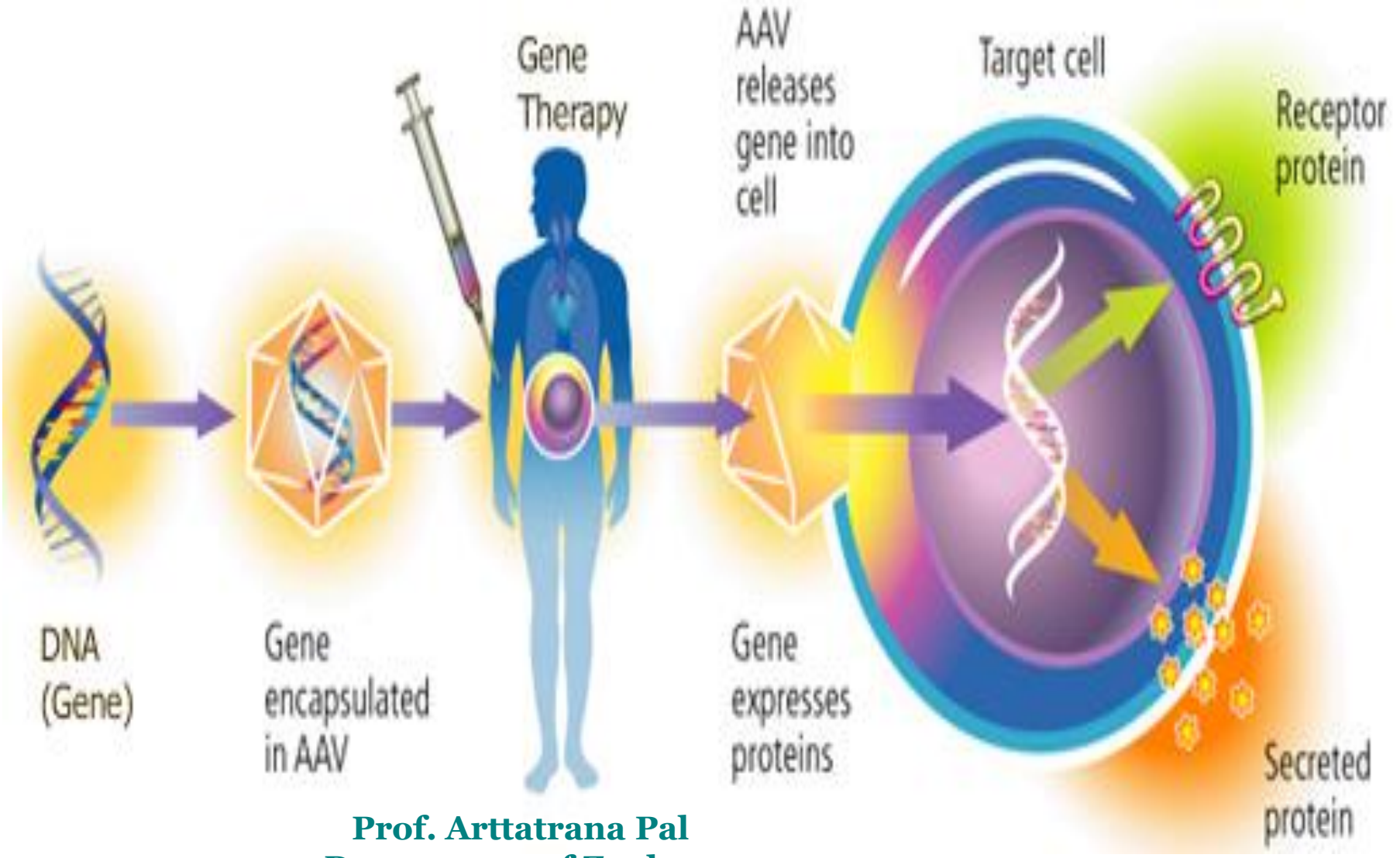
Gene Therapy- II



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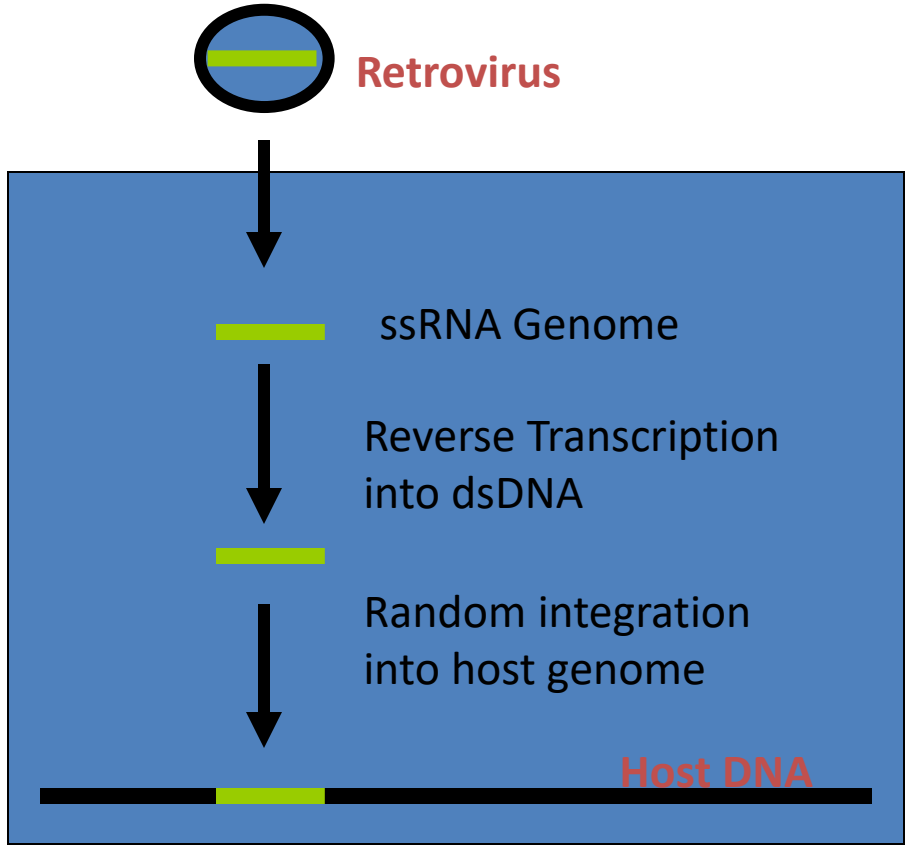
Retroviruses

(including Lentivirus, HIV and MMLV based vectors)

- **Single stranded RNA genome**
- **Lipid membrane enveloped**
- **Host range determined by envelope proteins**



Retroviral vectors

- Retroviral vectors are based on Moloney murine leukemia virus (Mo-MLV) which is capable of infecting both mouse and human cells

- The viral genes, gag, pol and env, are replaced with the transgene of interest and expressed on plasmids in the packaging cell line

- Because the non-essential genes lack the packaging sequence, they are not included in the virion particle

- To prevent recombination resulting in replication competent retroviruses, all regions of homology with the vector backbone is removed

Retroviral Genome

Long Terminal Repeat (LTR): **Necessary for integration into host genome**

[Long Terminal Repeats (LTRs): The Retroviral Promoter]

ψ (Psi): **packaging signal**

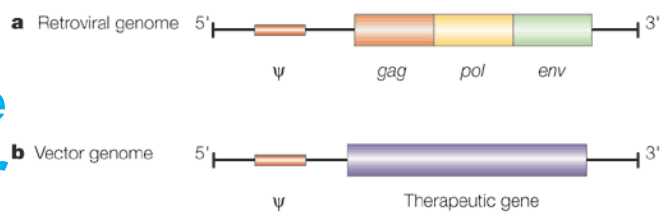
***gag*:** **Packages viral genome into viral particles**

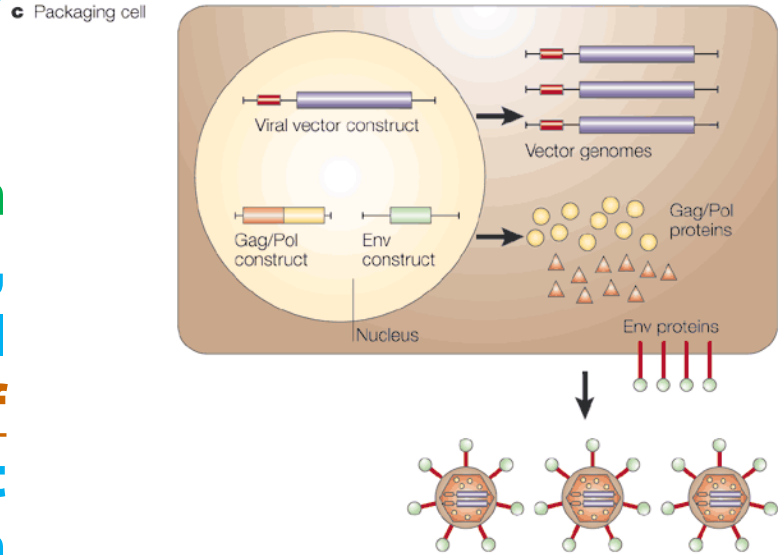
***pol*:** **viral polymerase necessary for viral replication**

***env*:** **viral envelope proteins, necessary for entry into host cells, dictate host range**

Retroviral vectors

Transcription could be under the control of LTRs or enhancer promoter elements might be engineered in with the transgene

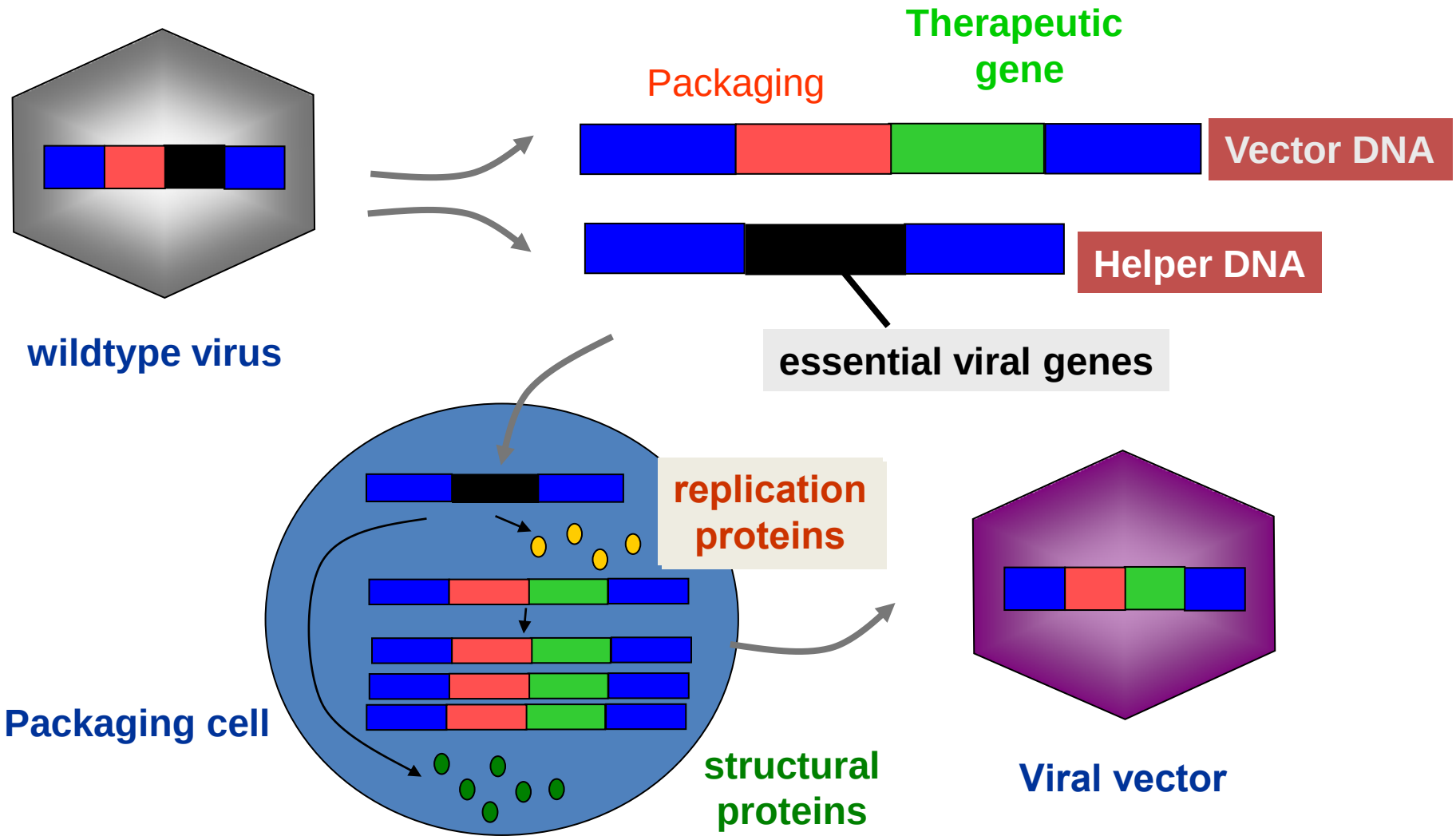




The chimeric genome is then introduced into a packaging cell, which produces all of the viral proteins, such as the products of the gag, pol and env genes, but these have been separated from the LTRs and the packaging sequence

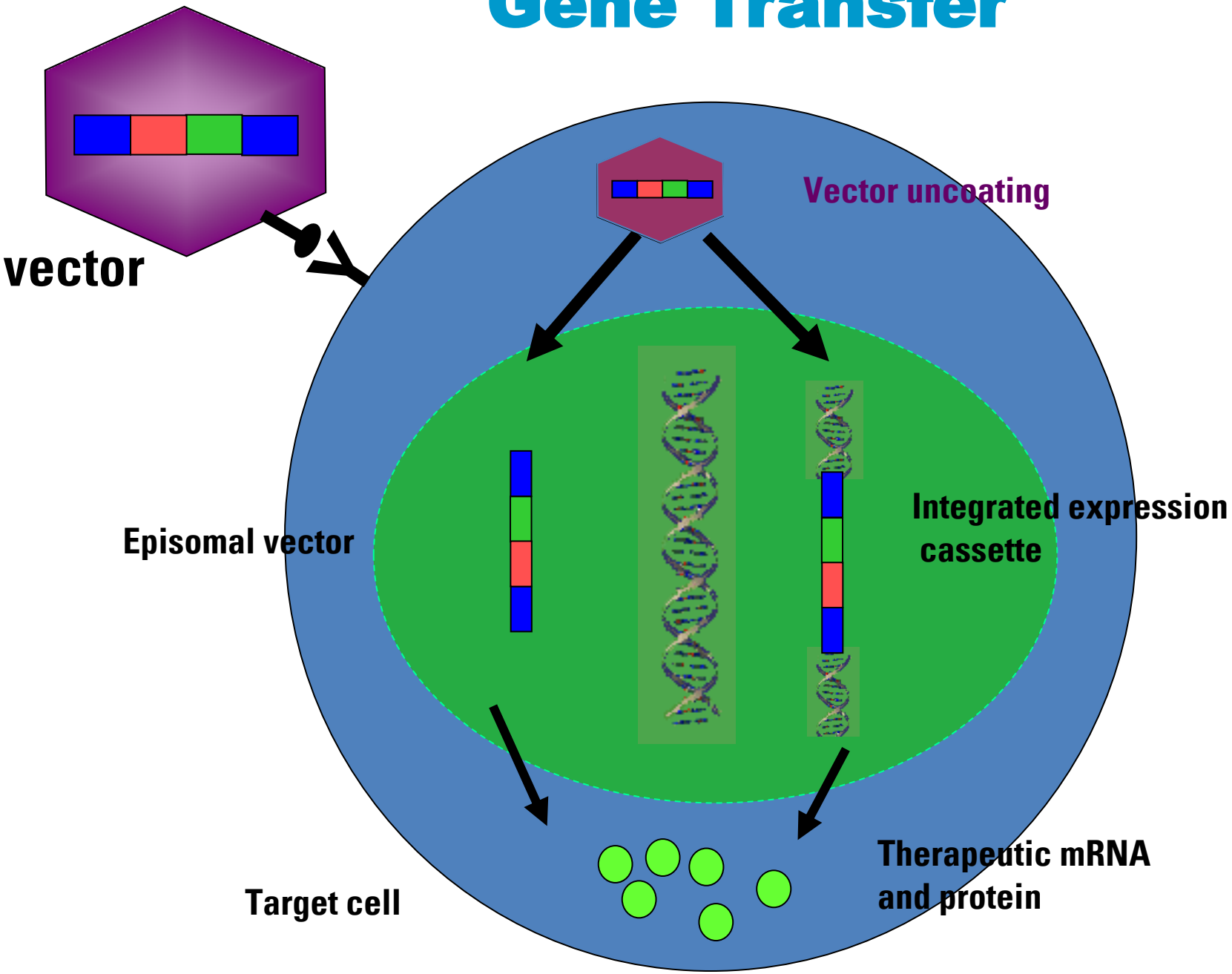
- **Only the chimeric genomes are assembled to generate a retroviral vector**
- **The culture medium in which these packaging cells have been grown is then applied to the target cells, resulting in transfer of the transgene**

Engineering a virus into a viral vector

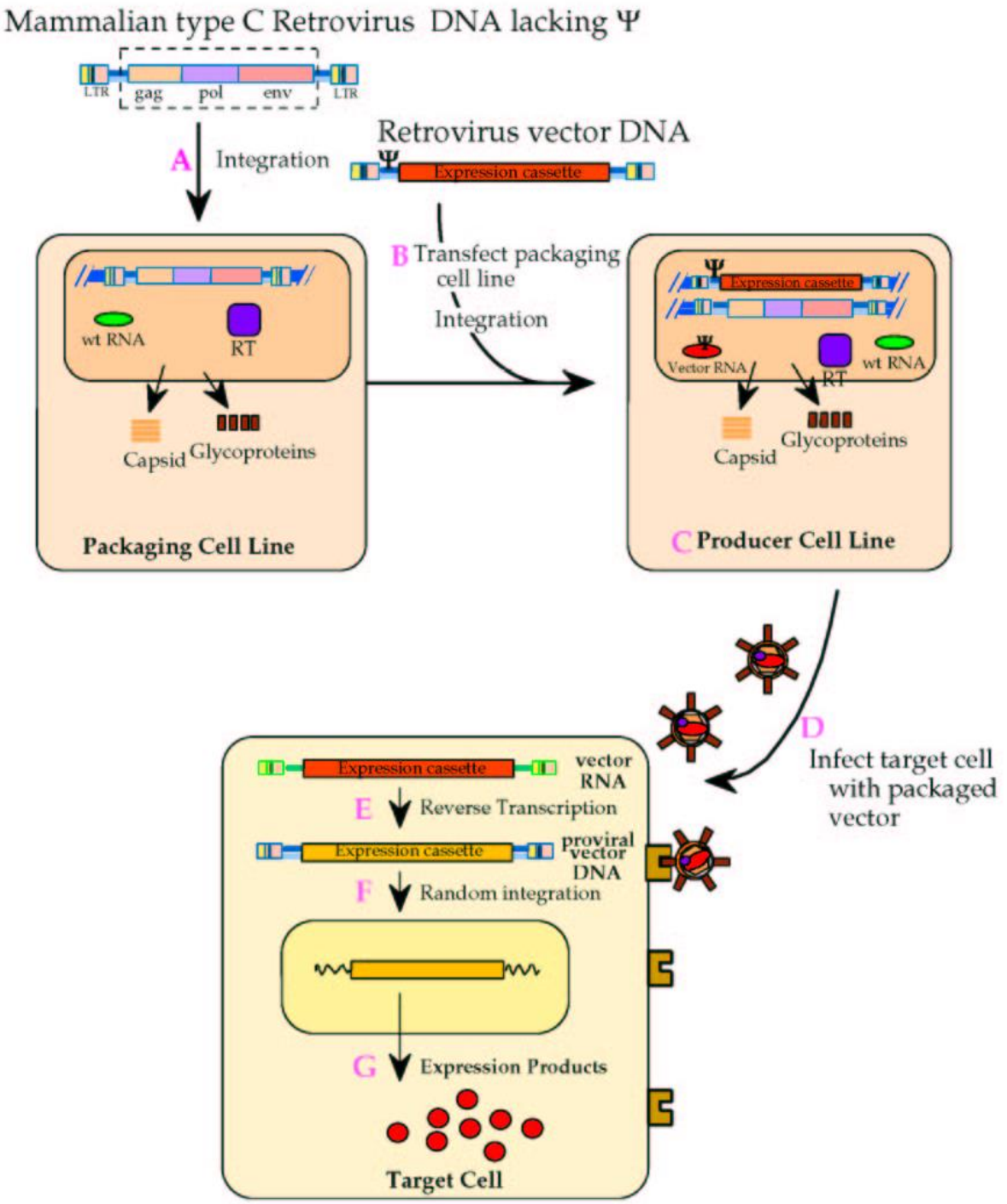


http://www.edu365.com/aulanet/comsoc/Lab_bio/simulacions/GeneTherapy/GeneTherapy.htm

Gene Transfer



Generation of Packaged Replication-deficient Retroviros Expression Vector



Characteristics of Retroviral vector

- Stable integration, potential long-term expression
- Excellent safety record
- Transduction resulted in therapeutic Factor VIII levels in preclinical models

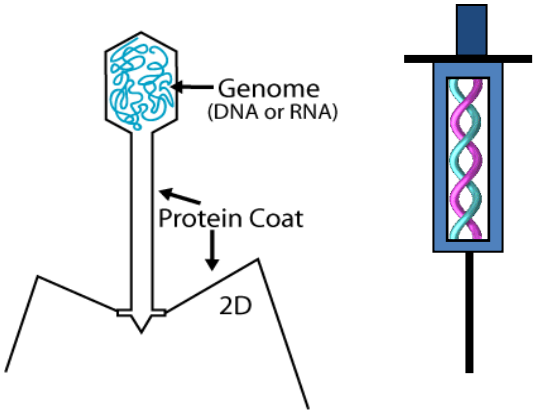
Retroviral vectors- Limitations

- A critical limitation of retroviral vectors is their inability to infect nondividing cells, such as those that make up muscle, brain, lung and liver tissue
- The cells from the target tissue are removed, grown in vitro and infected with the recombinant vector, the target cells are producing the foreign protein are then transplanted back into the animal (ex vivo gene therapy)
- Problems with expression being shut off, prolonged expression is difficult to attain
- Expression is reduced by inflammatory interferons acting on viral LTRs, as the retroviral DNA integrates, viral LTR promoters are inactivated
- Possibility of random integration of vector DNA into the host chromosome

Infectious Viruses: A Genetic "Syringe"

Viruses are composed of genetic material encapsulated in a protein coat

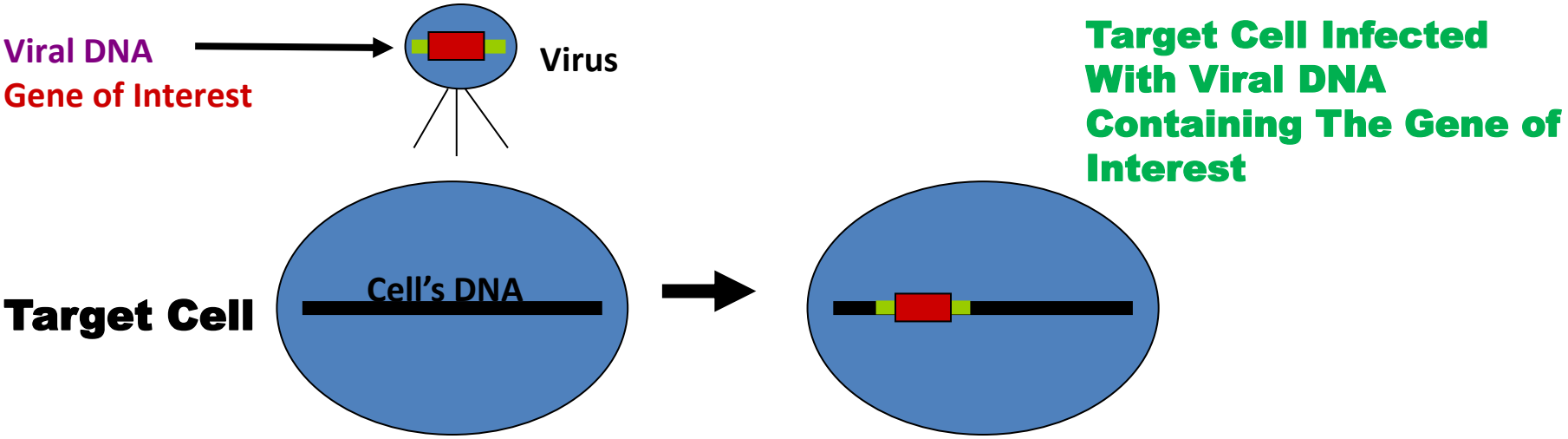
Viruses inject their genetic material into target cells



DNA Loaded Syringe

Viruses infect target cells with their genetic material

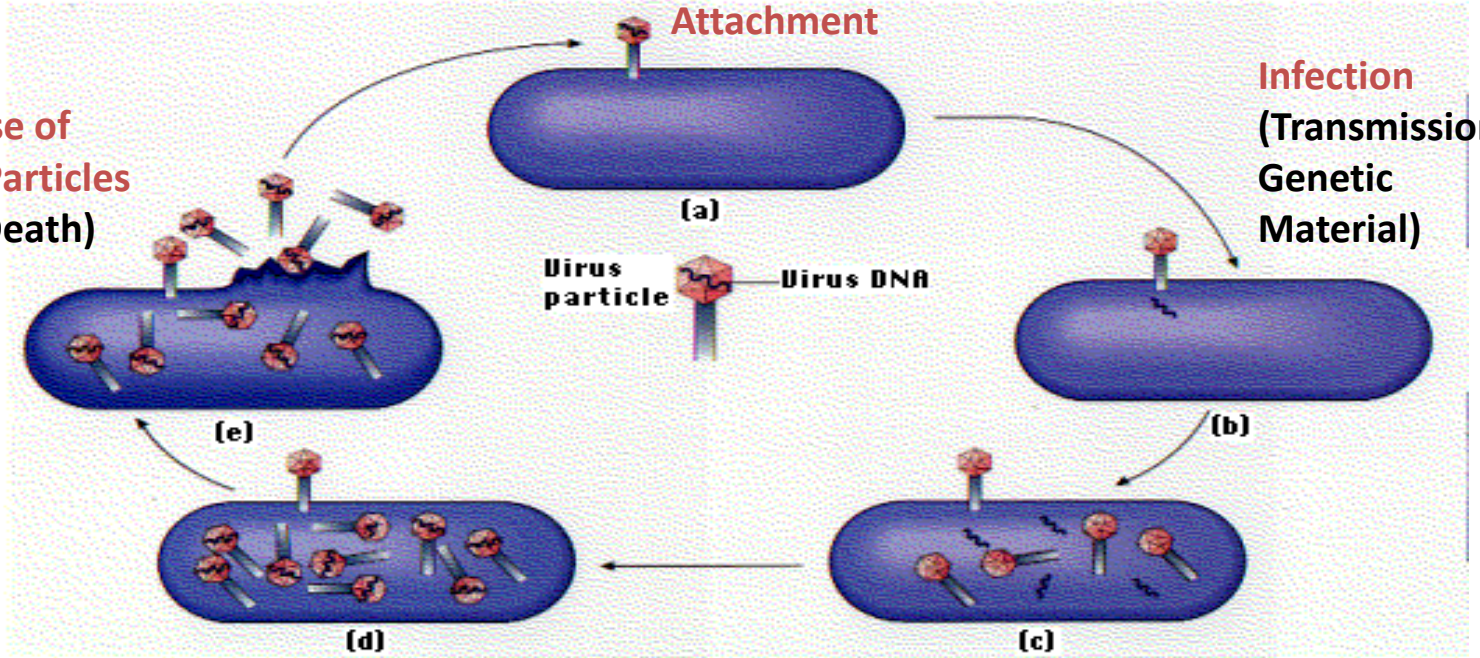
The viral DNA can be altered to contain a gene of interest (rDNA) to infect that gene into the target cell



Target Cell Infected With Viral DNA Containing The Gene of Interest

Safety Concerns Related to Infectious Viruses: A Genetic "Trojan Horse"

Viruses Cannot reproduce by themselves, so they infect cells with their genetic material to hijack the cellular machinery to produce more viruses. This process can result in cell death, tissue damage or even the death of the infected organism



Lentiviral Vectors

Belong to the retrovirus family but can infect both dividing and non-dividing cells

They are more complicated than retroviruses, containing an additional six proteins, tat, rev, vpr, vpu, nef and vif

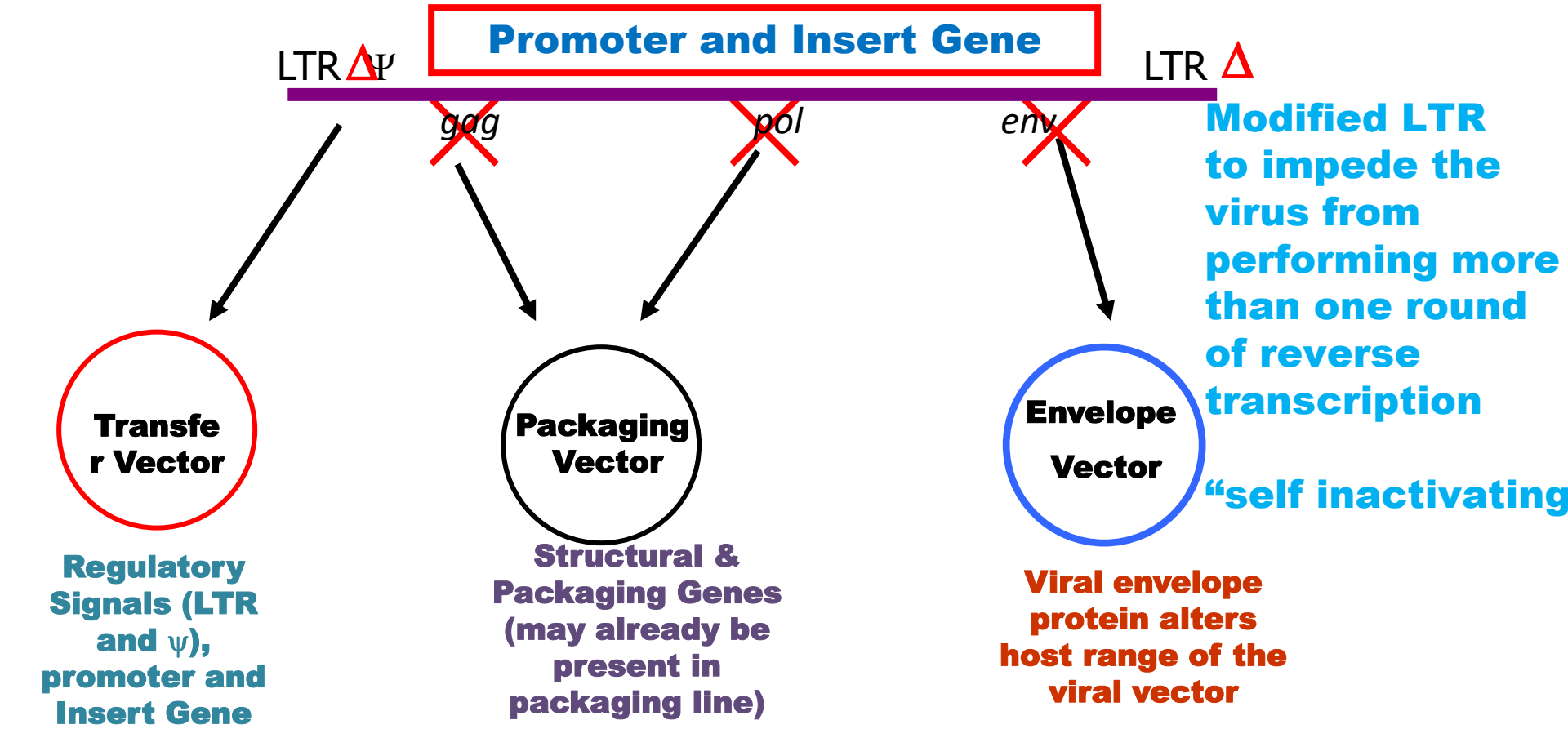
Human immunodeficiency virus (HIV) has been disabled and developed as a vector for in vivo gene delivery

Low cellular immune response, thus good possibility for in vivo gene delivery with sustained expression over six months

No potent antibody response

Design of Replication Incompetent Lentiviral Vectors

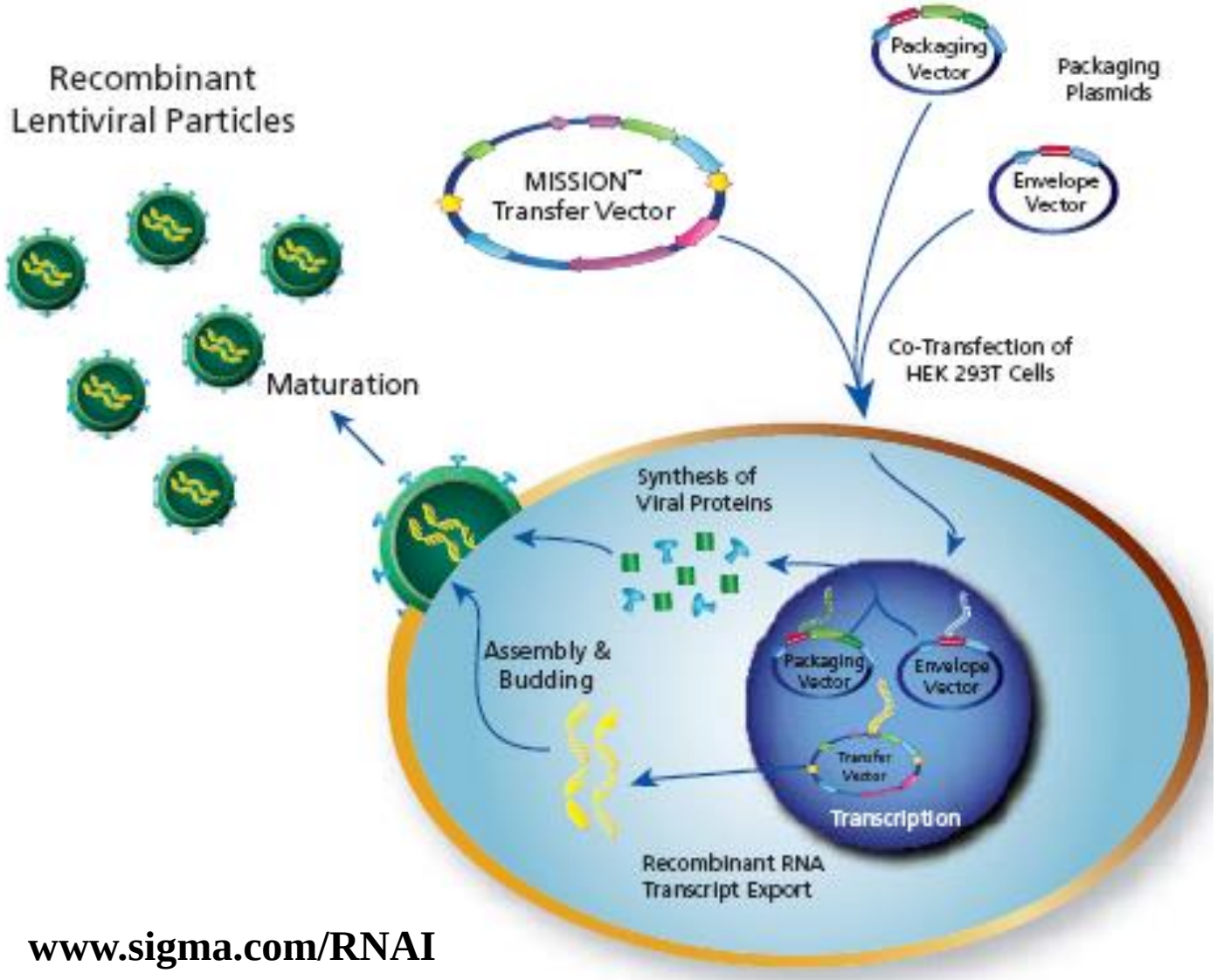
The viral vector is "gutted" as much as possible to create room for the insert gene and to divide the viral genome into cis- and trans- acting regions



Modified LTR to impede the virus from performing more than one round of reverse transcription

"self inactivating

Packaging Recombinant Lentiviral Particles



The three plasmids containing the viral genome components are transfected into the packaging line to create the infectious viral particles.

Multiple plasmids are used so multiple recombination events would be required to reconstitute a replication competent virus.

www.sigma.com/RNAI

Viral Psuedotyping: A Double Edged Sword

Tropism: The ability of a virus to infect a particular type of host cell

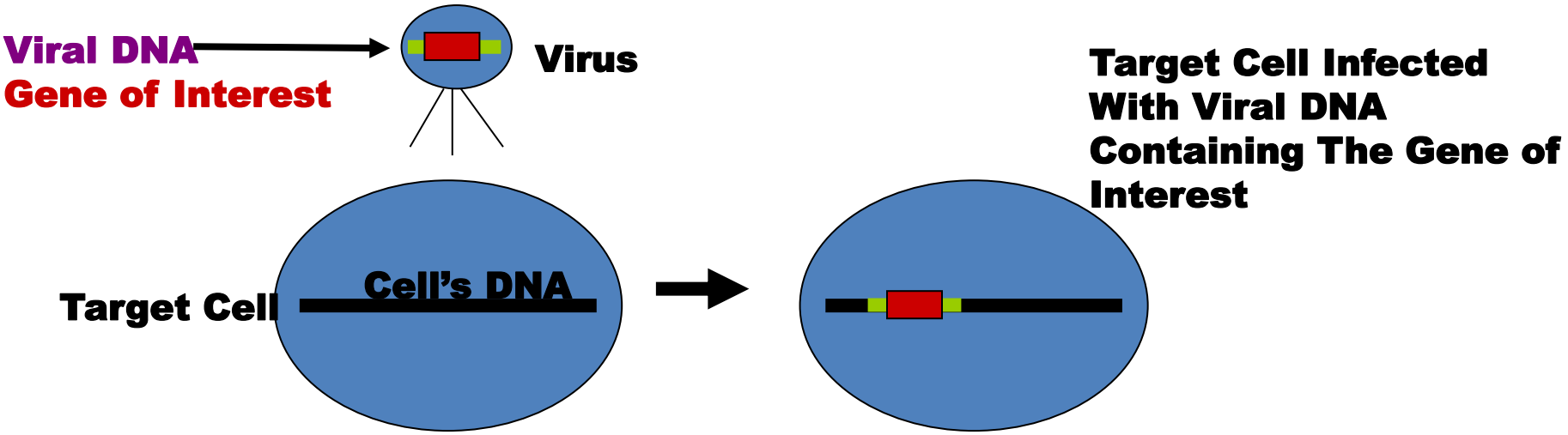
Psuedotyping: Altering the viral envelope protein to alter tropism, thus allowing the virus to infect cells it originally could not

Tropism	Host Range	Viral Envelope Protein	Receptor for Viral Envelope
Ecotropic	Mouse / Rat	Gap70	mCAT-1
Amphotropic / Dualtropic	Mammals	4070A / 10A1	Ram-1 / GALV
Pantropic	All Animals	VSV-G	Phosphotidyl serine Phosphotidyl inositol GM3 ganglioside

Special care should be used when working with pantropic or amphotropic viruses which can infect humans!

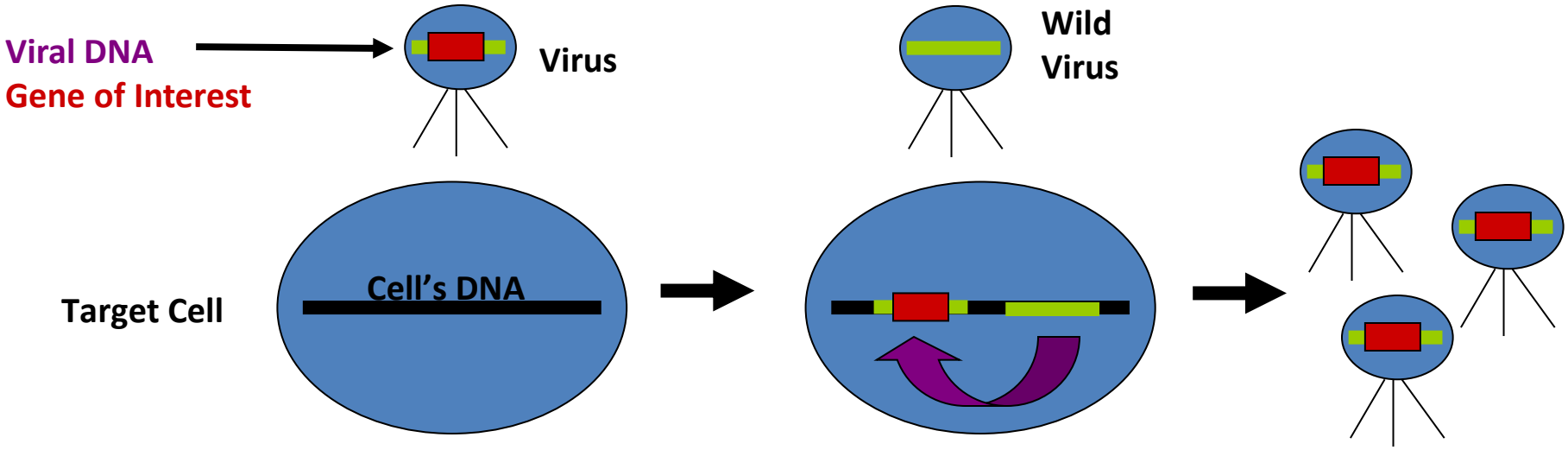
Replication Deficient Viral Vectors: Genetically Engineered So The Viral Infection Cannot Spread

The viral DNA does not contain the viral genes needed to make more viruses



No New Viral Particles are Created
Infection dose not spread

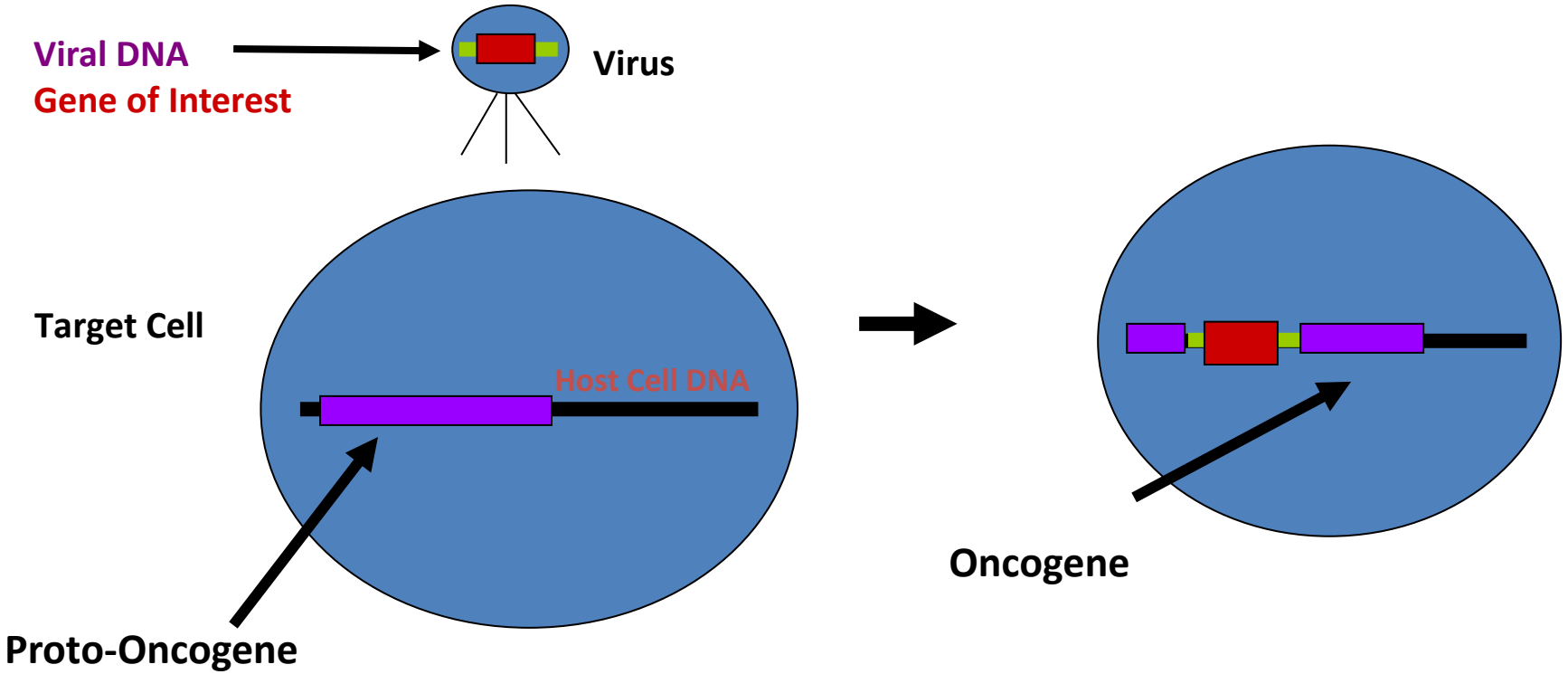
Rescue of Replication Deficient Viruses by super infection with Wild Viruses



Complementation:

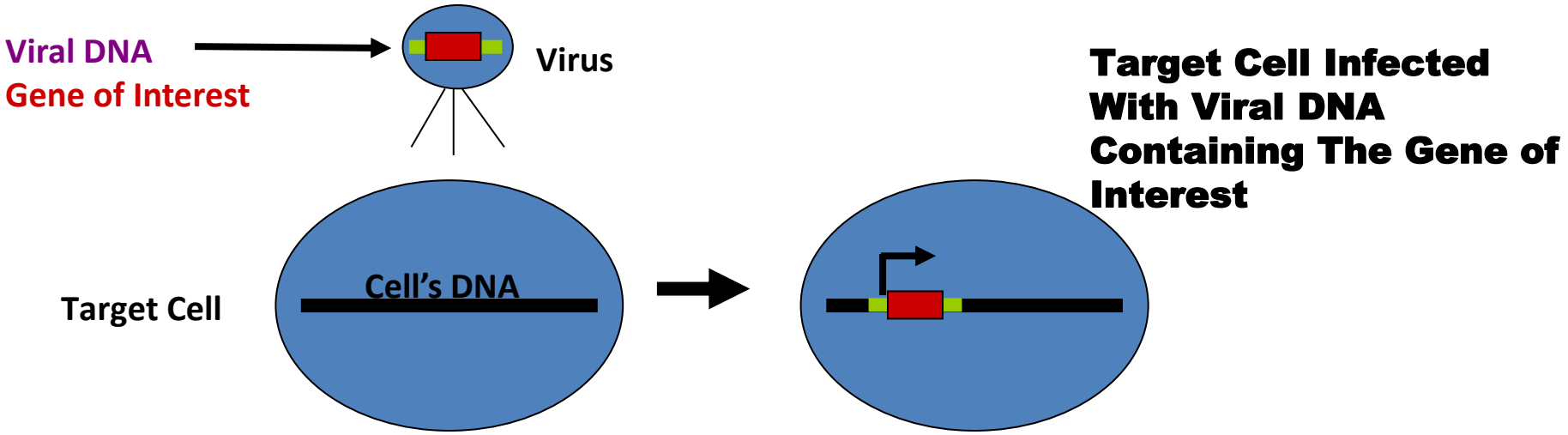
The genome from the wild virus provides the missing proteins needed for the viral vector to replicate. The super infected cell functions similarly to a packaging line

Risks Associated with Retroviruses:
Insertional Mutagenesis



Random integration of viral genome may disrupt endogenous host genes. Of special concern Is disruption of proto-oncogenes, which can lead to increased cancer risk

Risks Associated with Retroviral Vectors: Viral Transduction



Individuals infected with the viral vector may express the insert gene at the site of infection

Likely Symptoms of Lab Acquired Infections with Retro/Lentiviral Vectors

- Fever / flu-like symptoms
- Possible inflammation of infected tissues
- Random integration of viral genome into host genome can result in insertional mutagenesis and oncogenesis
- Expression of insert genes in infected tissues (oncogenes, inflammatory mediators and toxins are of special concern)

Common Methods of Deactivating Viruses

Lipid Enveloped Viruses

(Retro, Lenti, MMLV, HIV, Herpes Simplex, Flu, Hepatitis B and C)

Ethanol ← Cavicide
Quaternary Ammonium Compounds
Phenol
10% Bleach
Aldehydes (Paraformaldehyde, Gluteraldehyde)
Autoclave

Non-Lipid Enveloped

(Adenovirus, Adeno-Associated Virus)

10% Bleach
Aldehydes (Paraformaldehyde, Gluteraldehyde)
Autoclave

Please note:

- **Non-lipid Enveloped Viruses are Resistant to weaker disinfectants like ethanol and quaternary ammonium compounds**
- **10% bleach decomposes over time and has an approximate half life of 2 weeks. Recommend making fresh weekly**
- **Liquid disinfectants must be allowed the Appropriate contact time to be effective**

Risk Assessment

Risk assessment is a vital part of the IBC (Institutional Biosafety Committee**) review process as required by the NIH**

The purpose of a risk assessment is to determine the risk to researchers, the community and the environment

Steps to conduct a risk assessment:

- **Identify hazards**
- **Assess possibility for exposure**
- **Manage the risk**

Managing risk involves implementing controls to limit risk

Example of controls include:

Personal Protective Equipment (PPE): gloves, lab coat, eye and respiratory protection

Engineering: Biosafety Cabinet, centrifuge with sealed rotors or safety caps

Work Place Practices: Following the PI's approved biosafety protocol

Administrative: Training, supervision, lab inspections, vaccination, medical surveillance

Containing Risks Associated with Aerosols

Aerosol Producing Procedure	Method of Containment
Splash/Spray	biosafety cabinet, fume hood, splash shield
Vortexing	sealed tubes, biosafety cabinet
Centrifugation	sealed tubes, sealed rotor, safety cups
Homogenization	biosafety cabinet, fume hood, splash shield
Flow cytometry	fixation or BSL2+ containment
Injection/administration Into animals	biosafety cabinet, animal restraint
Cage cleaning (infected animals)	biosafety cabinet, etc.

Examples of Low Risk Work with Viral Vectors

Vector – Replication incompetent and self inactivating vector
Limited tropism (incapable of infecting humans)

Insert Gene – Is Not: toxic, oncogenic, immune modulatory, or increases tropism or pathogenicity

Procedures – limited to cell culture in a biosafety cabinet, centrifugation with sealed tubes and safety caps or sealed rotors

Volumes – 1-10 mL (easy to contain and transport)

Examples of High Risk Work with Viral Vectors

Vector – Replication competent vector, capable of infecting humans

Insert Gene – toxin or toxic at high levels, oncogene, immune modulation, increases viral tropism or pathogenicity

Procedures – aerosol production (homogenization, vortexing in open tubes, centrifugation without sealed tubes, safety caps or sealed rotors) injection/administration into animals

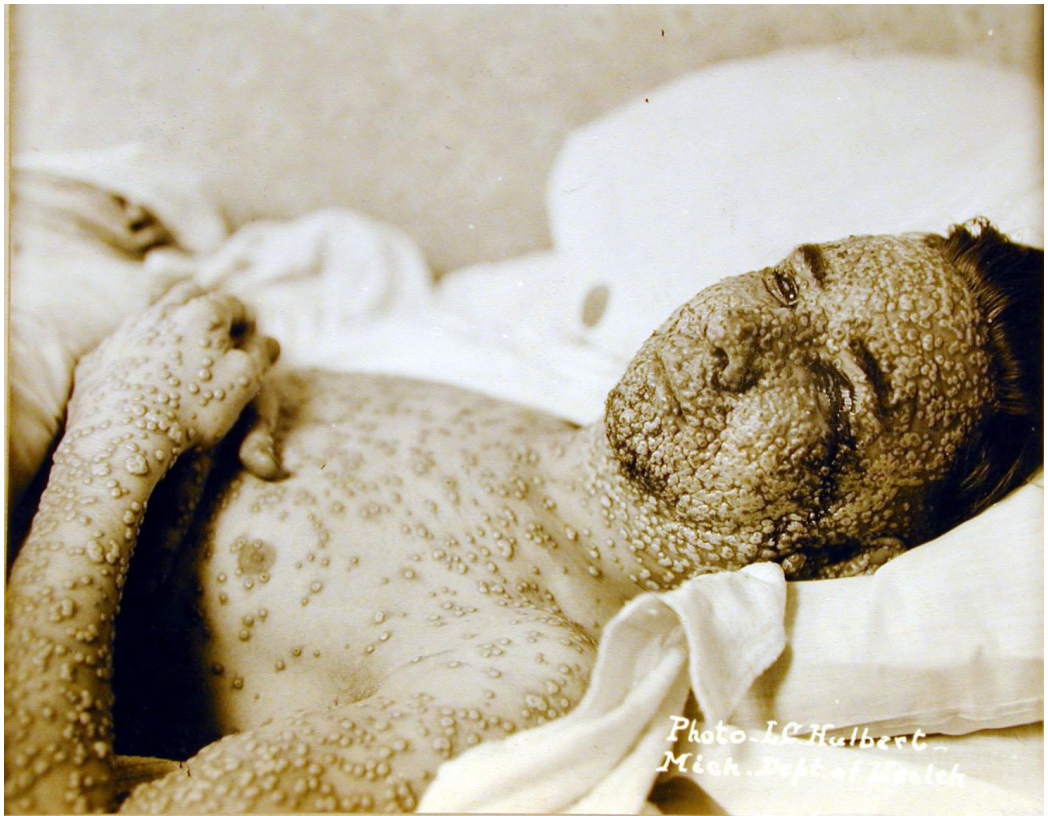
Volumes – Liters (requires bulkier containment and a cart to transport, higher likelihood of a spill)

Vaccinia virus vector

What is a Vaccinia Virus?

Vaccinia Virus is closely related to the pox-family of viruses, that was originally used as a vaccination and is currently being studied in more depth

Originated from pox lesions on infected cows



History

- Smallpox is an ancient disease, documented as endemic at least 2000 years ago

- First vaccine system - Edward Jenner, 1776 (Cowpox inoculum). Vaccinia virus is used later

- Worldwide eradication of smallpox declared by the WHO in 1976 due to widespread inoculation effort

Edward Jenner – 1798

General Characteristics of pox virus

- Large virus with double stranded DNA
- Cytoplasmic site of replication
- Encodes own multi-subunit RNA polymerase
- Genes expressed in a cascade fashion: early, intermediate, and late stages of gene expression
- Different gene stages have different promoter sequences and transcription factors

MORPHOLOGY OF THE VIRION

Have an brick-like shape; dimensions 400x200nm

Four major elements:

1. **Core (9 nm thick membrane, biconcave disk, a tightly compressed nucleoprotein)**

2. **Lateral bodies (unknown function)**

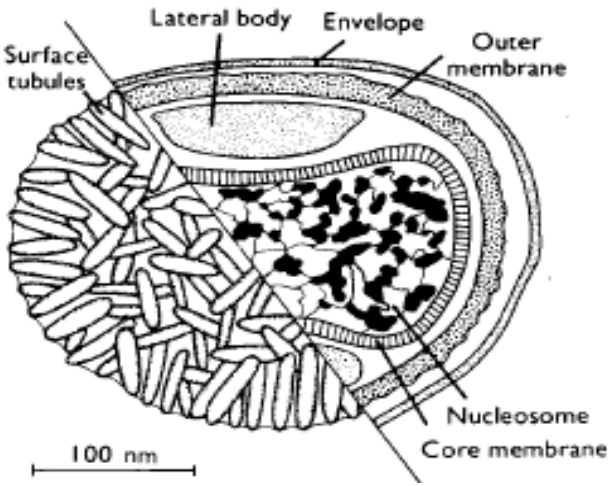
3. **Outer membrane (a protein shell 12nm thick, the surface consists of irregularly arranged tubules)**

4. **Envelope (an inconstant element, proteins are glycosylated and acylated)**

Core- nucleoprotein, is maintained in a superhelical configuration , and appears to occur in globuar structures interconnected by DNA-protein fibres , resembling the nucleosome structures of eukaryotic chromatin

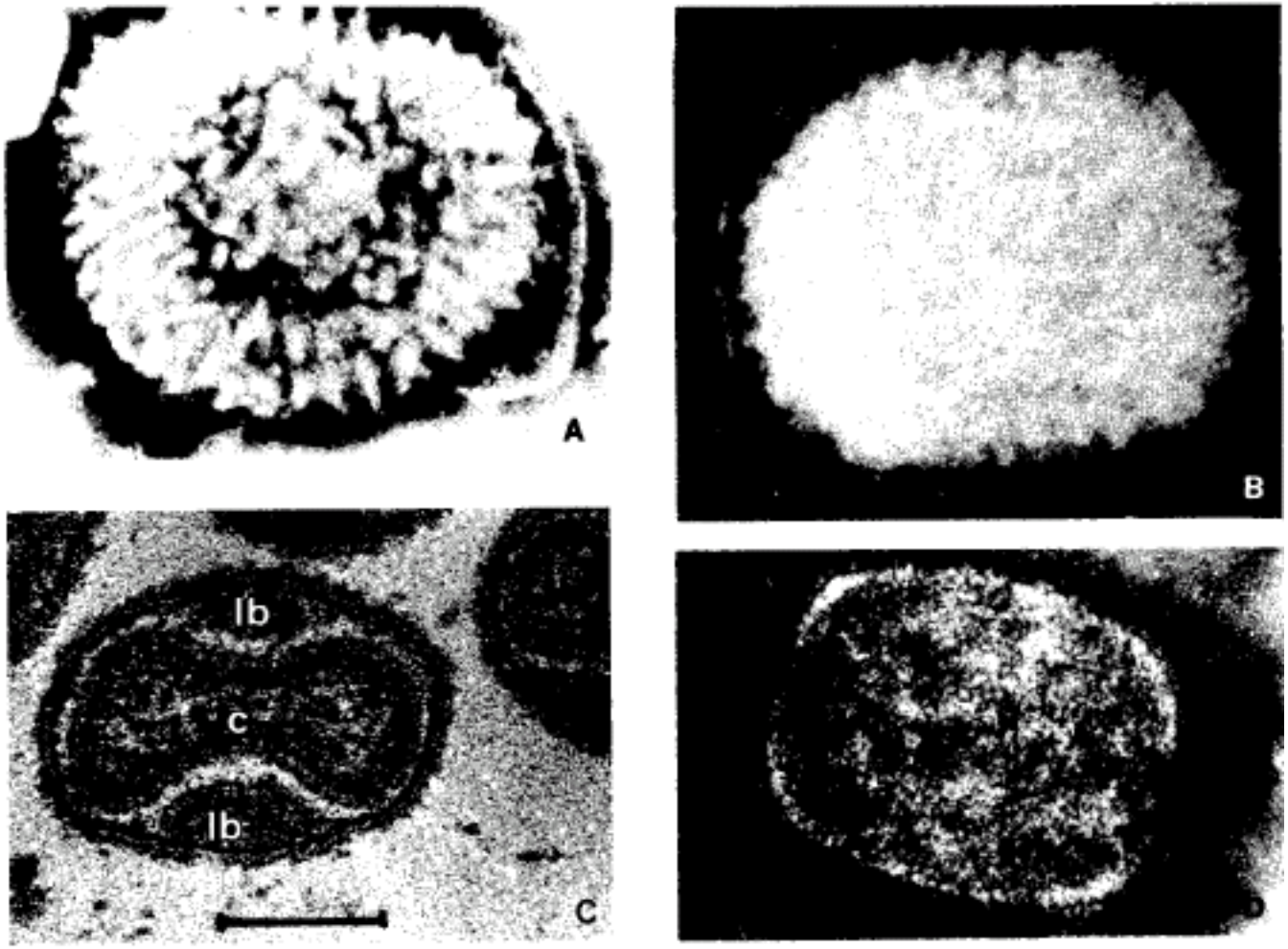
Virions are present in two infectious forms

1. **EEV (Extracellular Enveloped Virus)**- released from cells spontaneously, by exocytoses are enclosed within a lipoprotein envelope, which contains the haemoglutinin and other specific polypeptides - **CEV** (Cell Associated Enveloped Virus)



2. **IMV (Intracellular Mature Virus)** – released by cellular disruption, lacks envelope, "**naked virus**"

Vaccinia Virus – Electron micrographs



A. Non- enveloped virion (surface of outer membrane with tubular elements)
C. Thin section of non-enveloped virion (biconcave core)
B. Enveloped virion, found in extracellular medium
D. Viral core, released after treatment of virions with Nonidet

Fenner,F. et al. Smallpox and Its Eradiction. Genevea, Switzerland:WHO. 1998:1460

STRUCTURE OF THE VIRAL GENOME

Schemat of vaccinia virus DNA

- **Contains a single linear molecule of a double stranded DNA About 200 kbp long; guanine+cytosine content 36%**

- **When denatured the two sister strands form a large single-stranded circular molecule, being attached at each end of the genome by covalent links**

- **For the most part, the DNA sequences in the central part of the genome are unique, but the terminal fragments (inverted terminal repeats) cross-hybridize with each other and with the termini of other species of orthopoxvirus**

STRUCTURE OF THE VIRAL GENOME

- The ITR's include: an A+T-rich, incompletely base-paired, hairpin loop that connects the two DNA strands; set of short tandemly repeated sequences

- The ITR's are variable in length owing to deletions, repetitions, and transpositions

- Inverted repeats in vaccinia are 10 kbp long and in variola are 725 bp

- Variola vs. Vaccinia: genomes are highly conserved with >95% nucleotide identity, however towards the termini the sequences diverge

- Poxviruses that have been inactivated that don't damage their DNA can be reactivated

Vaccinia Virus Regulatory Cascade

EARLY
- Polymerase (DNA/RNA)
- Intermediate tx. Factors

INTERMEDIATE
- Late tx. Factors

LATE
- Structural genes
- Early tx. factors

Late Gene Transcription

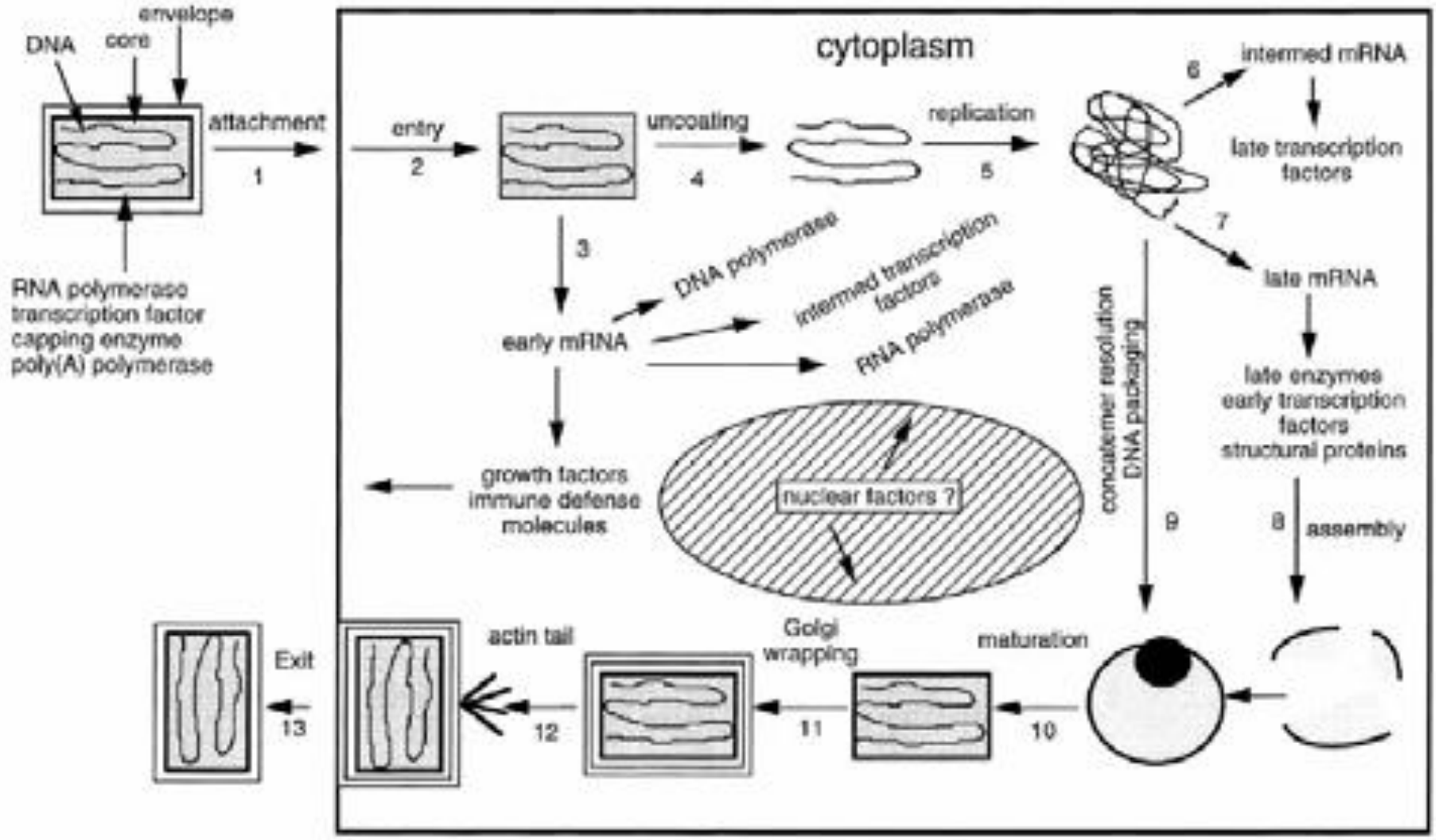
- Late Transcription Factors

Gene Protein Size Gene Expression

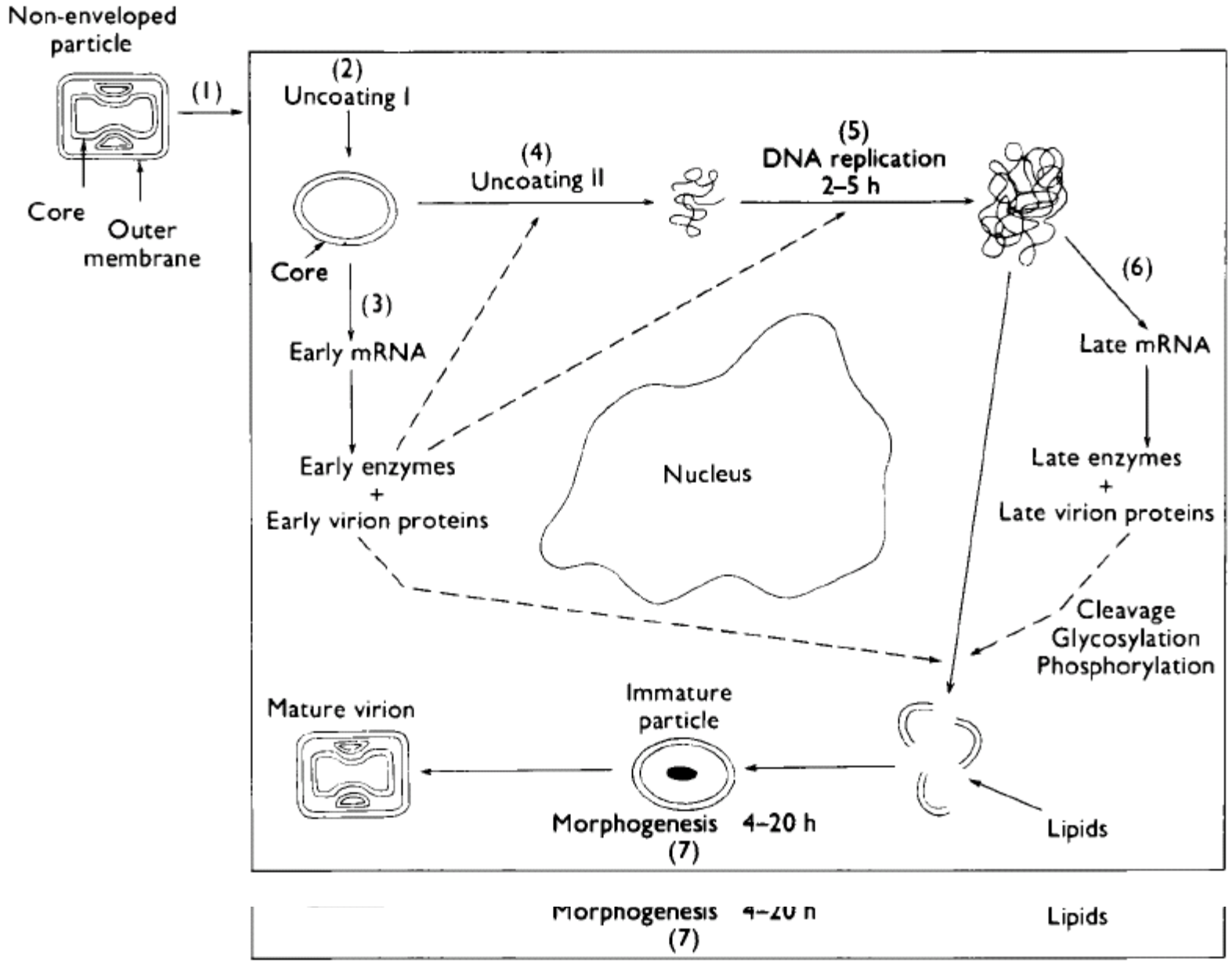
A1L 17kD Intermediate
A2L 26kD Intermediate
G8R 30kD Intermediate
H5R 36kD Early/Late

- Viral RNA Polymerase
- Cellular Viral Late transcription Factors-X (possibly two cellular factors)

Virus Lifecycle

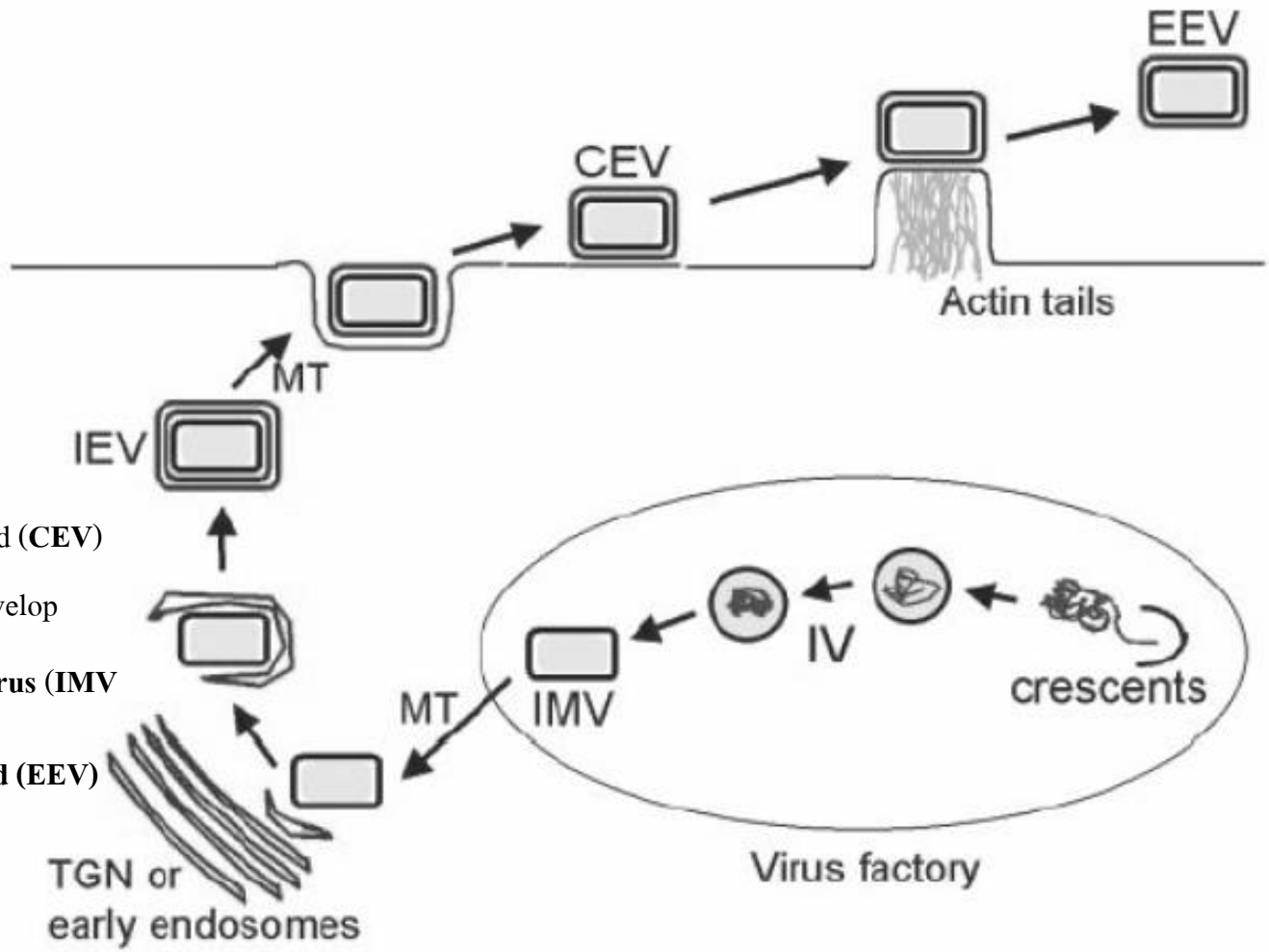


VIRAL REPLICATION – CELL



Fenner,F. et al. Smallpox and Its Eradiction. Genevea, Switzerland:WHO. 1998:1460

Virus Lifecycle



cell-associated enveloped (**CEV**)

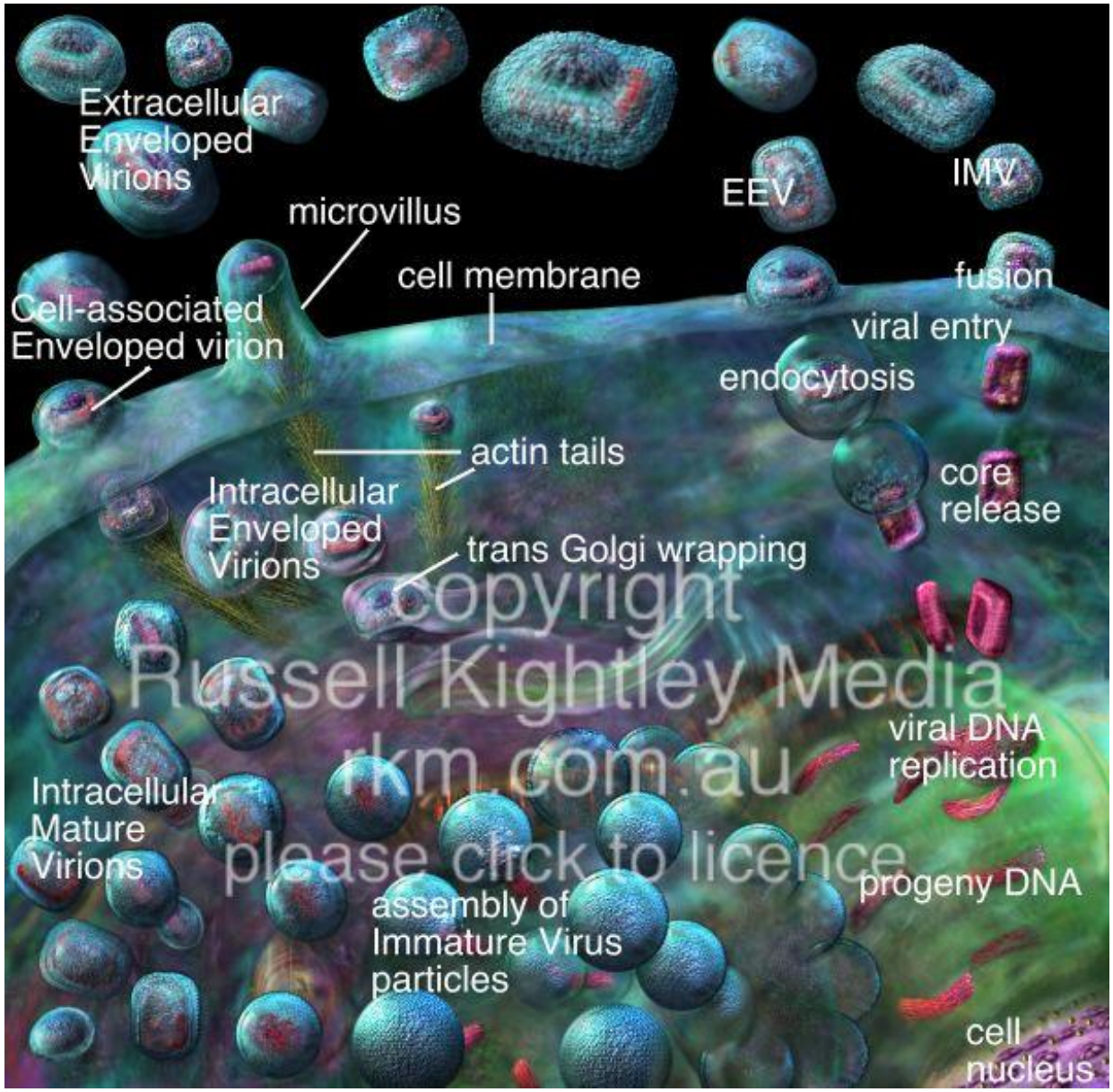
IEV= Intracellular envelop

intracellular mature virus (**IMV**

Extracellular enveloped (EEV)

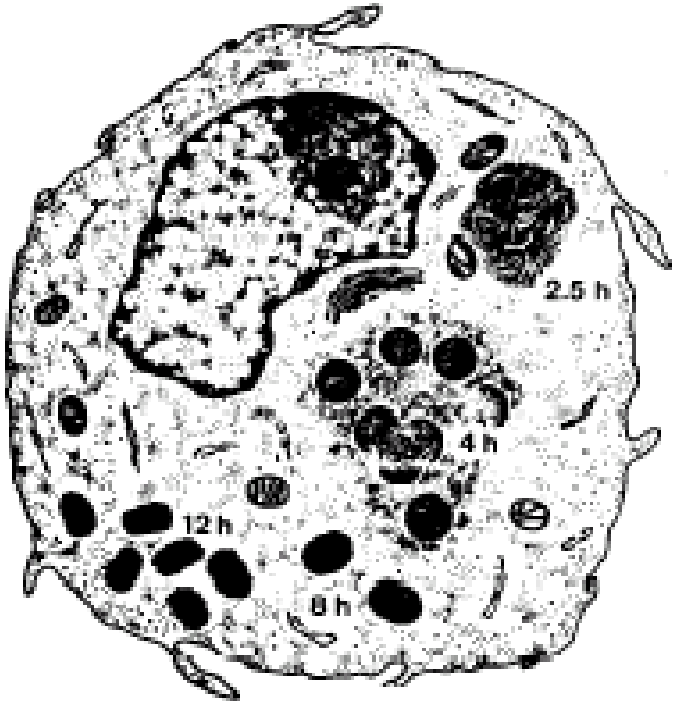
Fig. 1. Overview of VV morphogenesis. IMV are made in a virus factory and move on microtubules (MT) to the wrapping membranes derived from the *trans*-Golgi network or early endosomes. Here IMV are wrapped by a double membrane to form IEV that move to the cell surface on microtubules. At the cell surface the outermost IEV membrane fuses with the plasma membrane to form CEV that induce actin tail formation to drive the virion away from the cell. CEV may also be released to form EEV.

VIRAL REPLICATION – CELL CYCLE



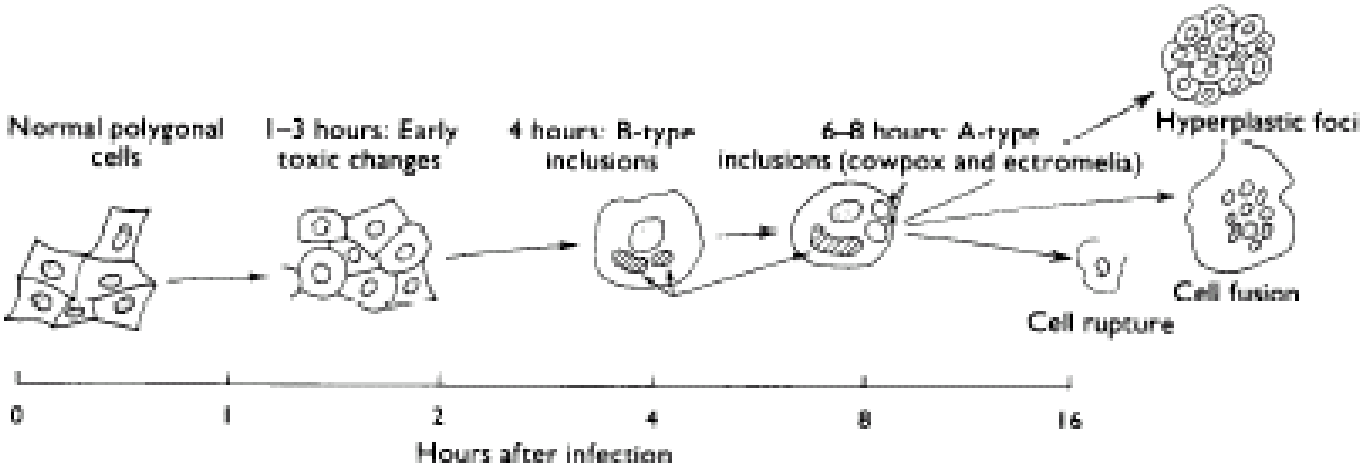
http://www.rkm.com.au/VIRUS/SMALLPOX/smallpox-life-cycle.html

CELLULAR CHANGES



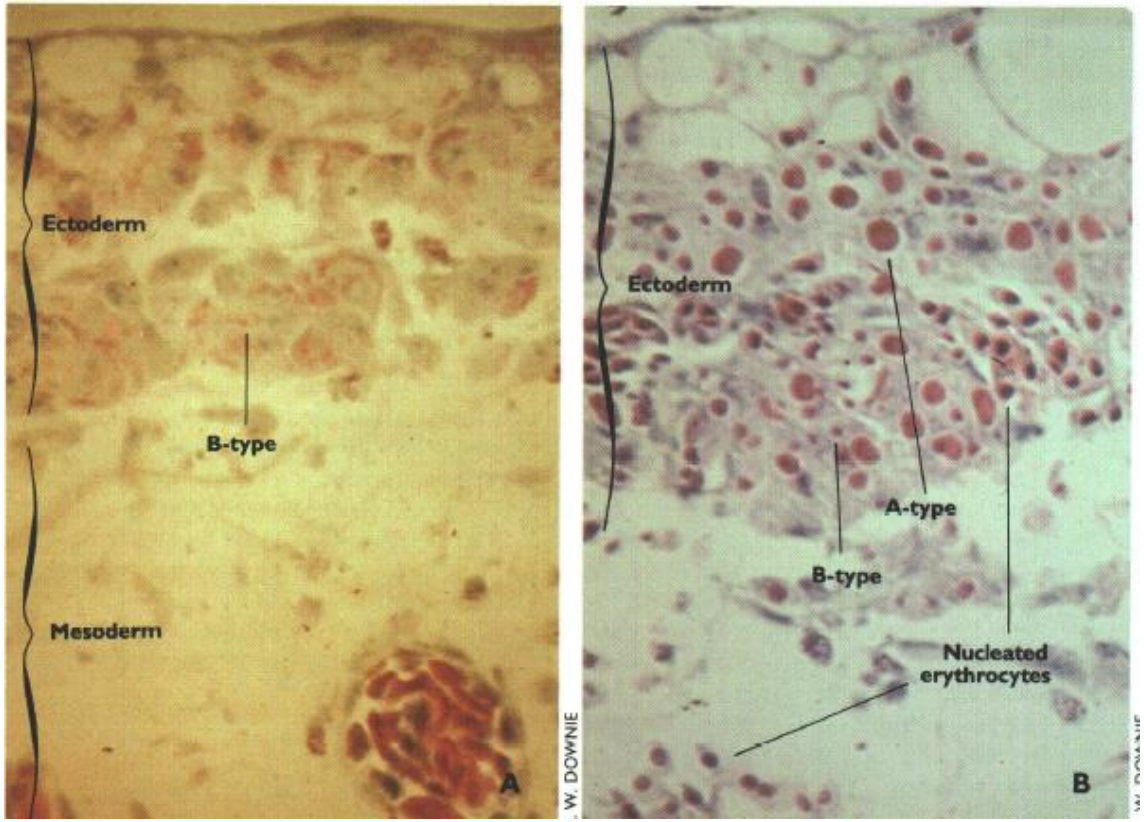
"Viral factory" visualized in stained cells as the B-type inclusion body, is first seen at 2.5h cupules first appear at 4 hours and some are completed as immature particles 6-8hours. From 8 hours onwards mature particles appear

"Toxic"changes may occur in the inf. cells, which in monolayer cultures become rounded and retract from each other



Fenner,F. et al. Smallpox and Its Eradiction. Genevea, Switzerland:WHO. 1998:1460

Cytoplasmic inclusion bodies in infected cells



B-type (Guarnieri bodies)- sites of viral replication produced by all orhopoxviruses

A-type – strongly eosinophilic, found in cells infected with: cowpox,ectromelia and raccoonpox virus; appear late in the infection and are not associated with viral replication (may contain mature virions)

Fenner,F. et al. Smallpox and Its Eradiction. Genevea, Switzerland:WHO. 1998:1460

VIRAL PROTEINS

- Encodes about 200 proteins

- The central part of the genome encodes for structural and functional proteins; Virulence genes are found near the inverted repeats

- Numerous virus-encoded enzymes, are packaged within the virus core, including:

  - multisubunit DNA-dependent RNA polymerase
  - RNA polymerase associated protein of 94kd (RAP94)
  - a transcription factor
  - capping and methylating enzymes
  - poly(A) polymerase

When RNApolymerase inactivated no replication and virus cannot replicate mRNA for early genes

VIRAL PROTEINS

These components are used to synthesize translatable mRNA

-Importatnt proteins for replication:

* **topoisomerase**
* **thymidine kinase – allows the incorporation of Thymidine into DNA**
* **thymidylate kinase – catalyzes the reversible phosphorylotransfer between ATP and TMP**
* **ribonuceoside reductase – converts ribonucleoside diphosphates (NDP's) into deoxyNDP's**
* **dUTPase – minimize the misincorporation of Uracil into DNA**
* **Uracil-DNA gylclosylase – removes the RNA base (Uracil) from DNA**
* **DNA ligase**

Non enzymatic -VIRAL PROTEINS

1. Membrane proteins:

A33R, A34R, A36R :*N-glycosylated, phosphorylated
* fromation of actin tail and microvilli, which facilitate viral dissemination

A36R : required for kinesin recruitment and is involved in microtubule-based motility of IEV's

A56R: Hemagglutinin, N- and O- glycosylated , promote cell fusion and cell to cell viral spread

A27L: required for the formation of IEV, fusion protein, microtubule –dependent movment, normal sized plaques , has additional role in the viral assembly

A28L: fusion protein; A28 deficient virions with normal amounts of A27 and A17 (binding partner) are unable to induce cell fusion

2. Core proteins:

F17R, L4R, A3L, A10L : account for ~70% of the viral core by weight, bind DNA

A36R- is thought to create a link between the actin and microtubule cytoskeleton (nature cell biol, vol3, nov2001 pg992- 998)

A28 protein not required for virion assembly, intracellular movment wrapping or exit form the cell

VIRAL IMMUNOMODULATORY STRATEGIES

1. **Poxviruses encode multiple classes of immunomodulatory proteins to inhibit diverse processes as:**
   - * apoptosis
   - * the production of interferon
   - * the production of chemokines and inflammatory cytokines
   - * the activity of complement, NK, CTL's, antibodies

The "holy grail" of virus infection is the generation of new progeny virions that can disseminate with minimal interference from the host's defence
Apoptosis –proteins that function either as ap inhibitors or serve as suicide caspase substrates

VIRAL IMMUNOMODULATORY STRATEGIES

2. The inhibitory proteins, produced by virus, fall into three main classes:

-Virokines

* resemble host cytokines
* secreted from infected cells to block hosts receptors
* vIL-10, vIL-18

-Viroreceptors

* mimic host cellular receptors
* altered cellular receptors that have lost their transmembrane sequnces and consequently are secreted from infected cells to sequester ligands
* vINF-Rs, vTNFRs

-Intracellular proteins

* target host signal transduction pathways
* inhibit inner antiviral pathways:
  - apoptosis – vFLIP's, serpins
  - proinflammatory cascades - TNF

VIRAL IMMUNOMODULATORY PROTEINS

Complement Regulatory Proteins

VCP – Vaccinia virus Complement control Protein, consists of short consensus repeats found in hosts' complement regulatory proteins

Inhibits the classic and alternative pathways of complement through binding and inactivating both C4b and C3b

VIRAL IMMUNOMODULATORY PROTEINS

SPICE- the smallpox inhibitor of complement enzymes
- molecularly engineered homologue of VCP (Rosengrad et al;.Univ. of Penn.)

Demostrated: the functional advantage of variola complement regulatory protein Over the vaccinia homologue

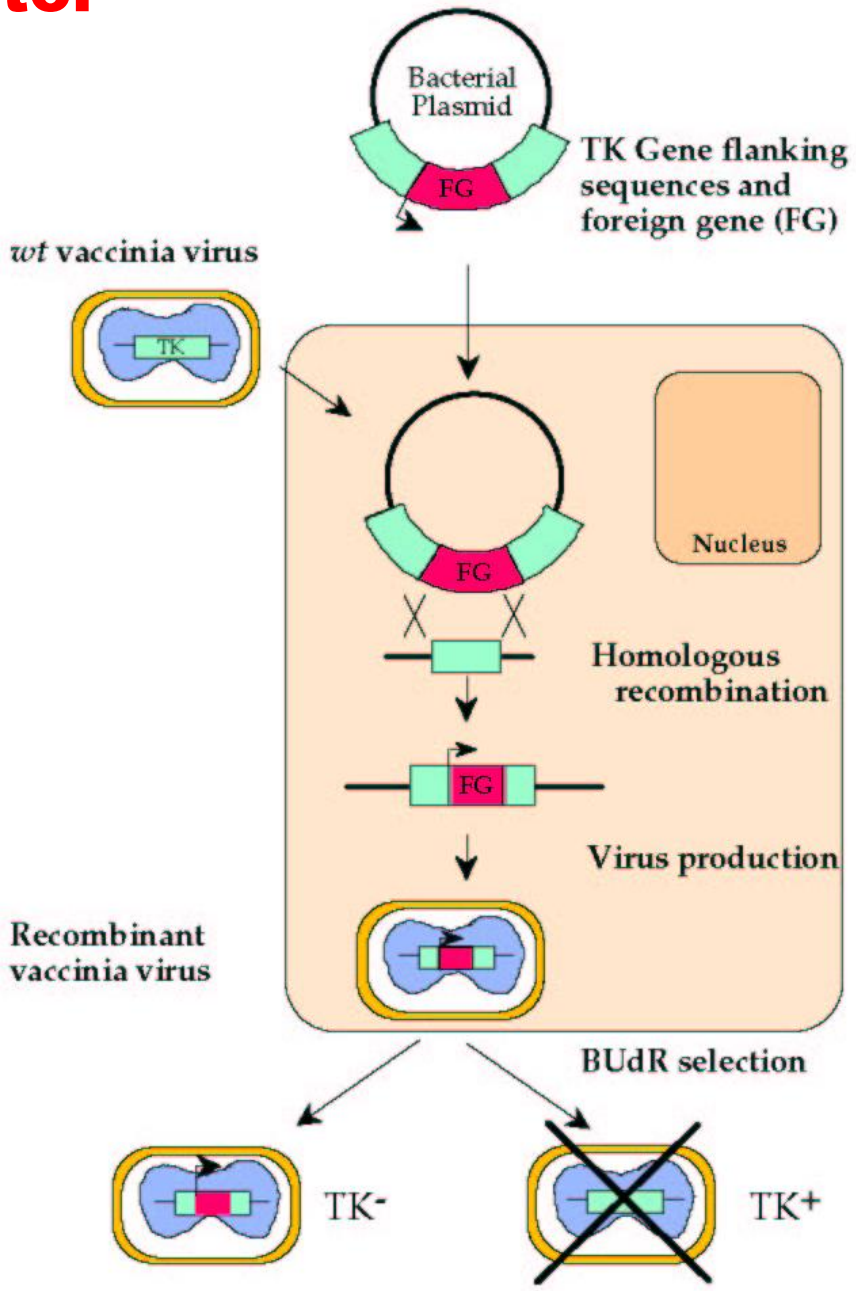
- More human complement specific than VCP
-100-fold more potent at inactivating C4b&C3b
-SPICE serves to inhibit the formation of the C3/C5 convertases necessary for Complement-mediated viral clearance

VIRAL IMMUNOMODULATORY PROTEINS

SPICE- provides the first evidence that variola proteins are particulary adept at overcoming human immunity, and the decreased function of VCP suggests one reason why the vaccinia virus vaccine was associated with relatively low mortality

Disabling SPICE may be useful therapeuticaly

Recombinant Vaccinia virus Expression Vector



Thank You