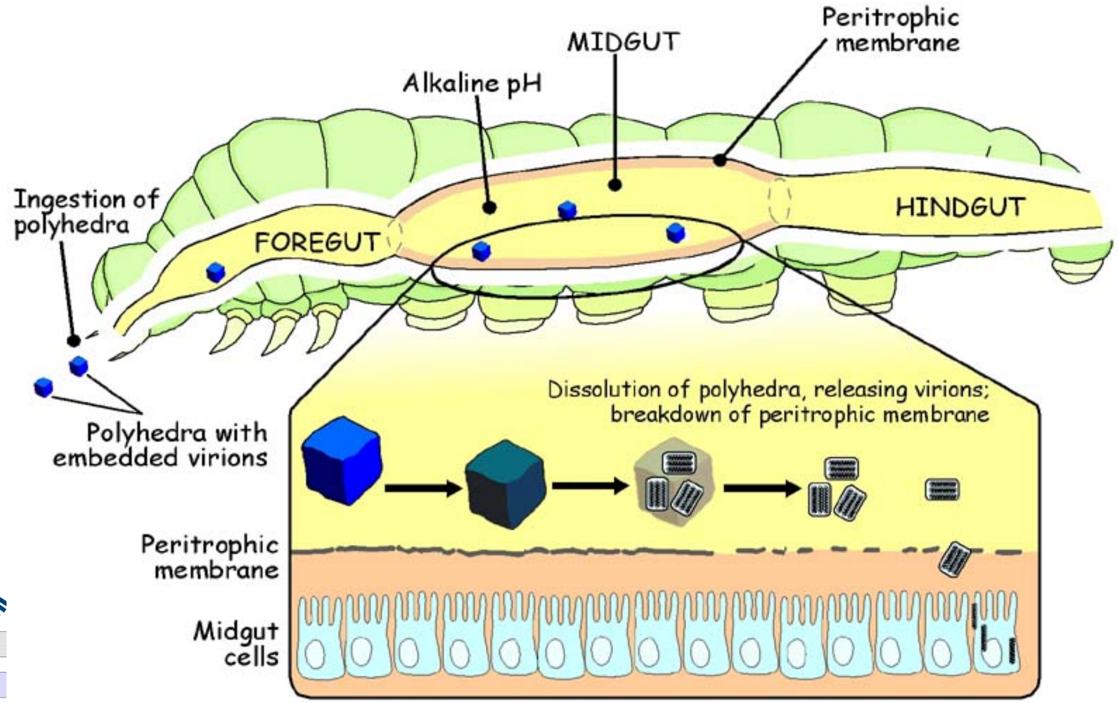


NPV infection of an insect host



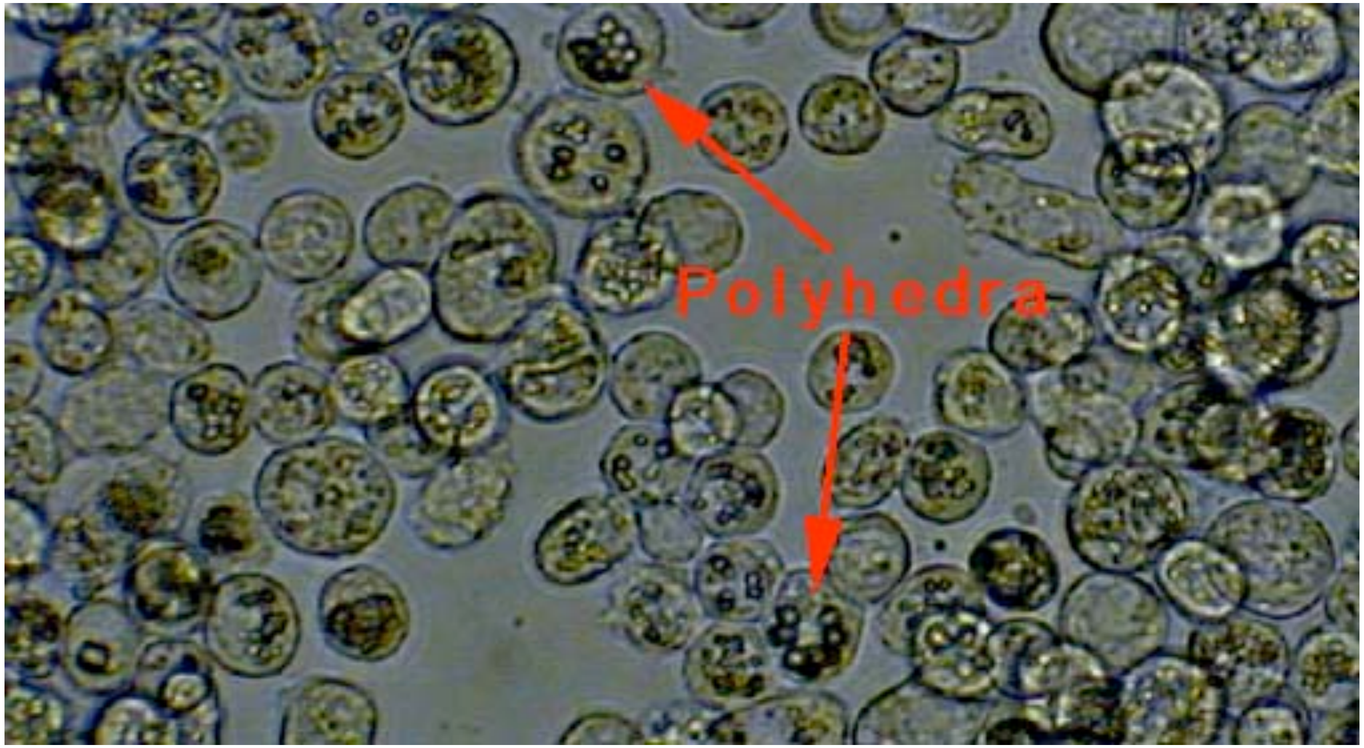
1. Insect feeding on virus-contaminated foliage
2. Close up of occlusion bodies (OBs)
3. Lumen of digestive tract (alkaline conditions)
4. Virus particles being released from OBs and attaching to brush border of gut cells
5. Replication of virus in insect cell

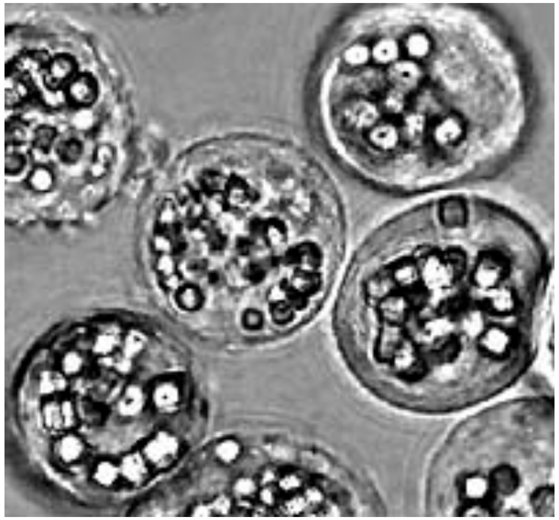
Virus
Occlusion body
Nucleus
Cytoplasm
Hemocoel
Gut lumen
Plant

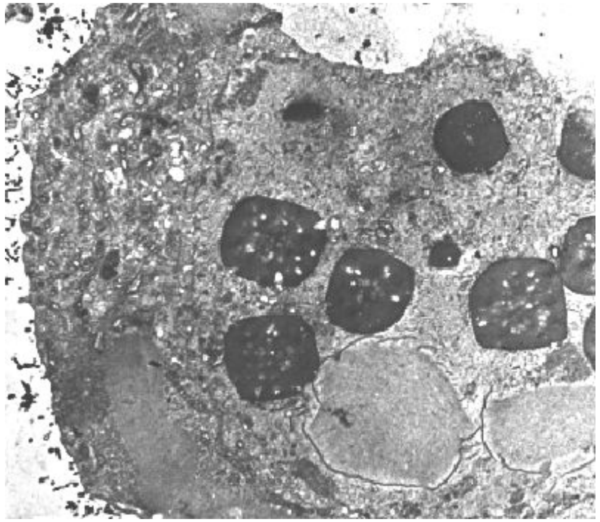


Baculovirus as a natural insecticide

A cabbage looper exposed to baculovirus, from two to seven days after infection


Polyhedra





Baculovirus vectors

- Why this system is suitable for the generation of viral vectors?
  - polyhedrin promoter is strong
  - polyhedrin accumulates to very high levels at 36-48 h. p. i. (up to 1 mg per 1-2 x10^6 cells, 30-50% of the total insect cell protein)
  - don’t need polyhedron “package” for *in vitro* virus propagation
  - virus lacking the polyhedrin gene have a plaque morphology that is distinct from that of wt virus (visual screening)
  - replace polyhedrin coding sequences with the gene of interest

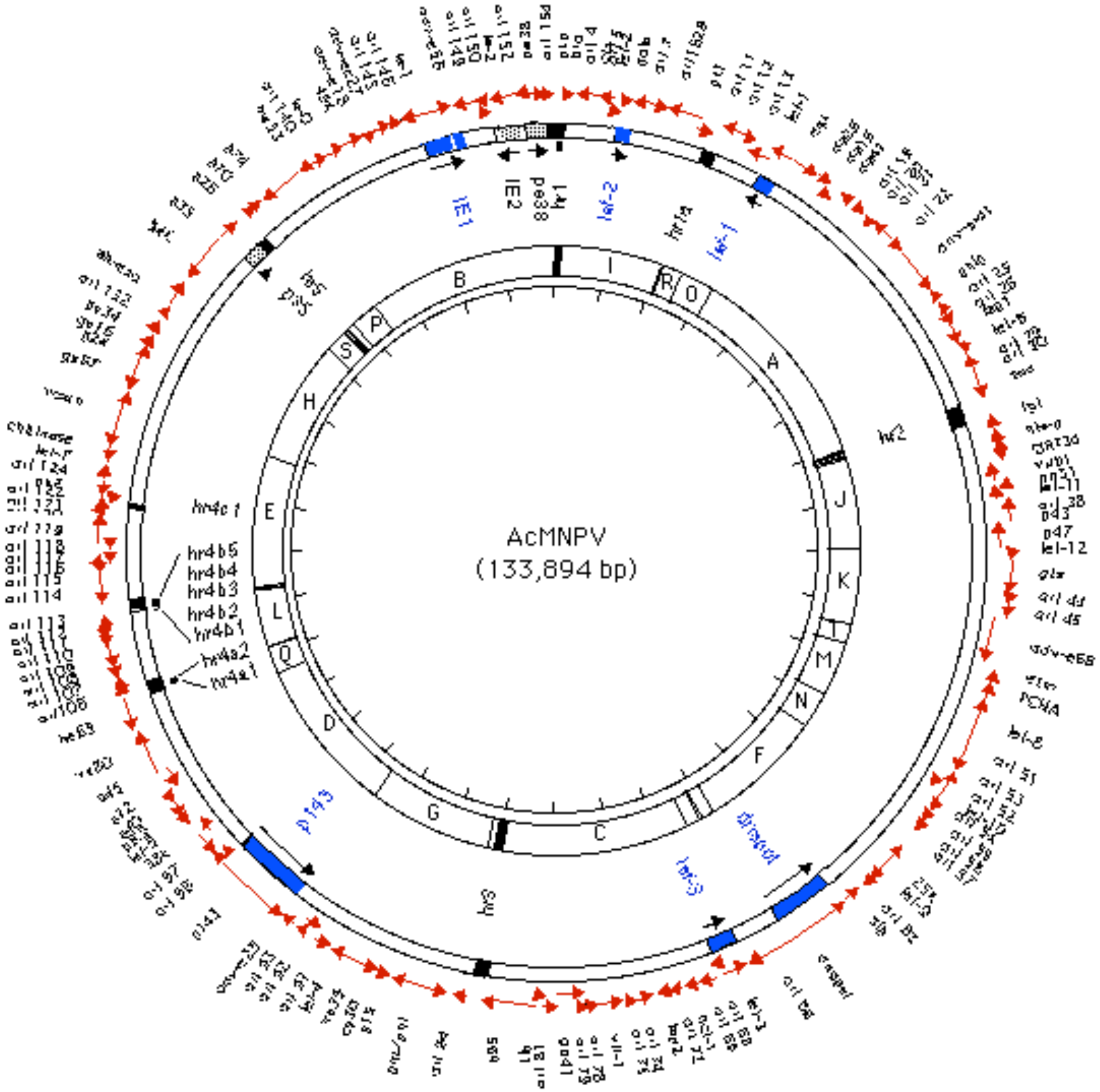
Baculovirus System

- Virus utilized
  - AcMNPV
    - *Autographa californica multiple nuclear polyhedrosis virus*
  - *A. californica* = alfalfa looper
  - AcMNPV infects more than 30 different insects
  - Commonly used cell line
    - Fall armyworm - *Spodoptera frugiperda* (*Sf9*)
    - Polyhedrin promoter very active in these cells

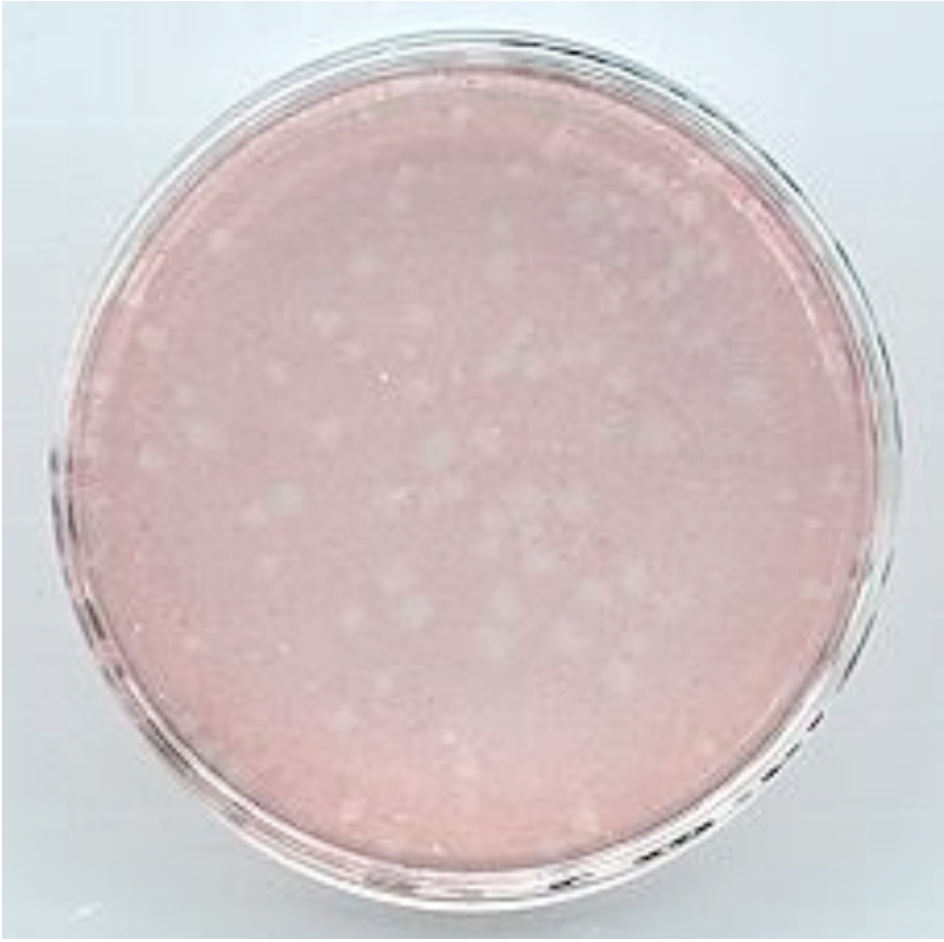
Baculovirus System

A. californica = alfalfa looper

AcMNPV genomic map

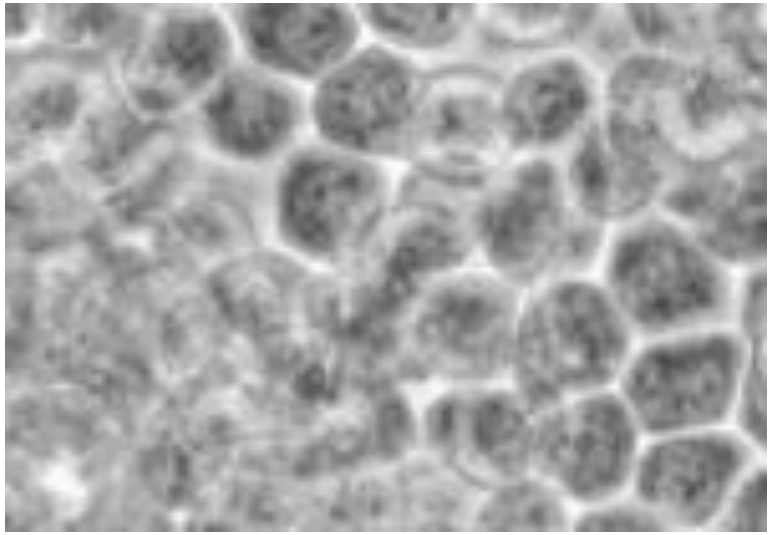


Routine growth of Sf9 cells in suspension culture. Incubation of infected cells is at 28°C with shaking at 150rpm for 1-4 days

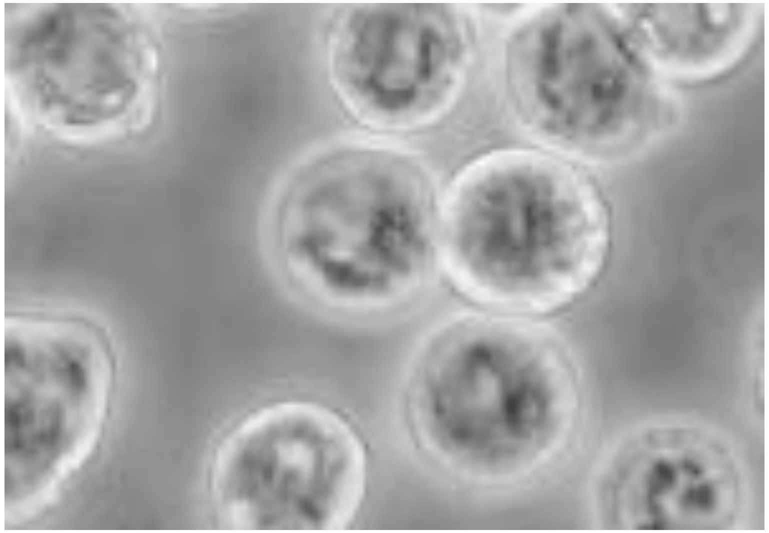


Baculovirus plaques. Zones of clearing (plaques) are generated by infection of Sf9 cells with individual baculovirus particles. Uninfected Sf9 cells surrounding the plaque are stained pink with neutral red.

Infected and uninfected Sf9 cells can be distinguished by morphology



Uninfected Sf9 cells. These cells continue to divide and form a confluent monolayer



Sf9 cells infected with **recombinant baculovirus**. These cells stop dividing and enlarge.

Sf9 cells infected with wt or recombinant Baculovirus can be distinguished by morphology

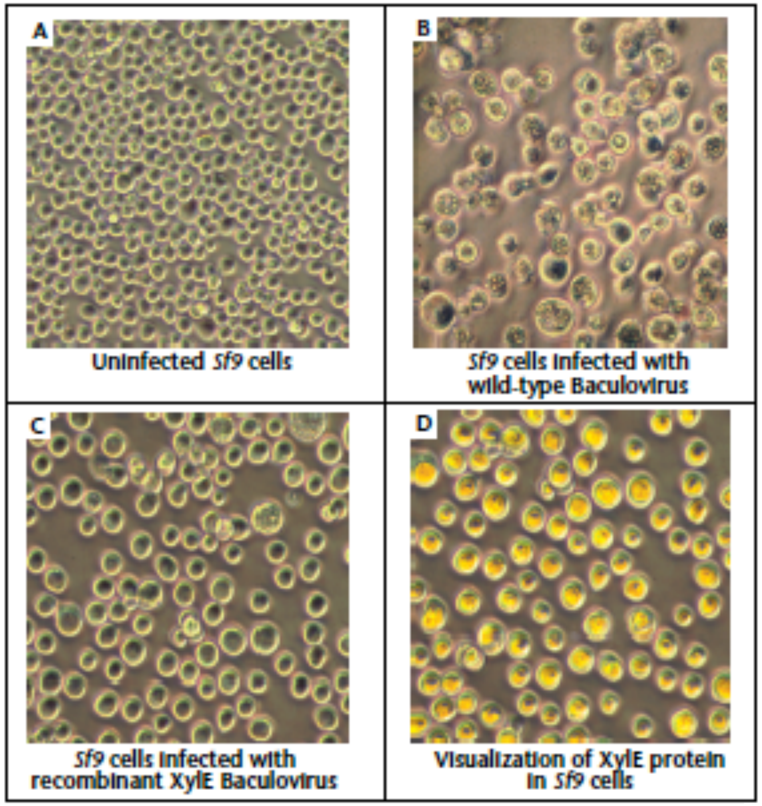
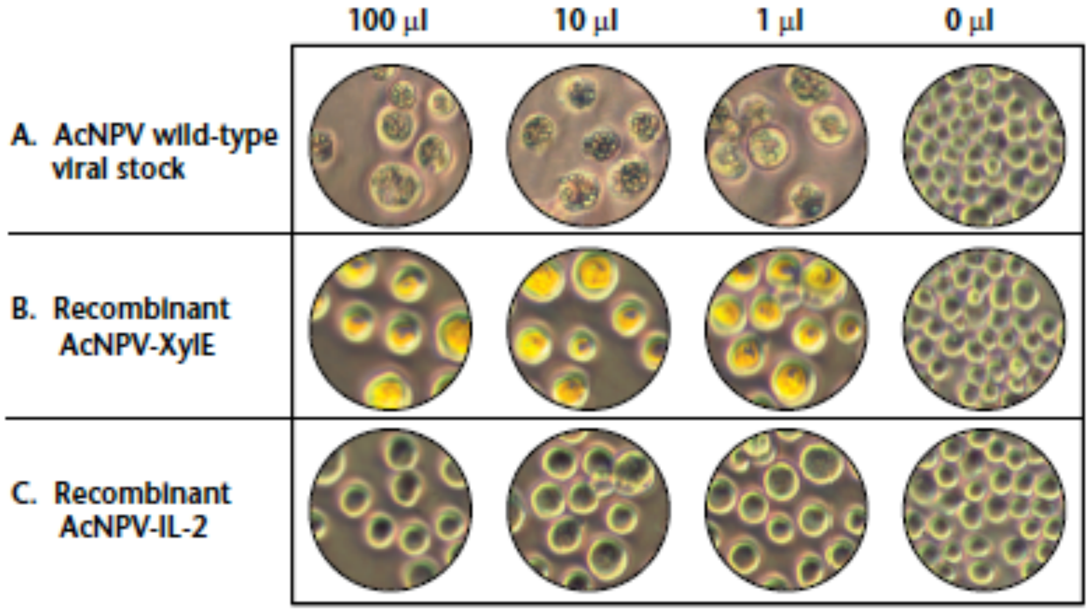
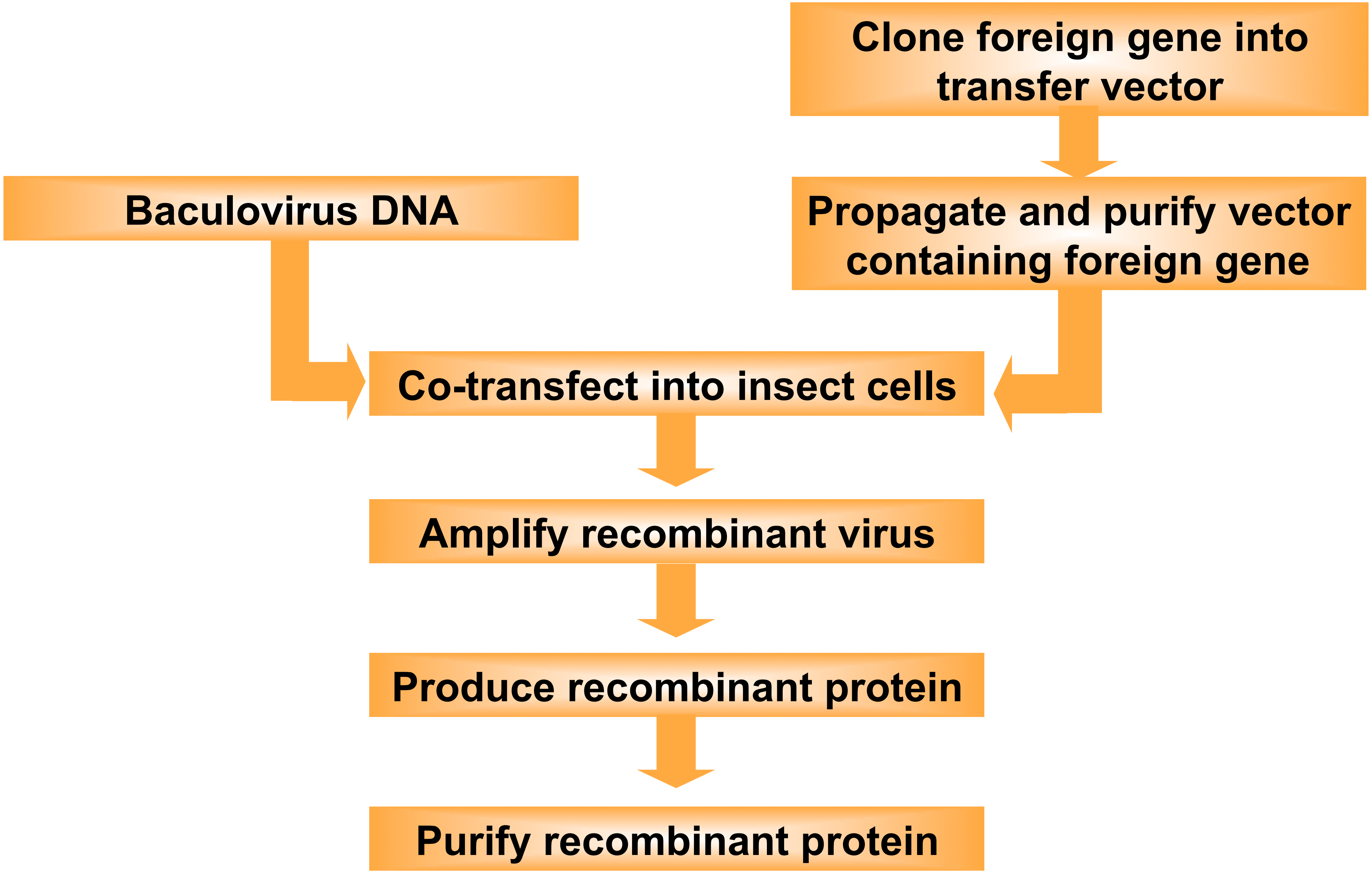


Figure 7. Comparison of uninfected and infected *Sf9* cell monolayers. *Sf9* cells uninfected (A), infected with wild-type (AcNPV) Baculovirus (B), or infected with recombinant Baculovirus containing the XylE gene (C and D). Cells infected with wild-type Baculovirus are occlusion body positive (B), whereas cells infected with recombinant virus are not (C and D). Cells expressing recombinant XylE turn yellow in the presence of Catechol (D).



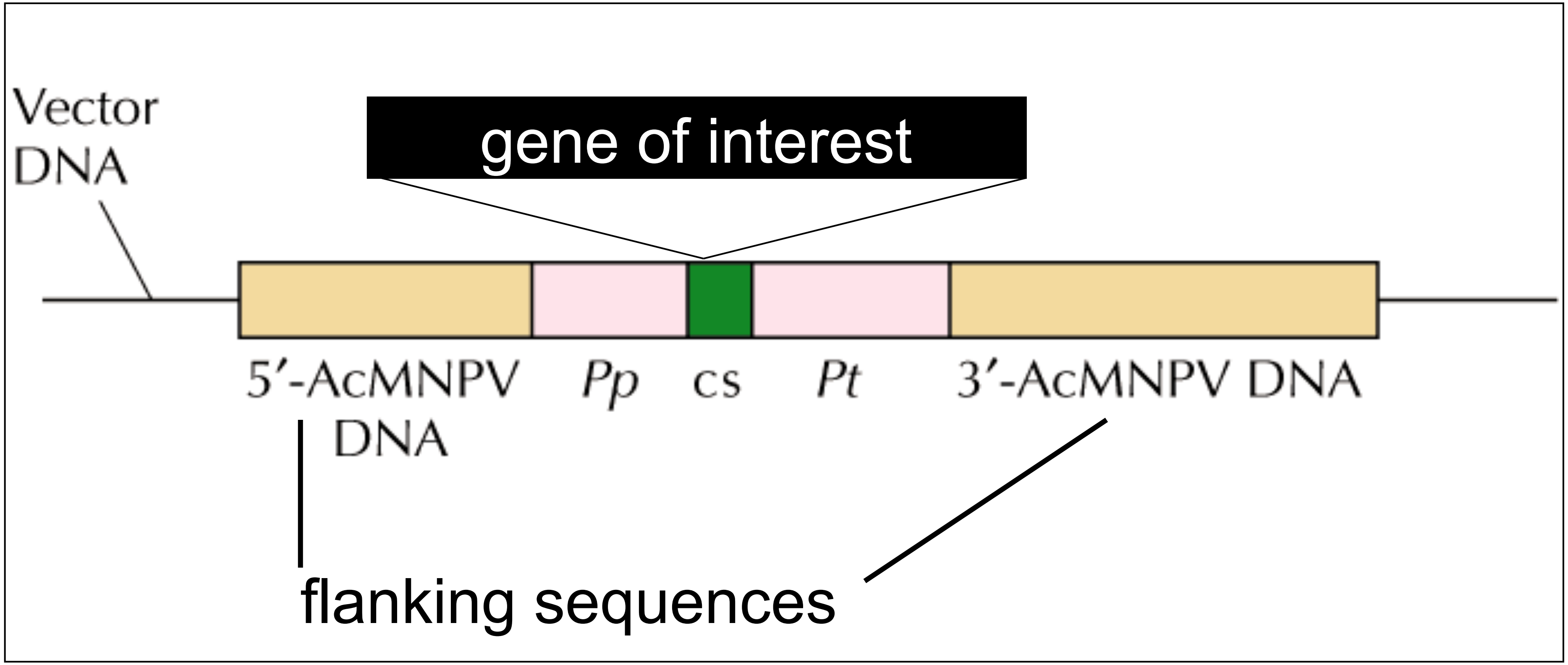
Flow chart for 1st Generation Baculovirus Expression System



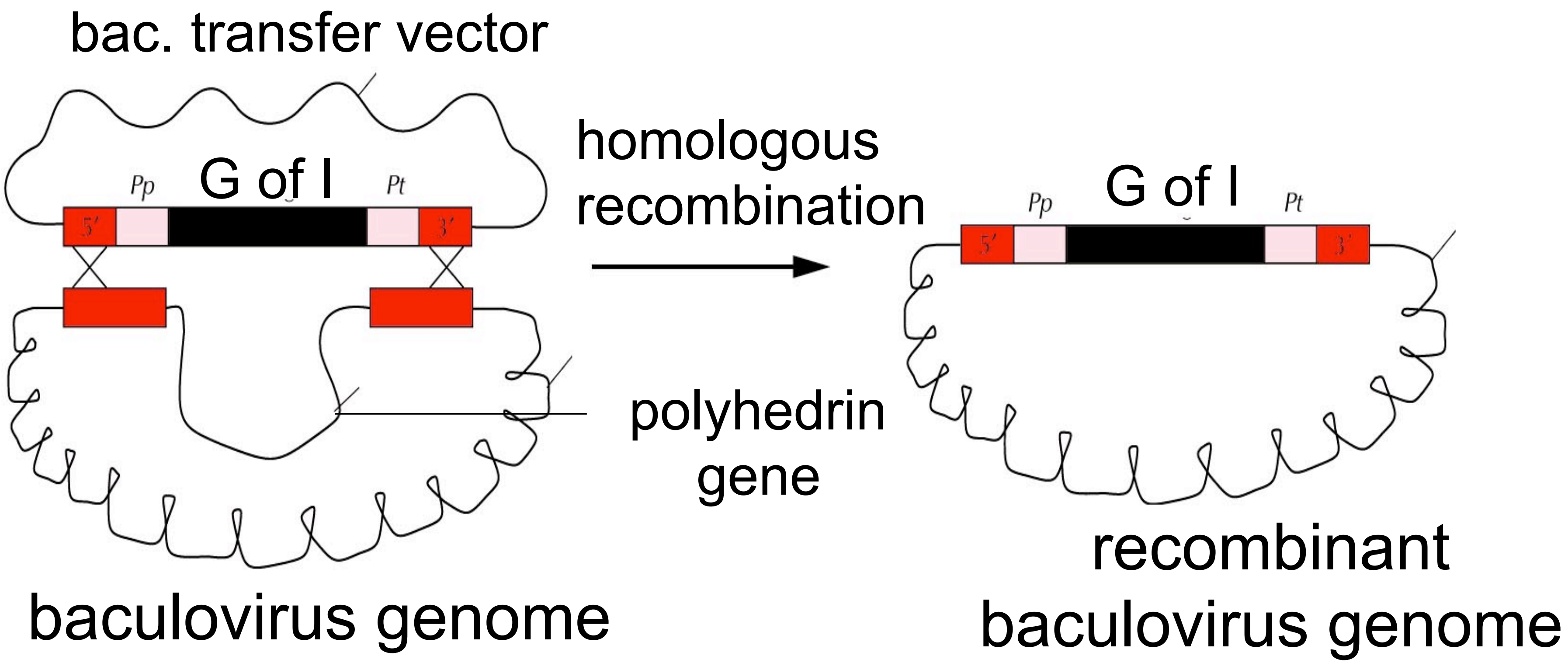
Procedure for Baculovirus Expression System

1) Clone gene of interest into bacterial transfer vector
2) Propagate transfer vector in E. coli
3) Co-transfect insect cell line with baculovirus AcMNPV DNA and transfer vector
4) Identify cells in which homologous recombination has taken place
5) Isolate recombinant virus
6) Infect new cells with recombinant virus
7) Harvest protein after 36-48 h post-infection

Baculovirus transfer vector



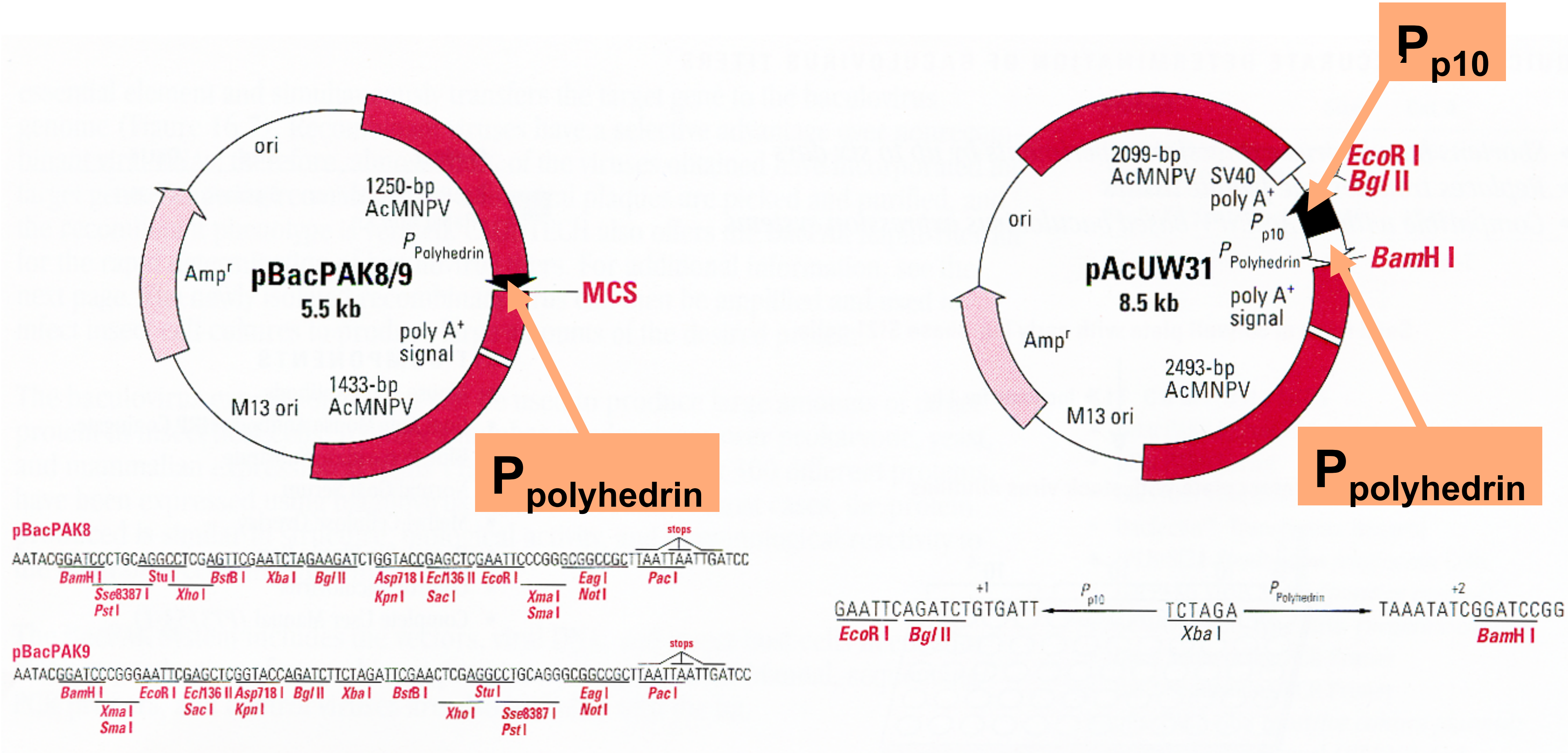
Homologous recombination between transfer vector and baculovirus genome



Baculovirus vector selection

Vector	Compatibility: BaculoGold™ DNA	Compatibility: AcRP23.lacZ DNA	Compatibility: AcUW1.lacZ DNA	Compatibility: AcNPV wild-type DNA	Promoter	Type	Fusion Protein	Features	Cat. #
Polyhedrin locus-based									
Single Promoter Plasmids									
pVL1392/3 (set)	•	•		•	Polyhedrin	very late	no	Standard polyhedrin locus vectors	21201P
pAcSG2	•	•		•	Polyhedrin	very late	site dependent	Recommended for large inserts, has an ATG	21410P
pAcMP2/3 (set)	•	•		•	Basic protein	late	no	Facilitates post-translational modifications	21209P
pAcUW21	•	•		•	p10	very late	no	Allows for in-larval expression, F1 origin	21206P
pAcGHLT-A, -B, -C (set)	•	•		•	Polyhedrin	very late	yes	GST-tag, 6xHis-tag thrombin cleavage site	21463P
pAcHLT-A, -B, -C (set)	•	•		•	Polyhedrin	very late	yes	6xHis-tag, thrombin cleavage site	21467P
pAcG1	•	•		•	Polyhedrin	very late	yes	GST-tag	21413P
pAcG2T	•	•		•	Polyhedrin	very late	yes	GST-tag, thrombin cleavage site	21414P
pAcG3X	•	•		•	Polyhedrin	very late	yes	GST-tag, factor X_a cleavage site	21415P
BioColors™ BV Control (set)	•	•		•	Polyhedrin	very late	yes	BioColors™ Genes	21518P
BioColors™ His (set)	•	•		•	Polyhedrin	very late	yes	BioColors™ Genes, 6xHis tag, thrombin cleavage site	21522P
Secretory									
pAcGP67 A, B, C (set)	•	•		•	Polyhedrin	very late	yes	Signal sequence	21223P
pAcSecG2T	•	•		•	Polyhedrin	very late	yes	Signal sequence, GST-tag	21469P
Multiple Promoter Plasmids									
pAcUW51	•	•		•	Polyhedrin, p10	very late	no	Simultaneous expression of 2 foreign genes; F1 origin	21205P
pAcDB3	•	•		•	Polyhedrin, p10	very late	no	Simultaneous expression of 3 foreign genes; F1 origin	21532P
pAcAB3	•	•		•	Polyhedrin, p10	very late	no	Simultaneous expression of 3 foreign genes	21216P
pAcAB4	•	•		•	Polyhedrin, p10	very late	no	Simultaneous expression of 4 foreign genes	21412P
p10 locus-based									
Single Promoter Plasmids									
pAcUW1			•	•	p10	very late	no	Standard p10 locus vectors	21203P
Multiple Promoter Plasmids									
pAcUW42/43 (pair)			•	•	Polyhedrin, p10	very late	no	Simultaneous expression of 2 foreign genes; F1 origin	21208P

Examples of Baculovirus Transfer Vectors



Improvement of selection and screening of recombinant baculovirus vectors

Replacement of a beta-galactosidase gene inserted in the virus genome with a foreign gene from the plasmid leads to the formation of colourless virus-infected cells in a plaque assay.

Advantages of Baculovirus Expression System

- Functional activity of the recombinant protein highly probable
- Post-translational modifications
- High expression levels (1 mg/1-2x10^6 cells)
- Capacity for large inserts
- Capacity to express unspliced genes
- Simultaneous expression of multiple genes
- Localization of recombinant proteins
- Ease of purification

Advantages of Baculovirus Expression System

Features	BEVS	Bacterial
Simple to use	**^**	**^**
Protein size	**unlimited**	**< 100 kDa**
Multiple gene expression	**^**	
Signal peptide cleavage	**^**	
Intron splicing	**^**	
Nuclear transport	**^**	
Functional protein	**^**	**sometimes**
Phosphorylation	**^**	**sometimes**
Glycosilation	**^**	
Acylation	**^**	

Disadvantages of Baculovirus Expression System

- Baculovirus system kills the cells - not continuous production – only transient
- Some proteins not modified correctly
- Insect cell culture is still expensive

Disadvantages of Baculovirus Expression System

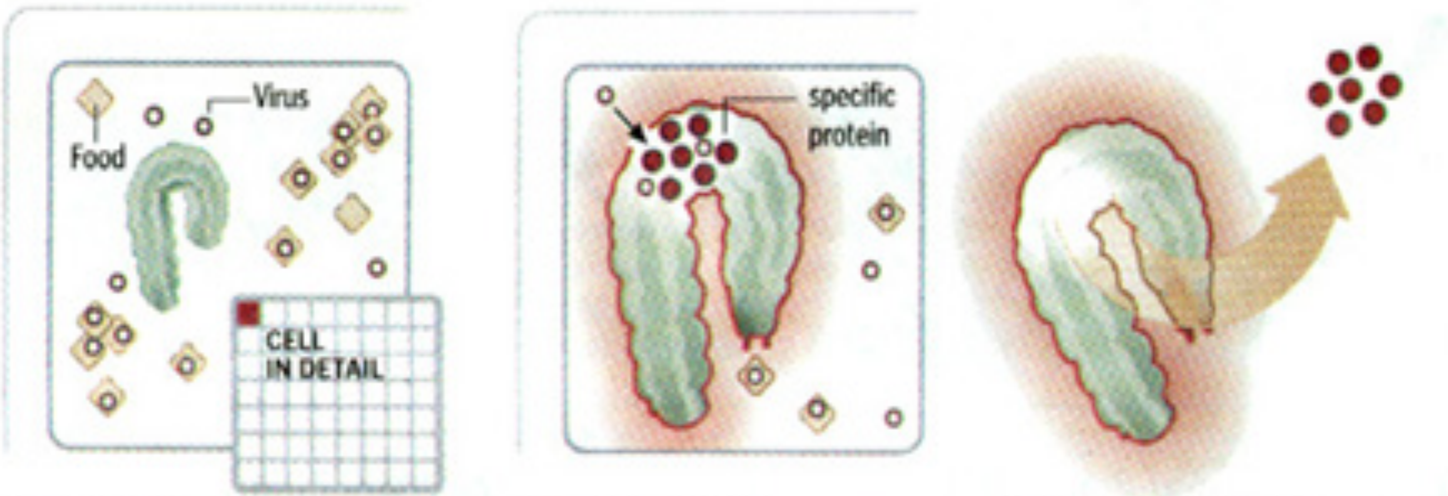
- Baculovirus system kills the cells in 4-5 days
  - not a continuous production - batch production
  - modify by continuous input of fresh cells,
  - OR
  - remove productive viral aspects
  - transfect insect cells with strong viral promoter driving the gene of interest
  - grow transfected cells indefinitely with continuous production of protein

Disadvantages of Baculovirus Expression System

- Insect cell culture is still expensive
  - one can shift to insect larvae
  - "low-cost protein factories"
  - comparable yields with lower costs
  - using caterpillars to produce proteins
  - NOT for human therapeutics, but other commercially important proteins

Caterpillars engineered to produce proteins

Chesapeake PERL Inc.'s method to efficiently manufacture proteins on a mass scale:



Week 1: Infection

The caterpillar hatches from the egg. It sits among food in a container made of 56 individual cells. At week's end a recombinant insect virus encoding a specific protein is sprayed into each cell.

Week 2: Production

As the caterpillar grows, it eats the virus-infested food and becomes infected. The recombinant virus directs it to produce the specific protein as well as a protein that causes it to glow.

End of week 2: Harvest

The caterpillar emits an intense glow signaling it's ready for harvesting. The caterpillars are ground up, and the specific proteins are separated and purified from caterpillar cadavers.

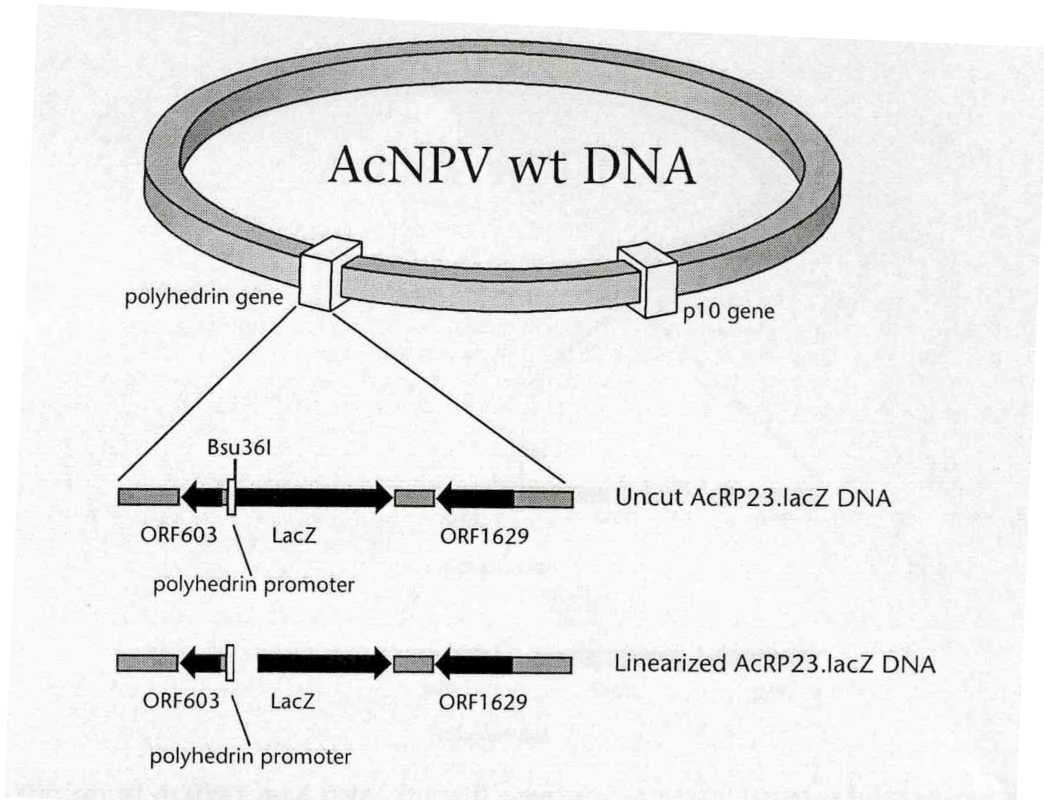
SOURCE: Chesapeake PERL Inc.

BY KRISTOPHER LEE—THE WASHINGTON POST

Disadvantages of Baculovirus Expression System

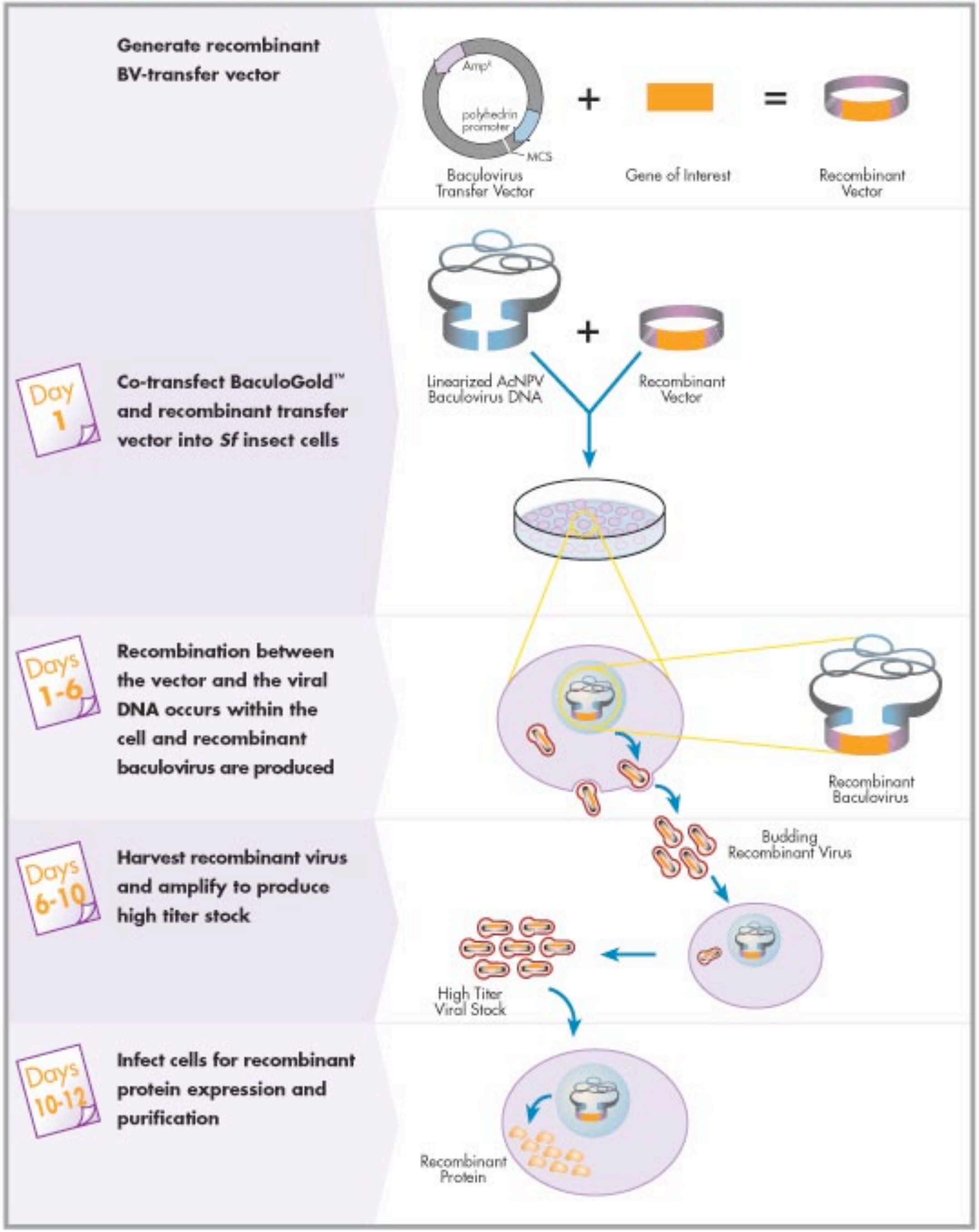
- Some proteins not correctly modified
  - “very late” expression from polyhedrin promoter
  - not all proteins correctly modified
  - now use strong promoter from earlier in life cycle (basic protein promoter, L gene)
- Also, linearized AcMNPV prior to transfection increases freq. of recomb. genomes
- [30% vs. 0.1%]

A linearized AcMNPV Baculovirus DNA

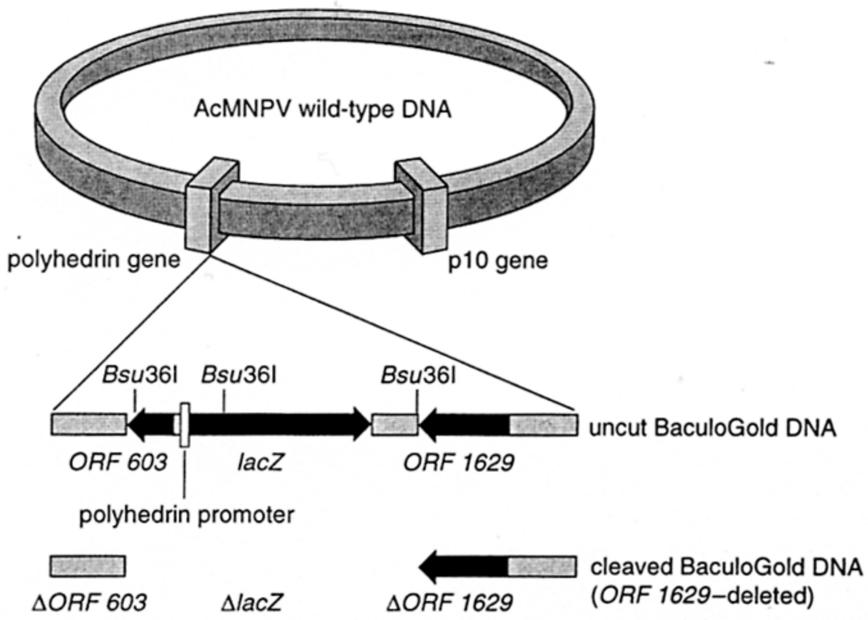


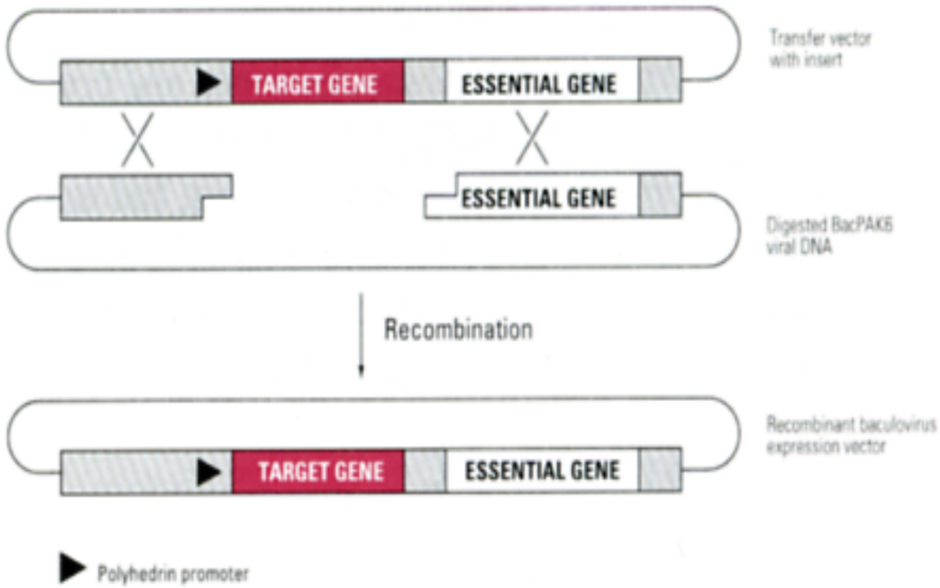
30% of all amplified virus particles will be recombinant

Procedure for BEVS with a linearized baculoviral DNA



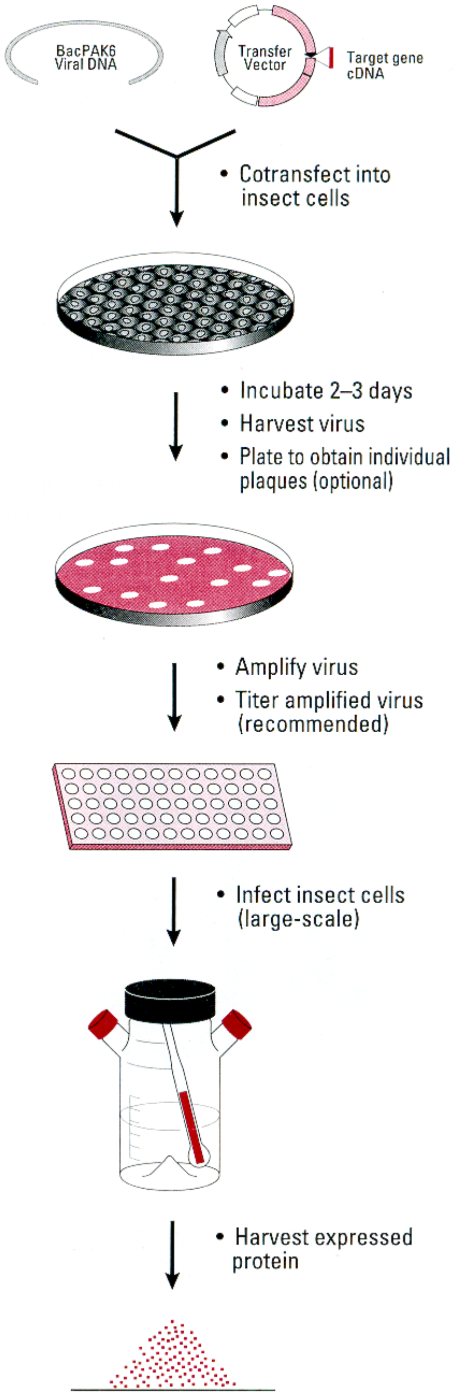
Recombinantion of transfer vector and modified AcNPV Baculovirus DNA

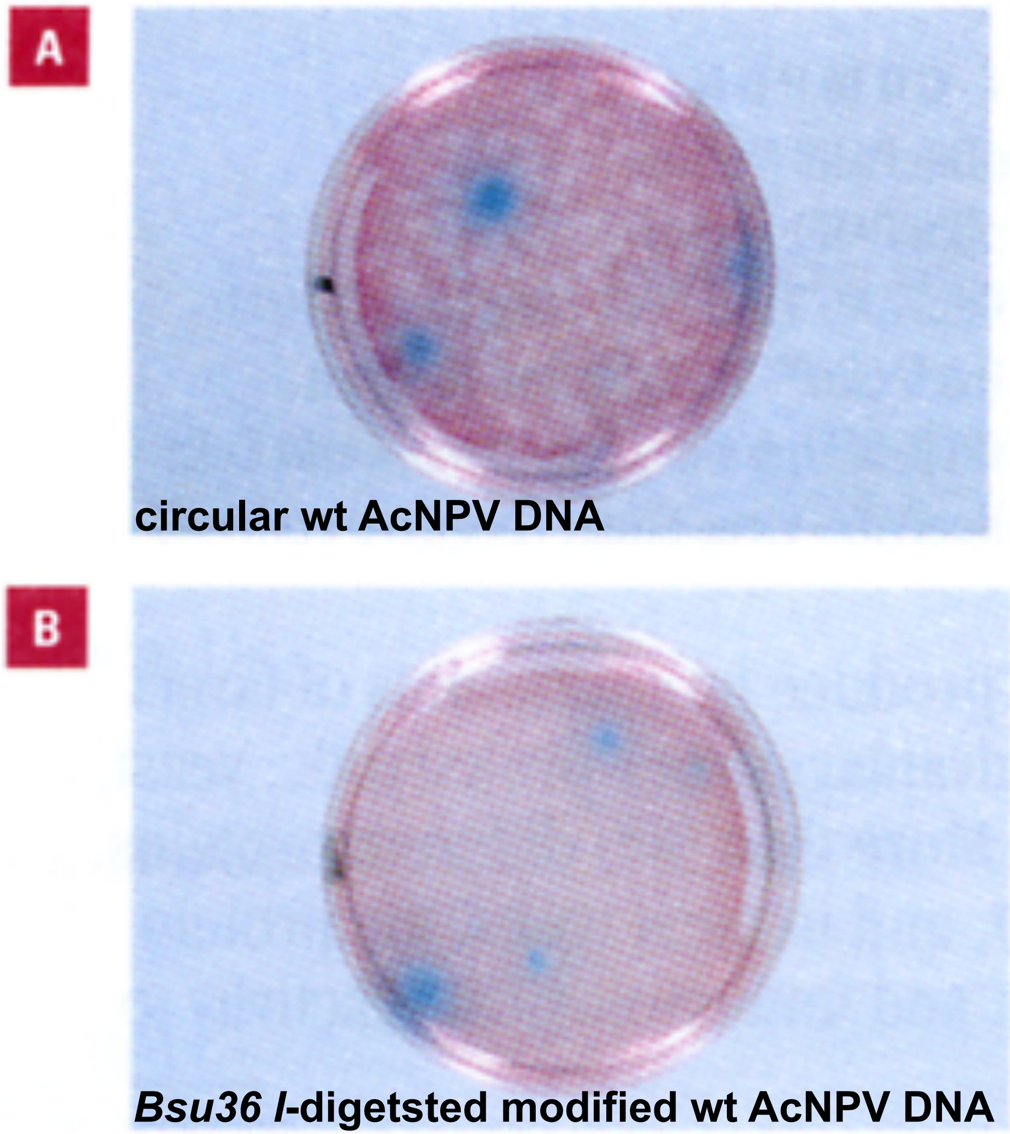




>99.9% of all amplified virus particles will be recombinant

Flow chart of an AcNPV modified DNA Baculovirus system

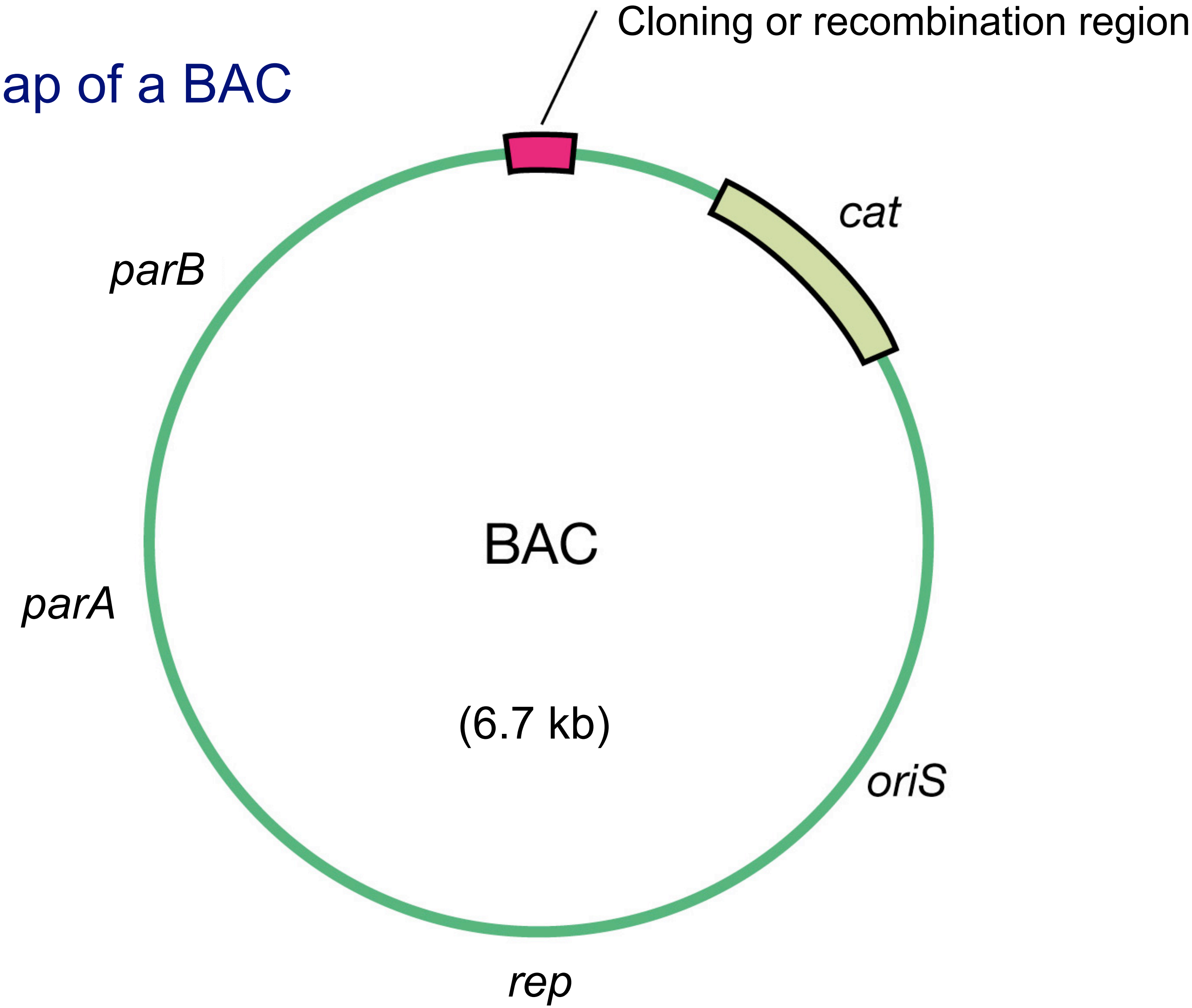




Insect- *E. coli* Shuttle Vector

- Shuttle Vector
  - Transfection of insect cells required only for production of recombinant protein
  - All other manipulations done in *E. coli*
- Baculovirus plasmid or Bacmid

Genetic map of a BAC

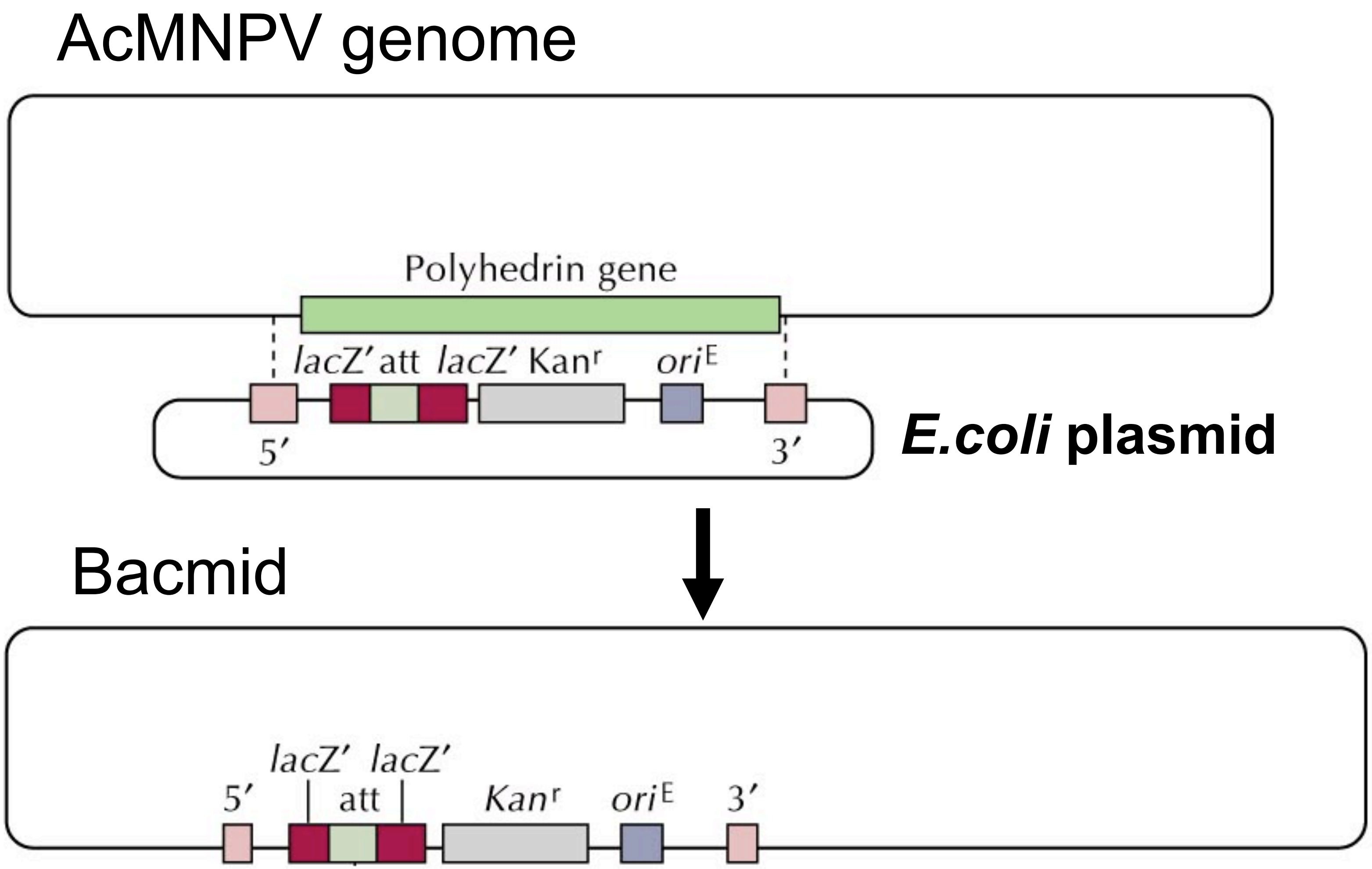


Foreign DNA of >300 kb can be inserted and stably maintained in BAC vectors

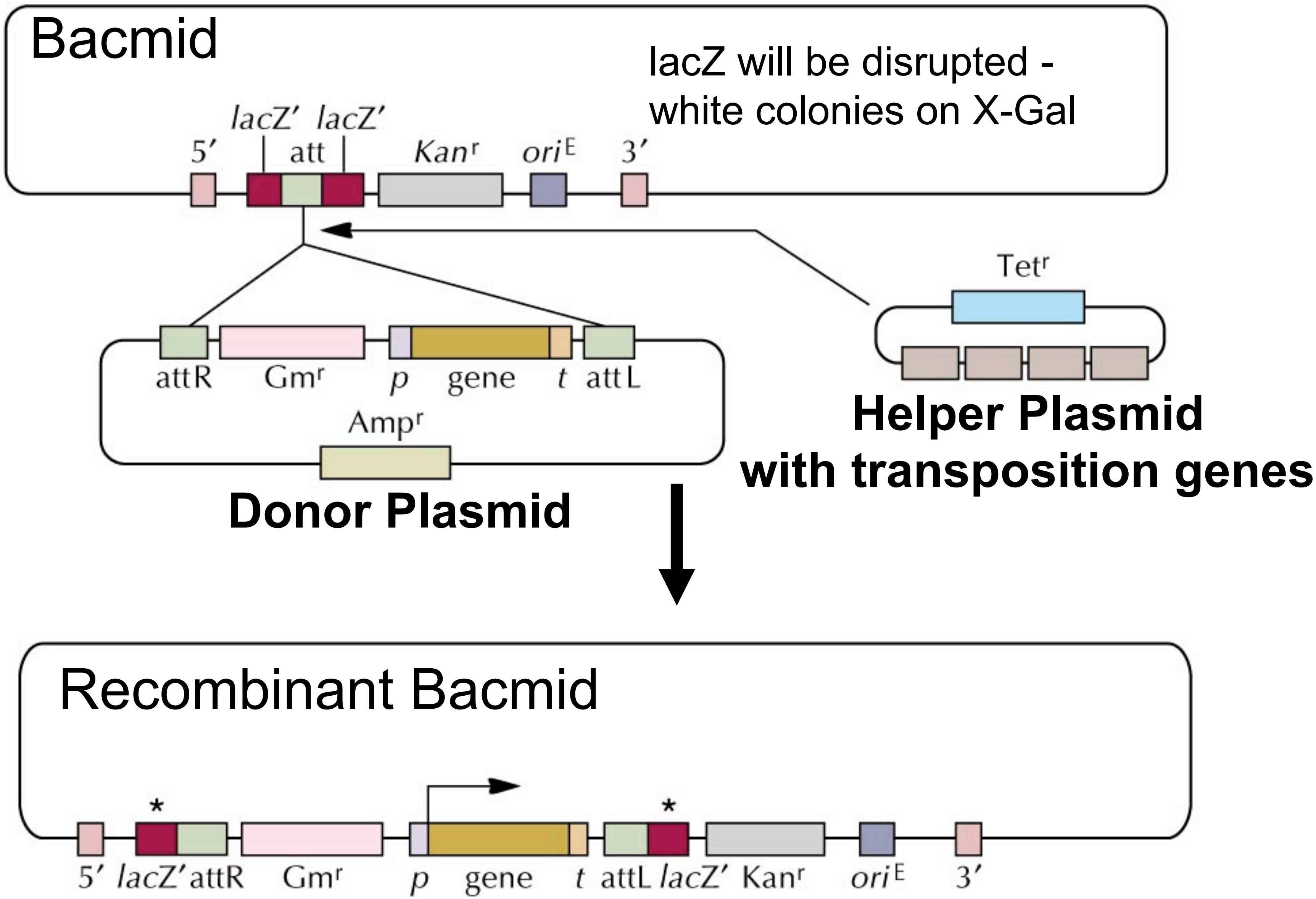
AcMNPV Bacmid

- Formed by integration of *E. coli* plasmid into AcMNPV genome
  - double crossover event
  - *ori* of replication, select. marker, etc.
- Target gene cloned into a second plasmid
  - Transfer vector donor plasmid
- A third plasmid provides proteins to move gene of interest into bacmid
  - Helper plasmid

Generation of a AcMNPV Bacmid

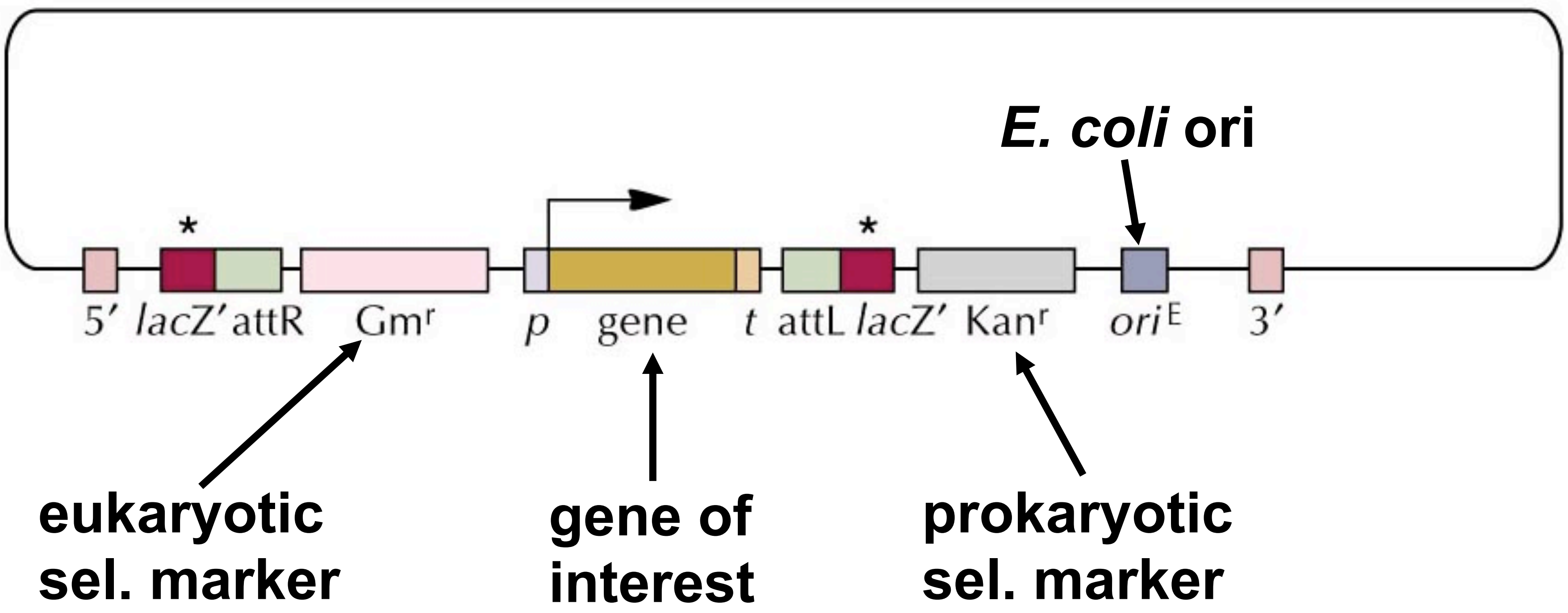


Generation of a recombinant AcMNPV bacmid

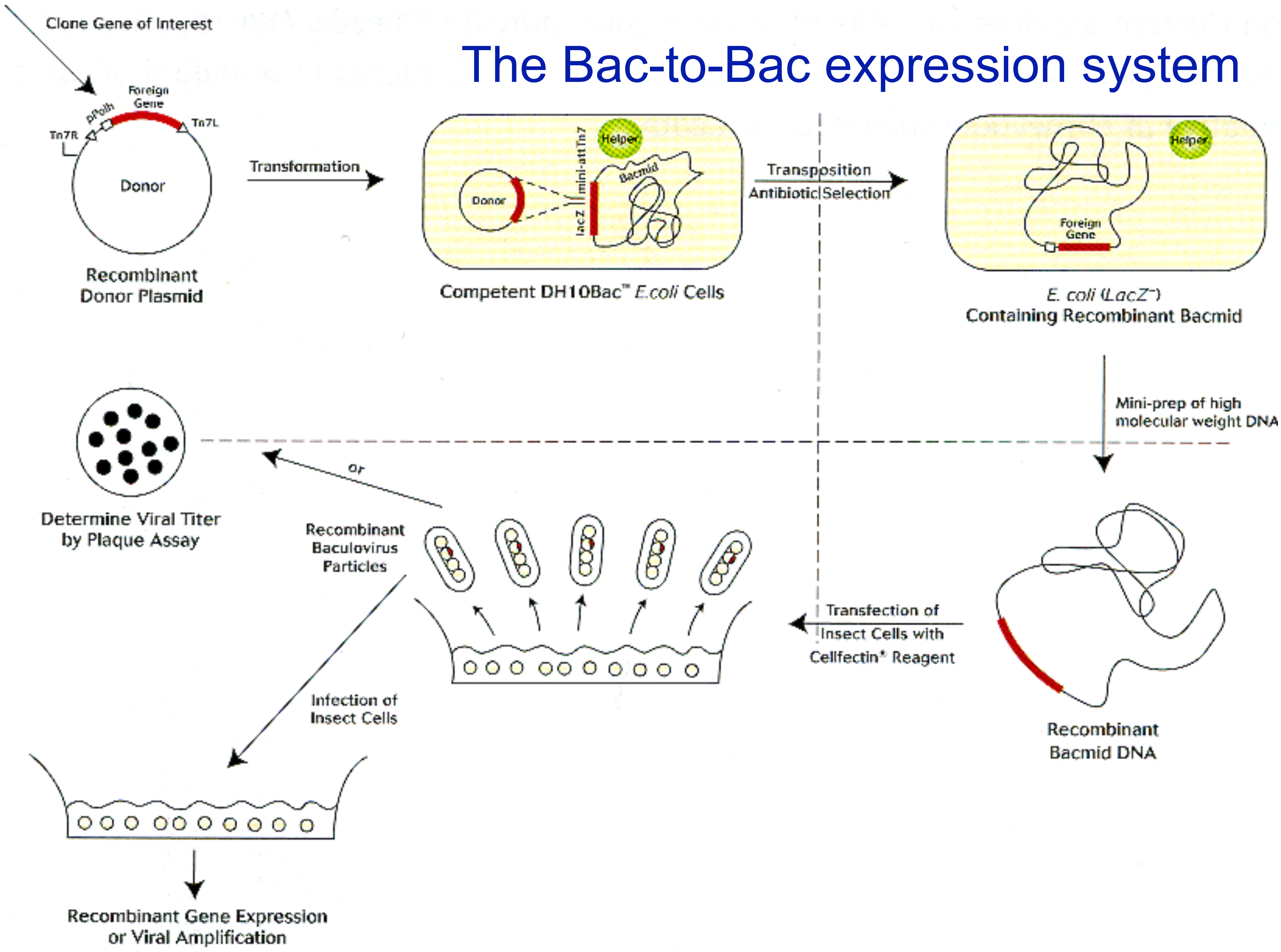


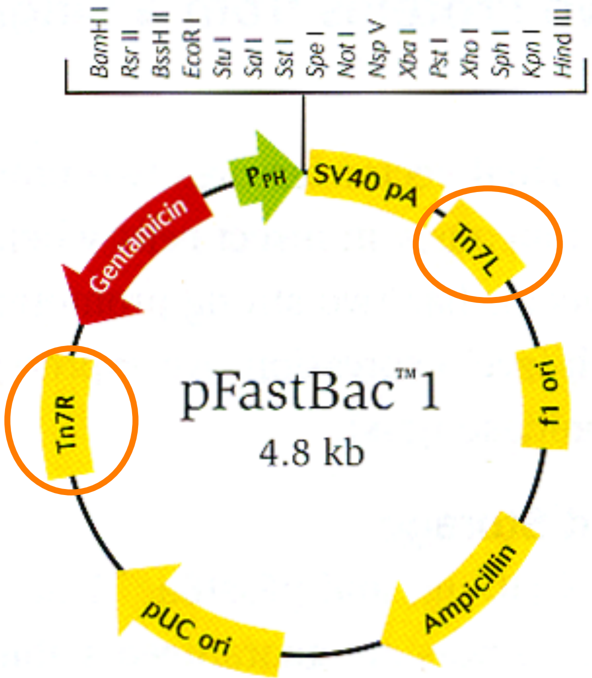
Recombinant AcMNPV Bacmid

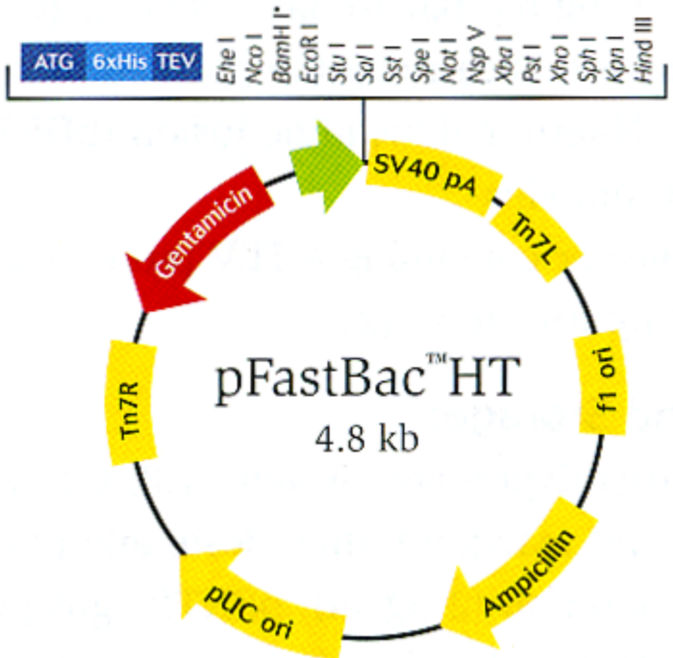
This system relies on generation of recombinant baculovirus by **site-specific transposition** in *E. coli* rather than **homologous recombination** in insect cells.

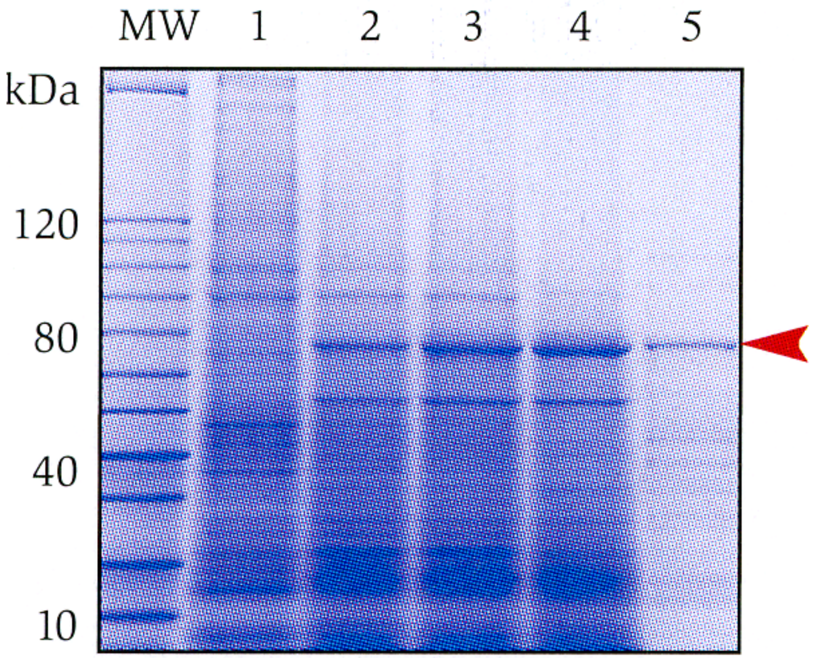


The Bac-to-Bac expression system



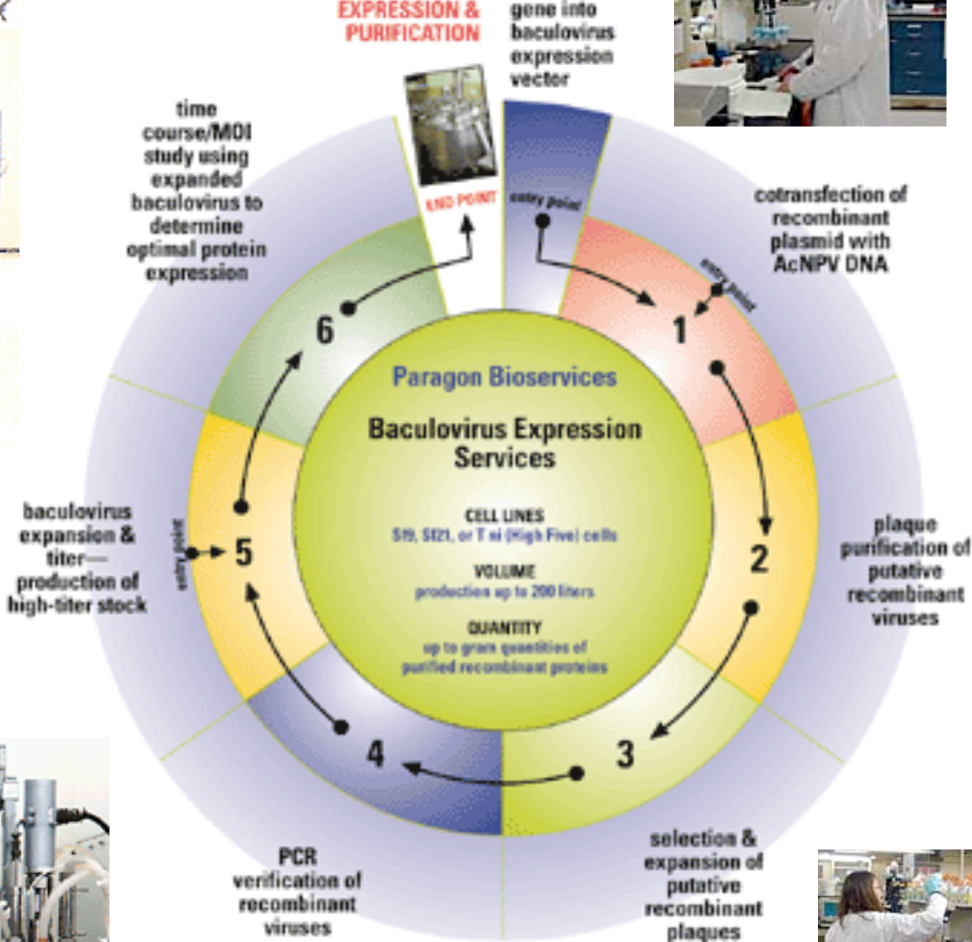


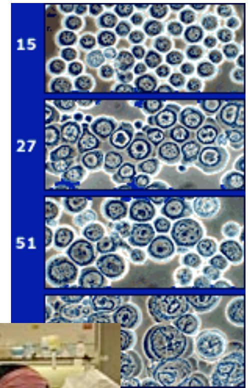




MW: 10 kDa Protein Ladder
Lane 1: Uninfected Sf9
Lane 2: Sf9 infected cells
Lane 3: Sf21 infected cells
Lane 4: High Five™ infected cells
Lane 5: 2 µg purified β-glucuronidase

BEVS for large scale protein expression



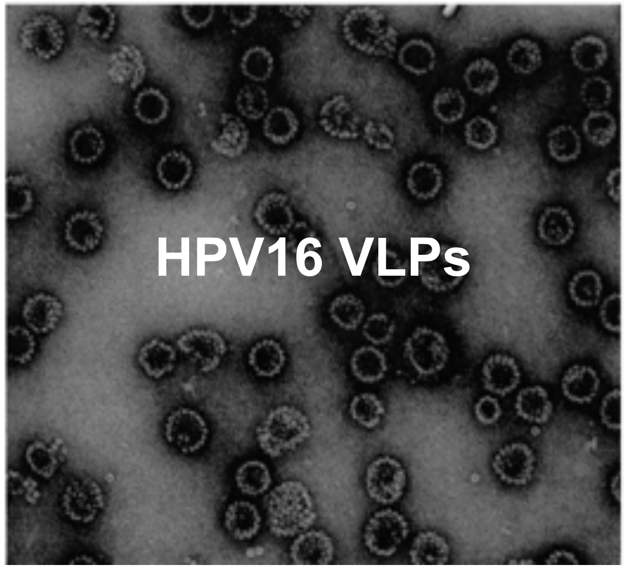


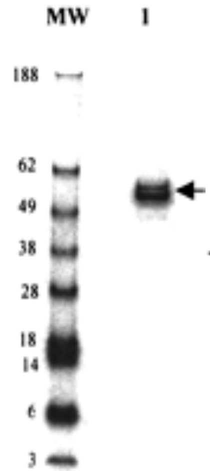
Suggested reading: J.Nat.Cancer Inst. 93, 284 -291,2001

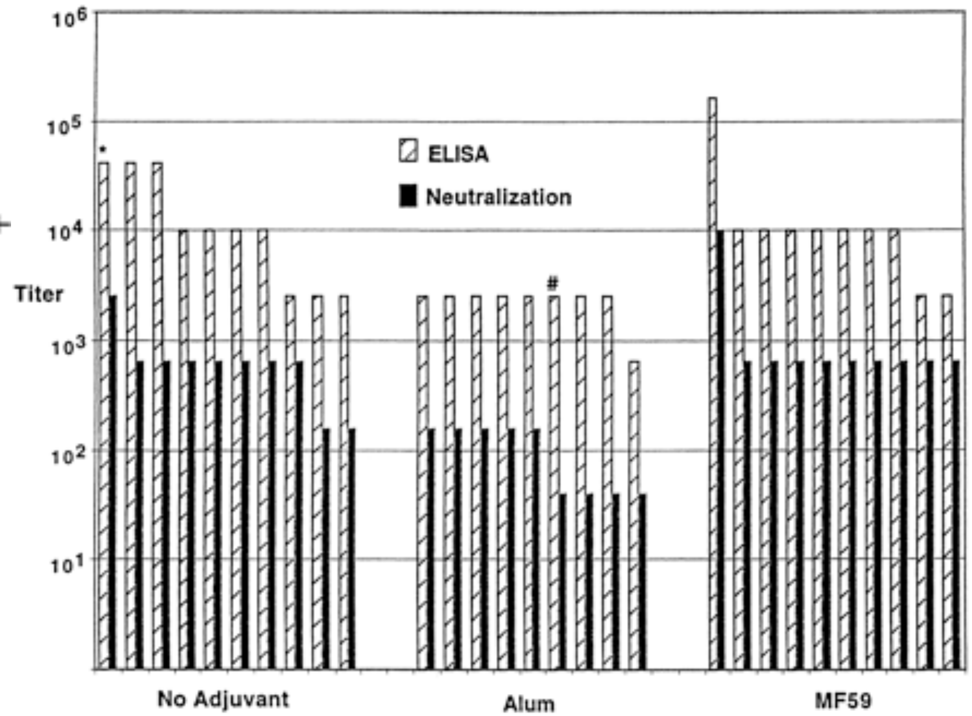
ARTICLES

Safety and Immunogenicity Trial in Adult Volunteers of a Human Papillomavirus 16 L1 Virus-Like Particle Vaccine

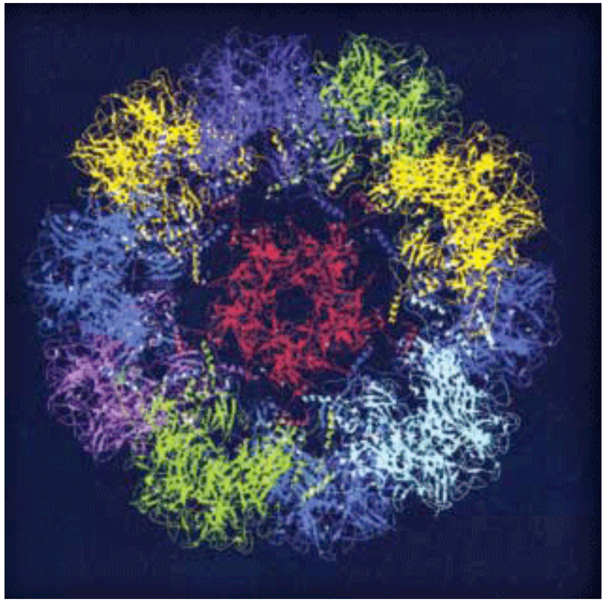
Clayton D. Harro, Yuk-Ying Susana Pang, Richard B. S. Roden, Allan Hildesheim, Zhaohui Wang, Mary Jane Reynolds, T. Christopher Mast, Robin Robinson, Brian R. Murphy, Ruth A. Karron, Joakim Dillner, John T. Schiller, Douglas R. Lowy







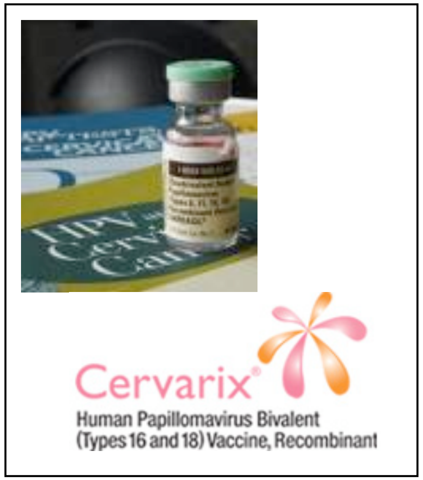
The recombinant HPV vaccines



Virus-like Particles (VLPs) Assembled from
the L1 Protein of Human Papillomavirus 16

baculovirus

yeasts



I vaccini contro il virus Hpv attualmente disponibili in Italia sono due:

•**Gardasil**, vaccino tetravalente, che protegge contro i genotipi 16-18 di HPV, responsabili di circa il 70% dei casi di carcinoma uterino, e i genotipi 6 e 11, responsabili del 90% dei condilomi, autorizzato all'immissione in commercio da Aifa con delibera del 28.02.2007 (costo al pubblico 171,64 euro).

•**Cervarix**, vaccino bivalente, attivo contro i genotipi 16 e 18, responsabili di circa il 70% dei casi di carcinoma uterino, autorizzato da Aifa con delibera del 29.10.2007 (costo al pubblico 156,79 euro).

•I vaccini sono somministrati gratuitamente dalle ASL alle bambine tra gli undici e i dodici anni, con la somministrazione per via intramuscolare di una dose iniziale e due richiami, entro i sei mesi dalla prima.

•Nel 2008 sono state vaccinate 280.000 bambine nate nel 1997; nel 2009 sono state vaccinate le nate del 1998 e così via per gli anni successivi.

•Il vaccino è inoltre disponibile a pagamento in farmacia, previa indicazione e prescrizione del medico, ed è destinato alle donne fino a 25 anni che non hanno ancora contratto l'infezione da HPV.