

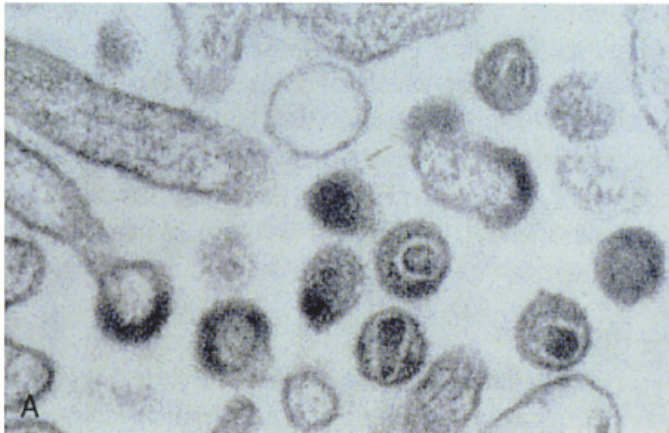
VIROLOGY

Engineering Viral Genomes: **Retrovirus Vectors**

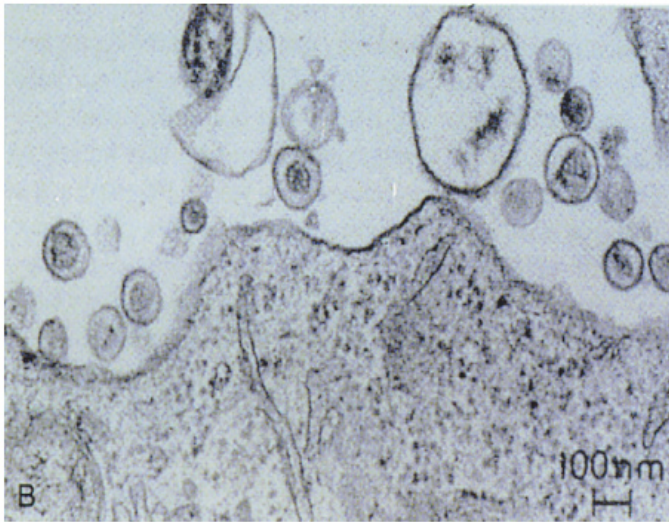
Family *Retroviridae*

<i>Genus</i>	<i>Examples</i>	<i>Features</i>
Alpharetrovirus	Rous sarcoma virus, Avian leukosis virus	oncogene present in many members
Betaretrovirus	Mouse mammary tumor virus	mammary carcinomas
Gammaretrovirus	Moloney murine leukemia virus, Feline leukemia virus	oncogene present in many members
Deltaretrovirus	Human T-cell lymphotropic viruses (1, 2 and 5), bovine leukemia virus	T- and B-cell lymphomas
Epsilonretrovirus	Walleye dermal sarcoma virus	sarcomas
Lentivirus	Human immunodeficiency virus (HIV-1, HV-2), simian immunodeficiency virus (SIV)	AIDS
Spumavirus	simian foamy virus, human foamy virus	benign

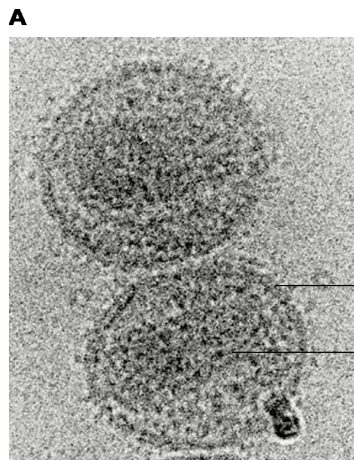
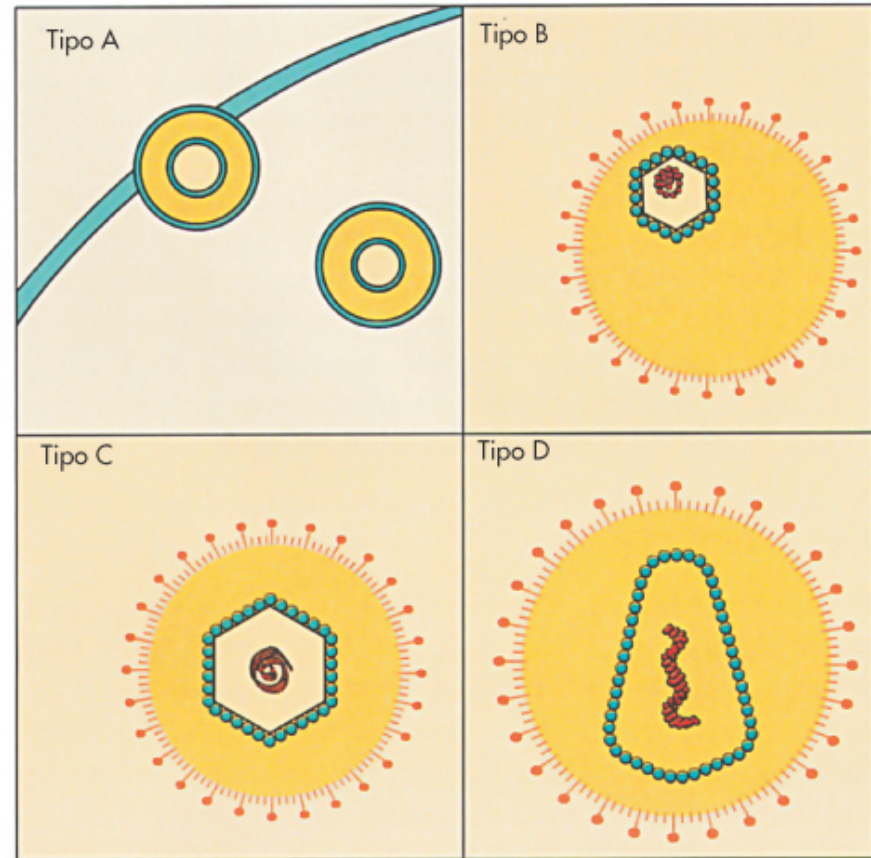
Morphology of Retroviruses



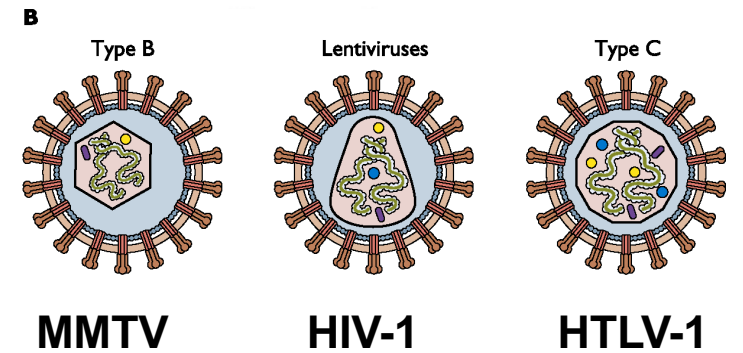
HIV-1



HTLV-1

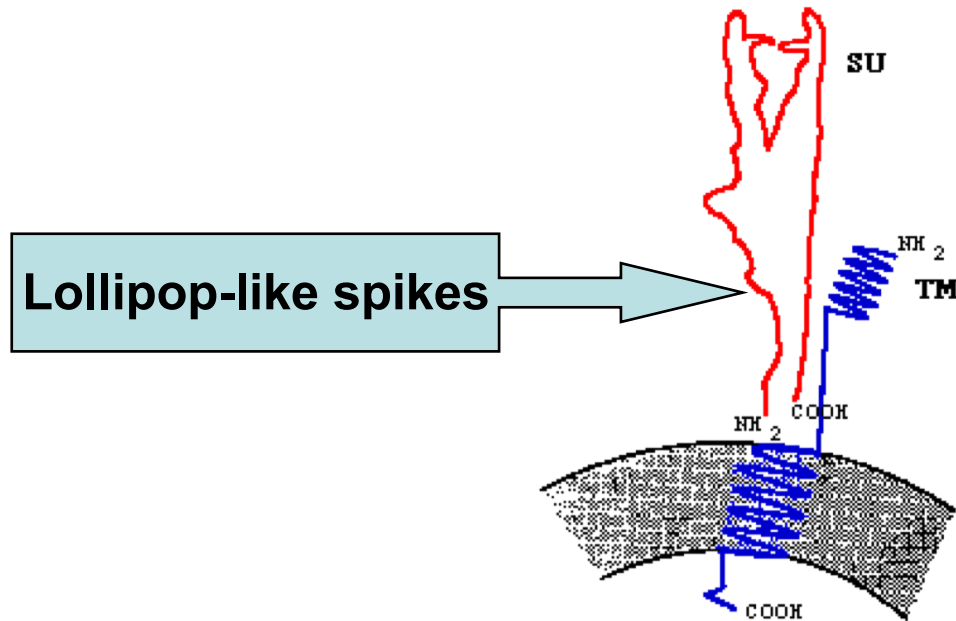
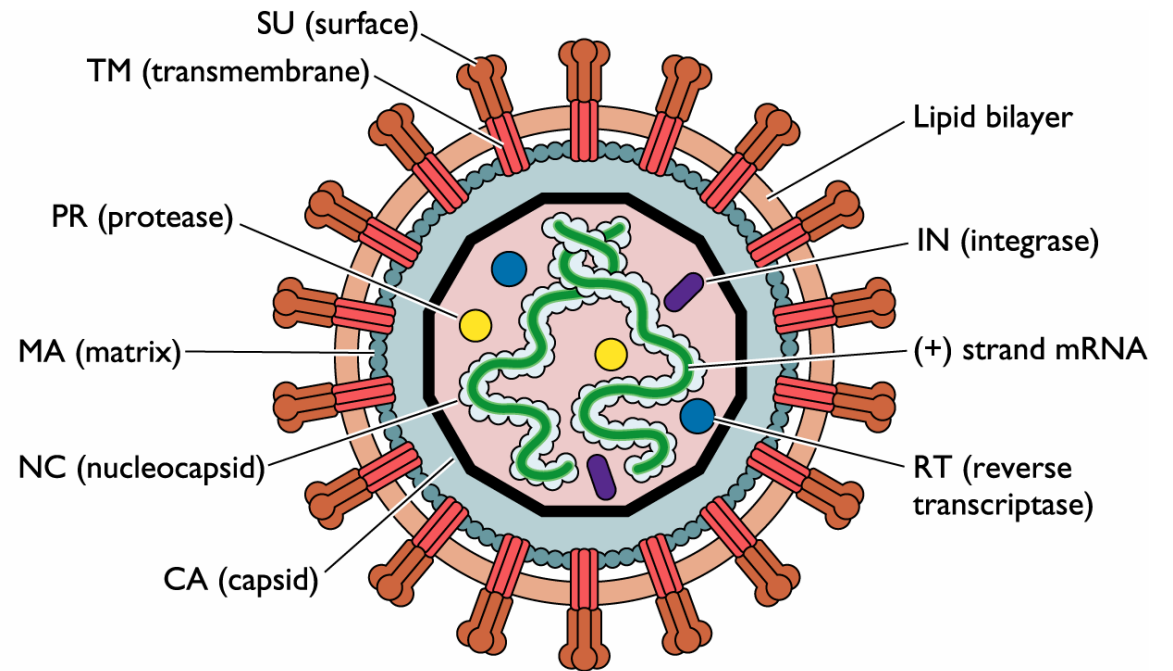
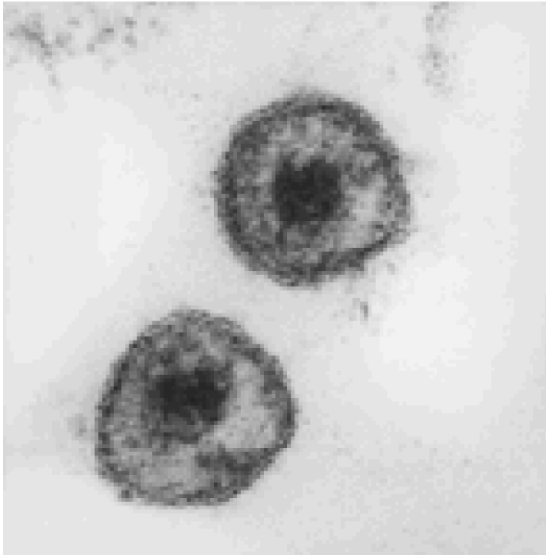


Envelope
Core



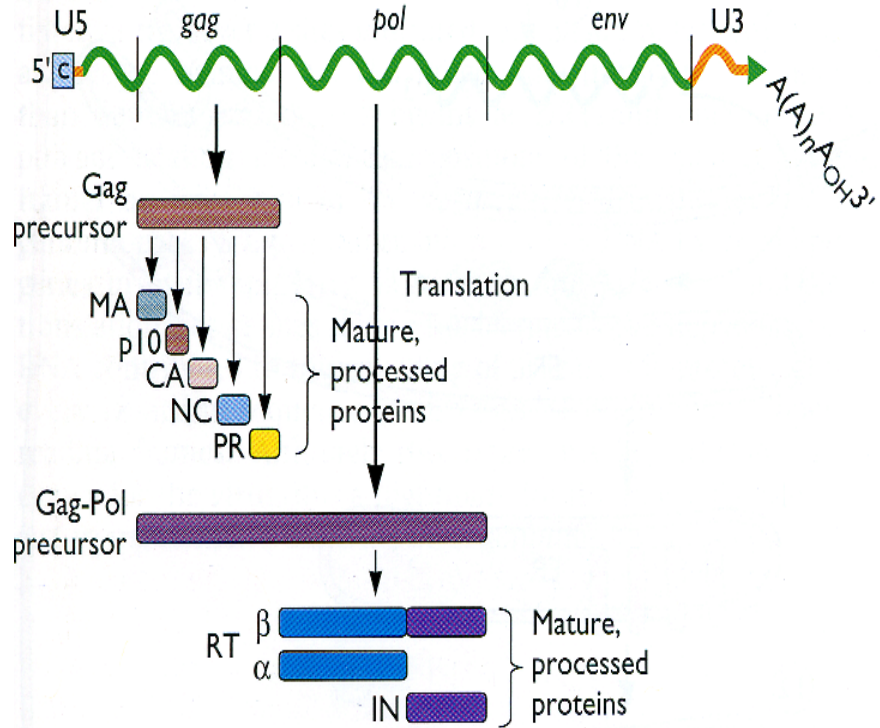
Structure of a Retrovirus

A

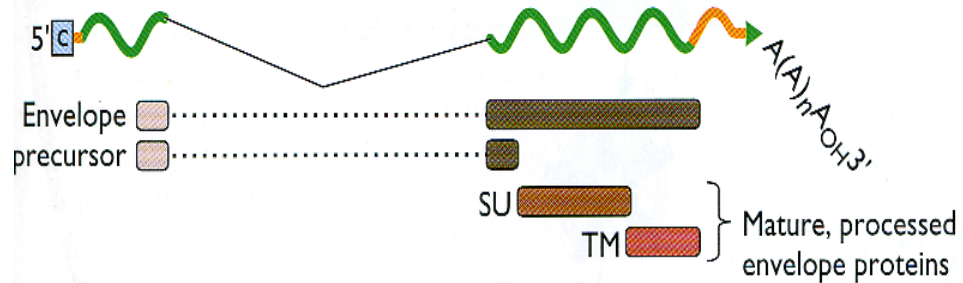


Genome expression

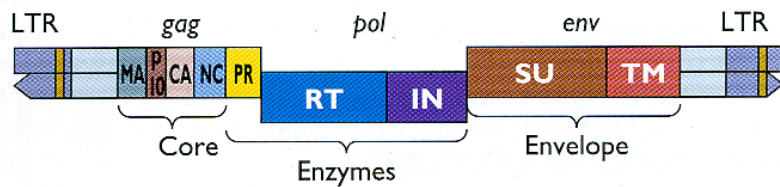
Genomic RNA, Gag-Pol mRNA, pre-mRNA

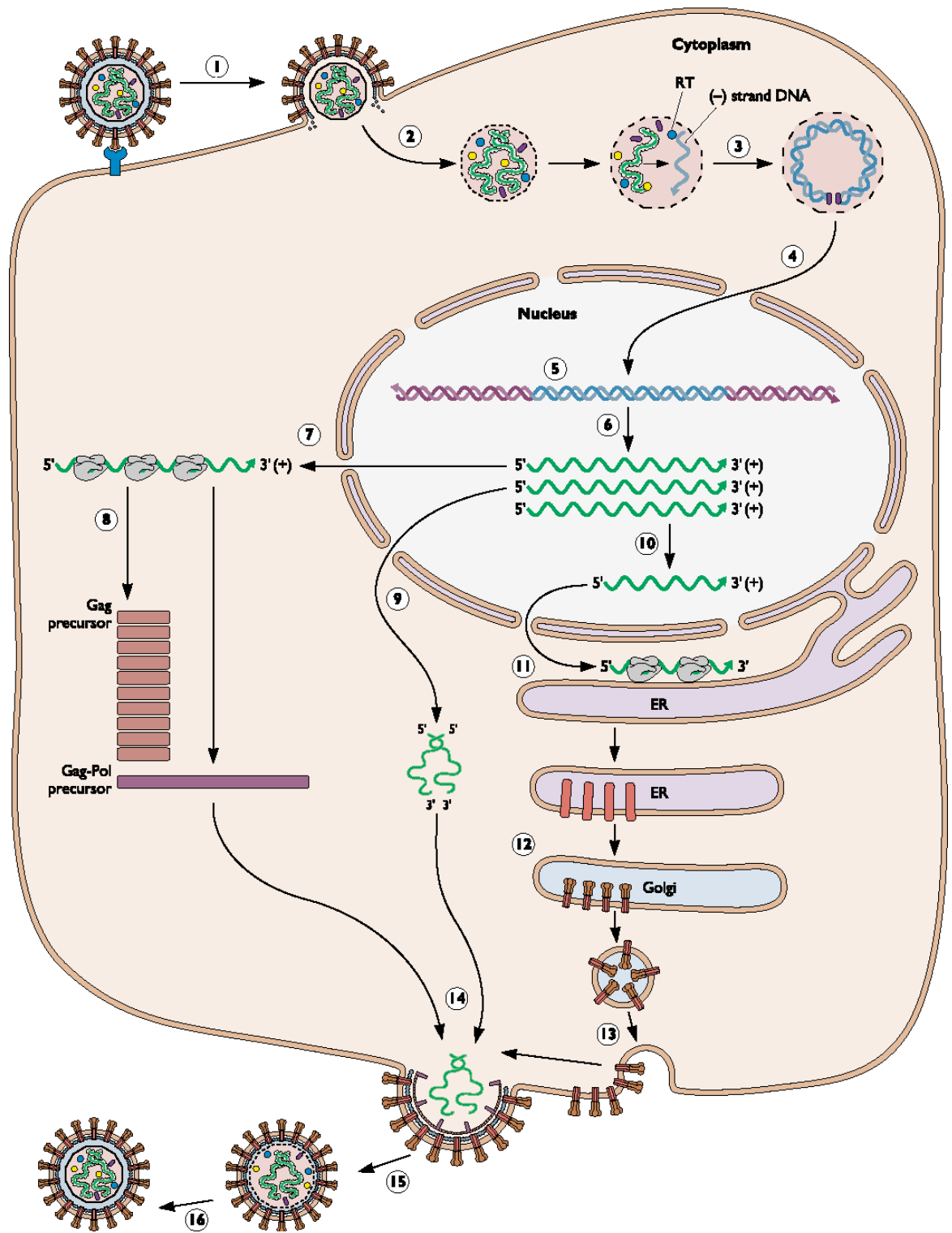


Singly spliced Env mRNA

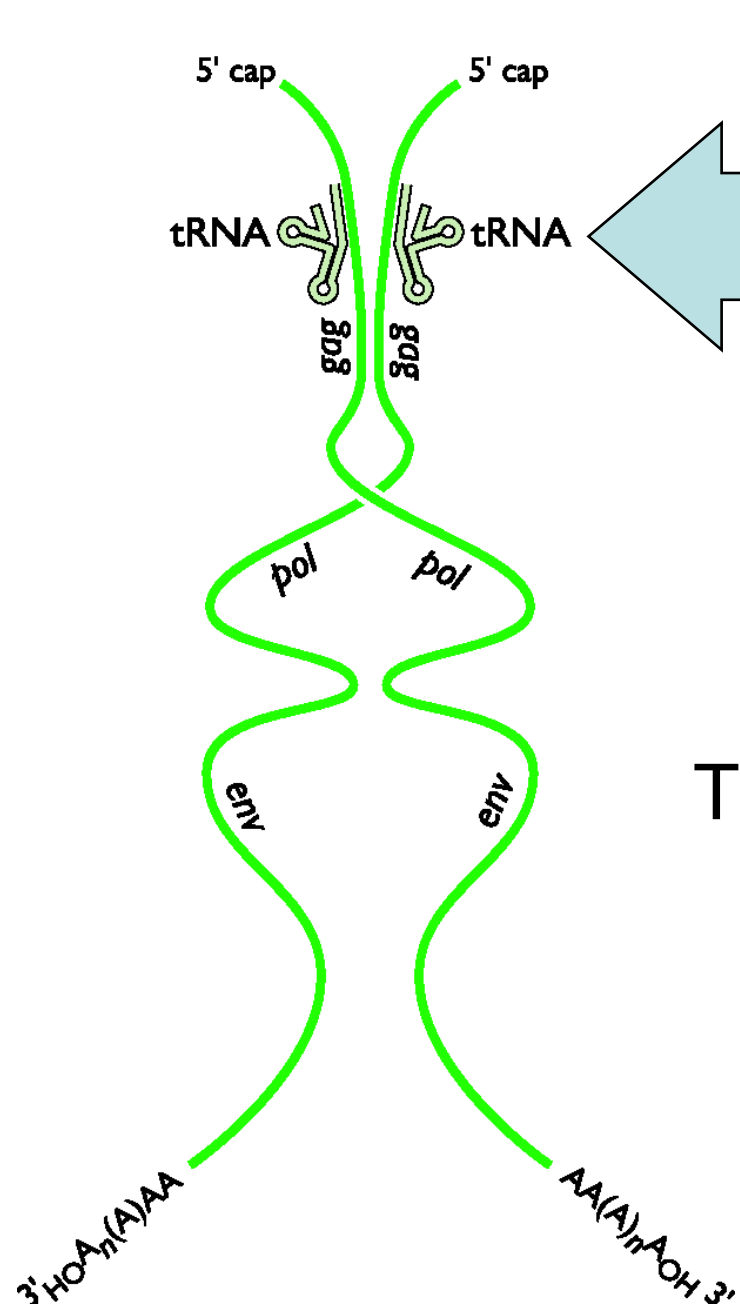


B Simple retrovirus (ALV)





Retrovirus
replicative cycle

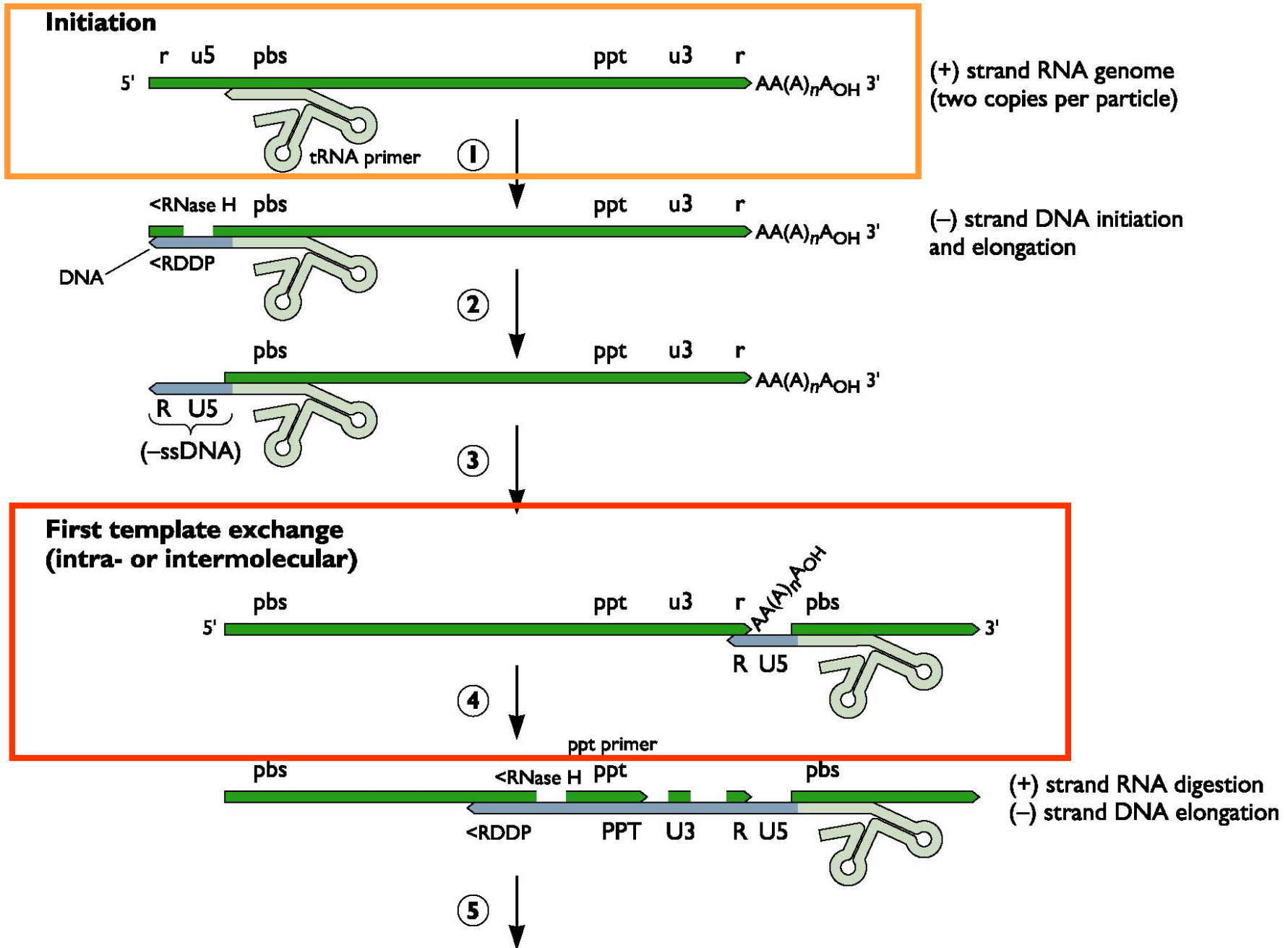


Most mammalian retroviruses use tRNA^{PRO}, tRNA^{Lys3}, tRNA^{Lys1,2}

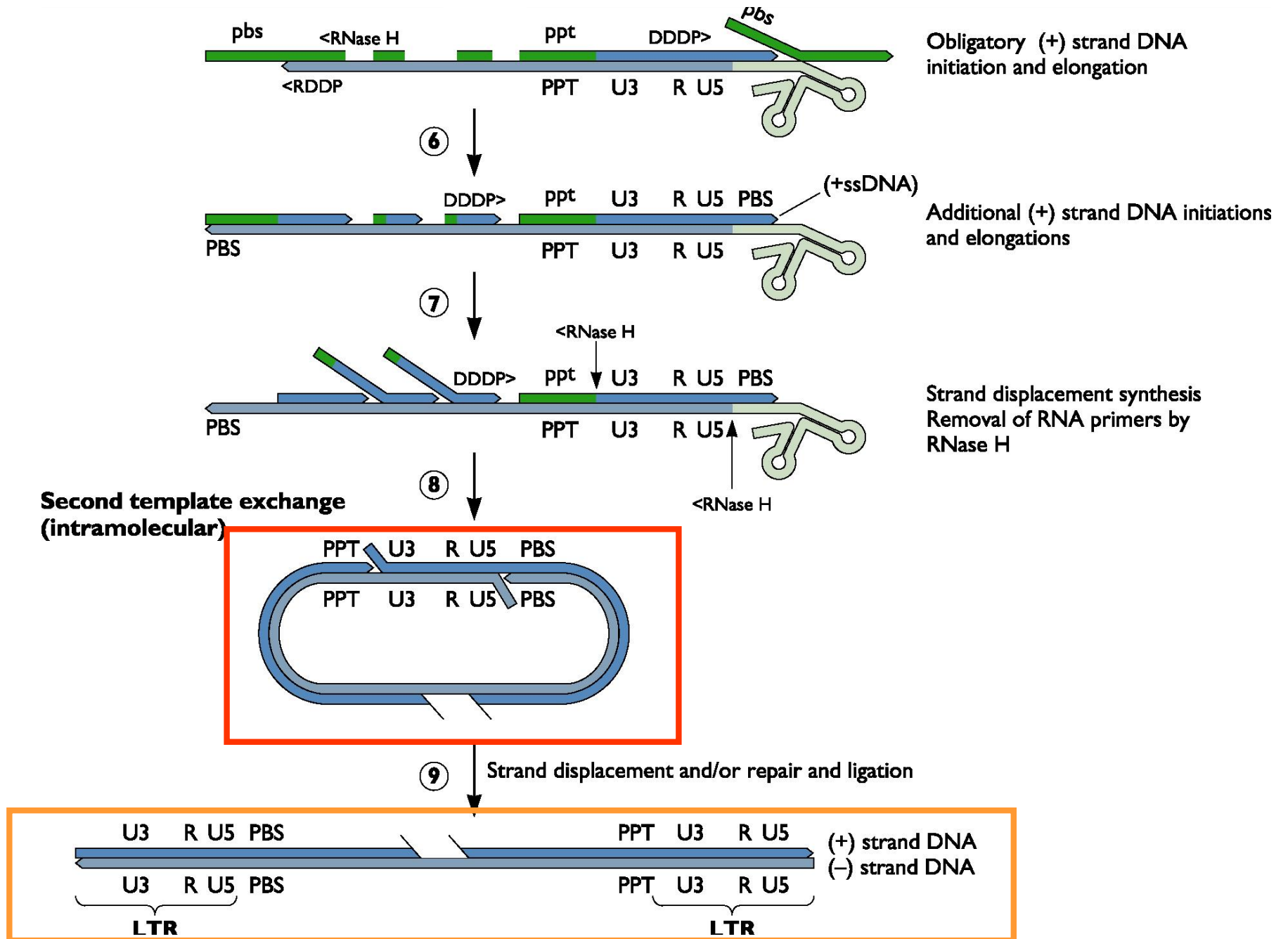
The partially unfolded tRNA is annealed via at least 18 nt at its 3' end to a site on RNA genome called the **primer-binding site (pbs)**

The diploid retroviral genome

The reverse transcription process -1

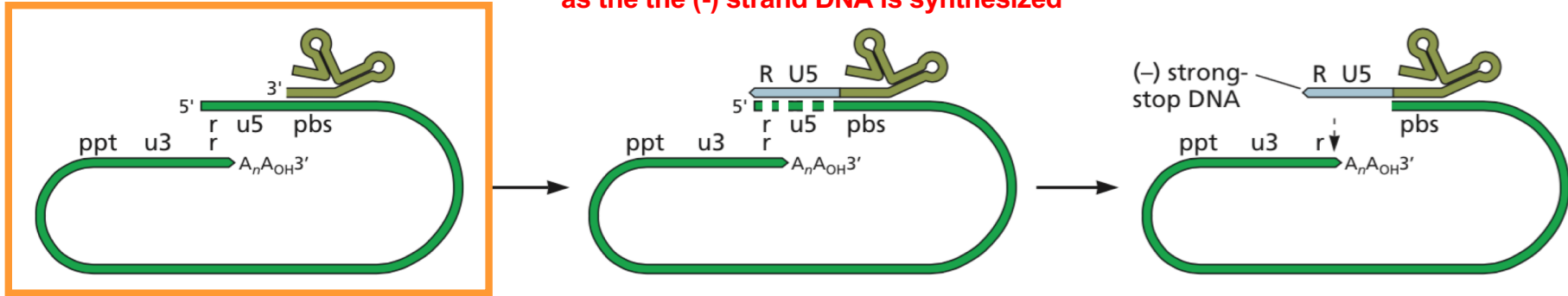


The reverse transcription process -2



1 Initiation of (-) strand DNA synthesis

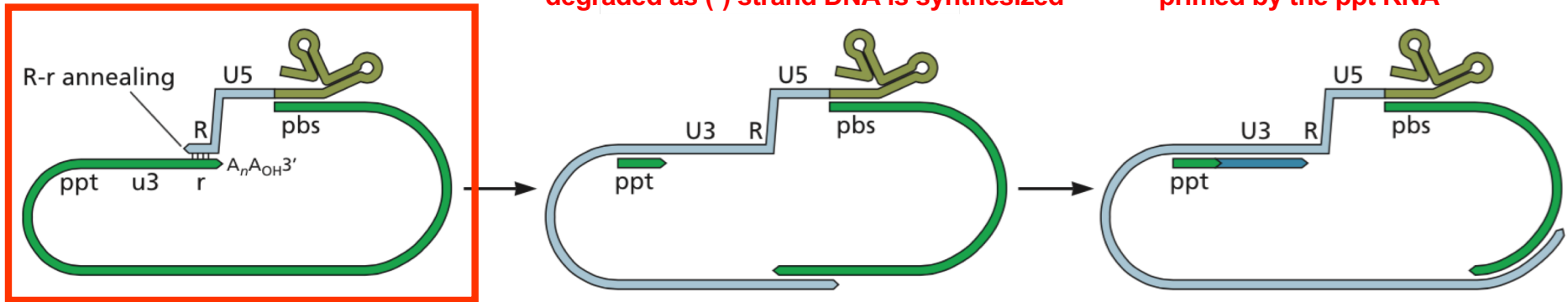
The 5' end of the viral RNA genome is degraded by the RNase H activity of RT as the (-) strand DNA is synthesized



2 First template exchange

The RNA genome continues to be degraded as (-) strand DNA is synthesized

(+) strand DNA synthesis begins primed by the ppt RNA

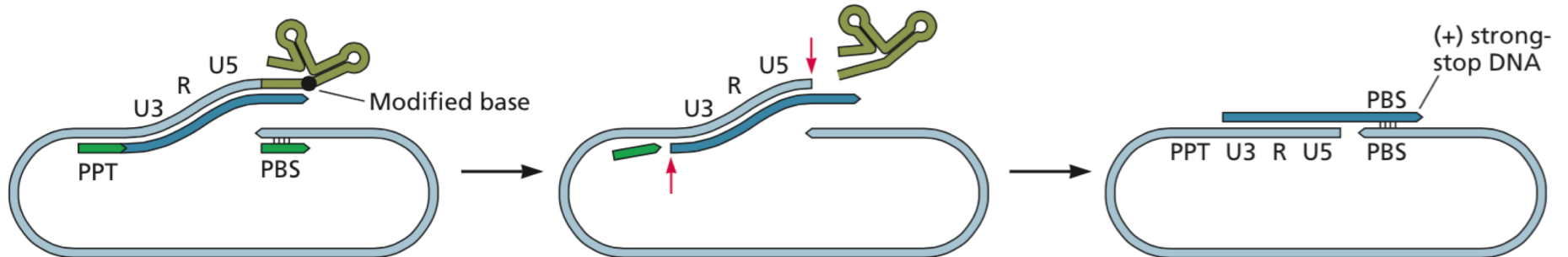


3 (+) strand DNA synthesis

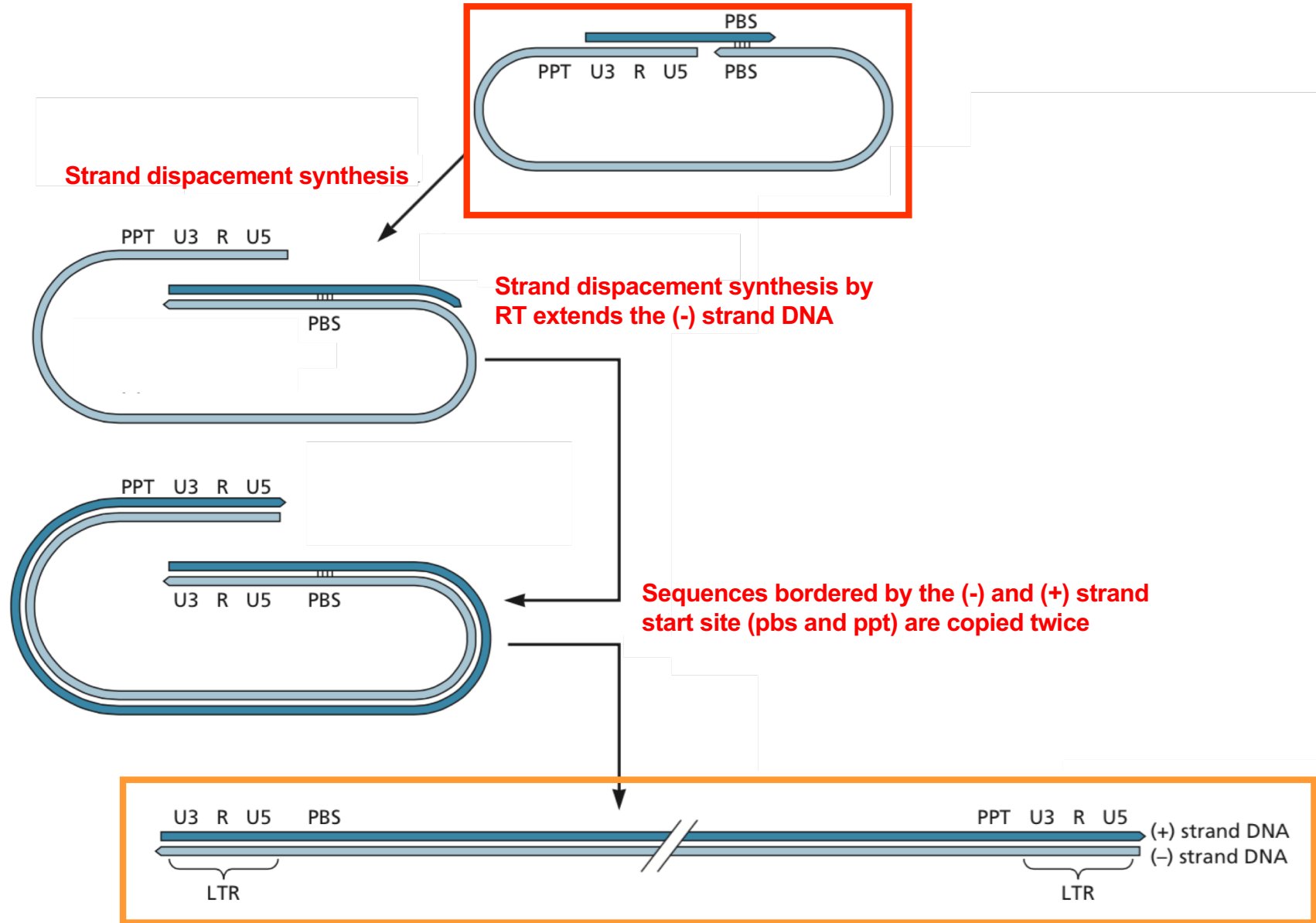
The PBS sequence is copied twice: from the RNA genome and from the tRNA primer (+)

RNase H activity of RT removes both primer RNAs

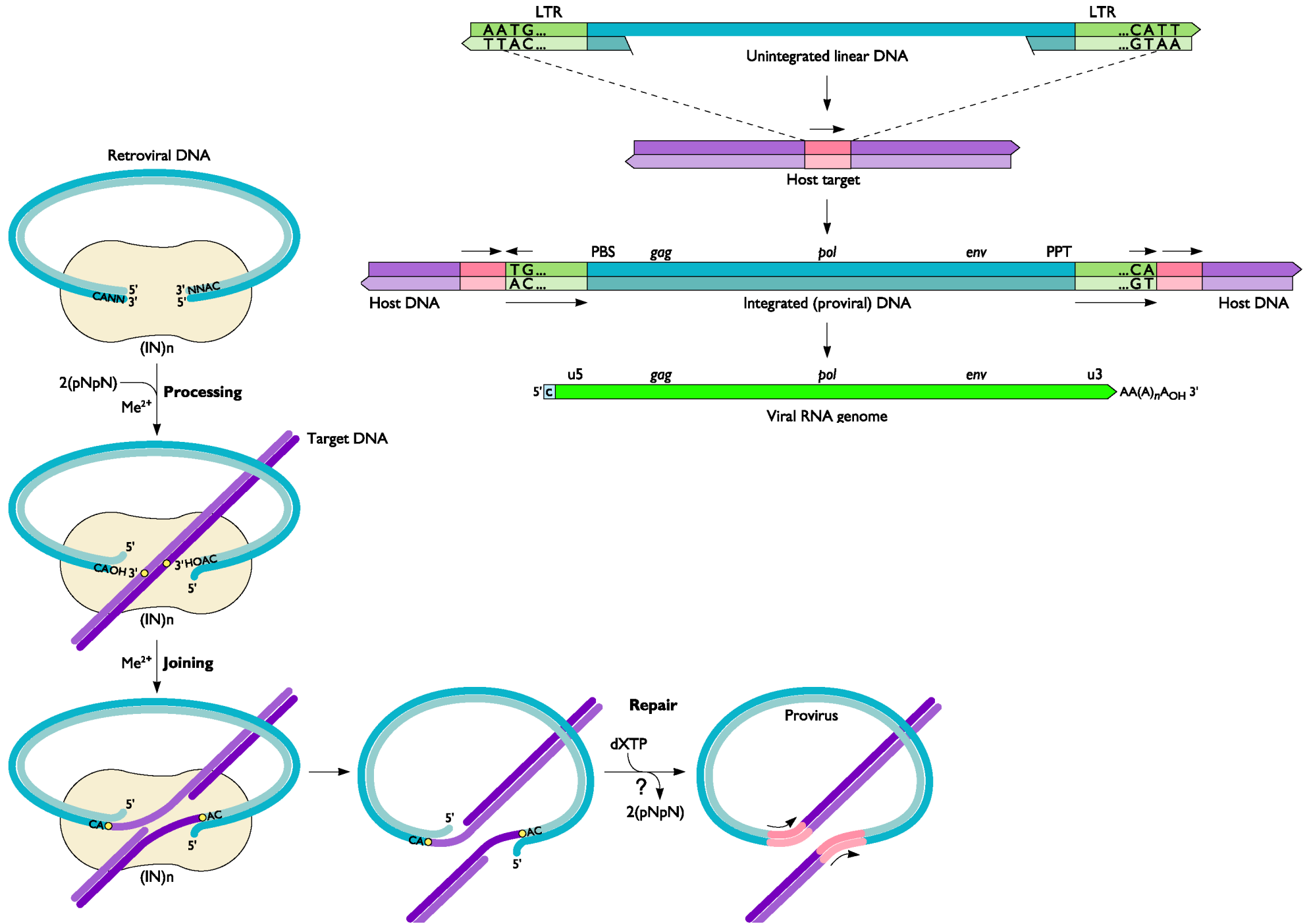
DNA ends are juxtaposed by annealing at complementary PBS sequences



4 Second template exchange is facilitated by annealing of PBS sequences

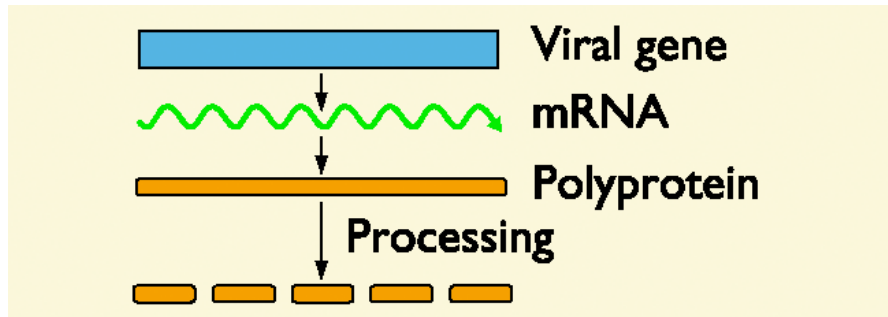


Characteristics of retroviral integration

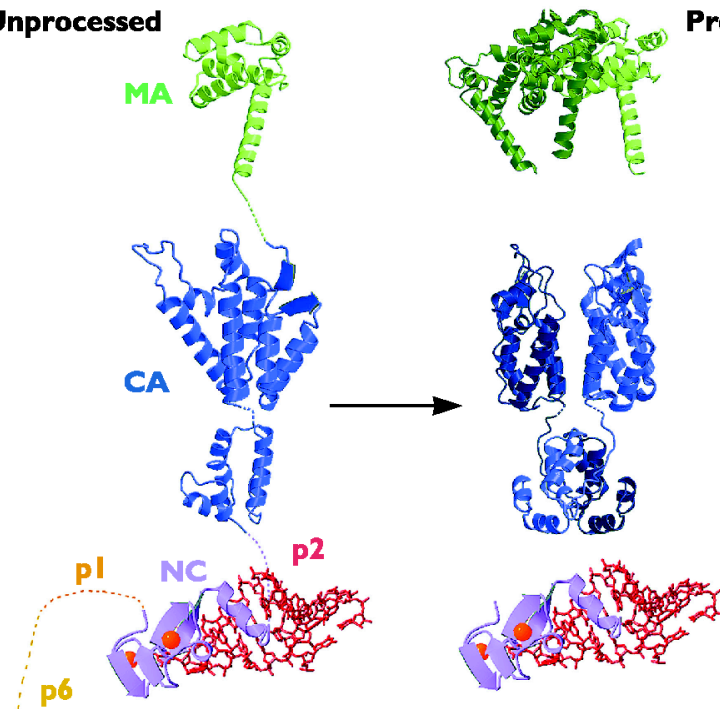


Translation strategies of Retroviruses

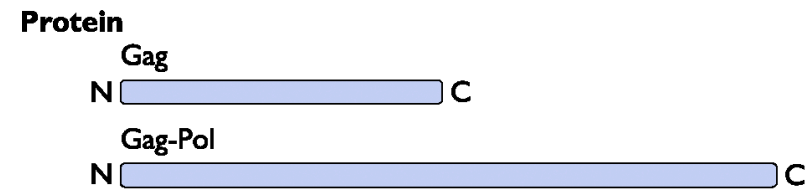
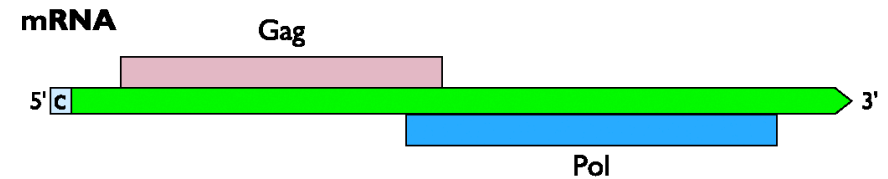
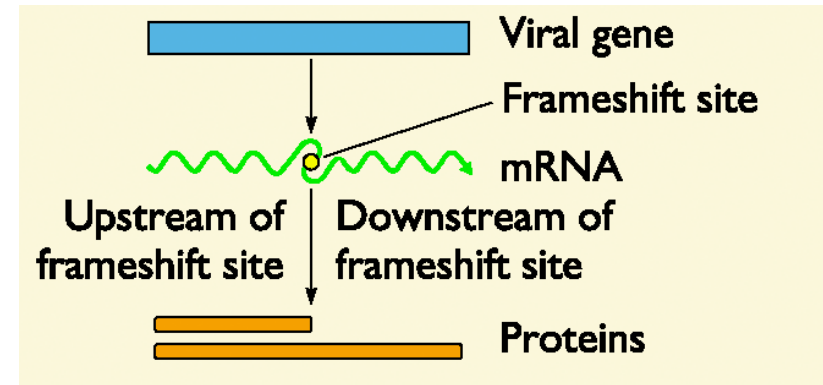
The synthesis of multiple proteins



Unprocessed Processed

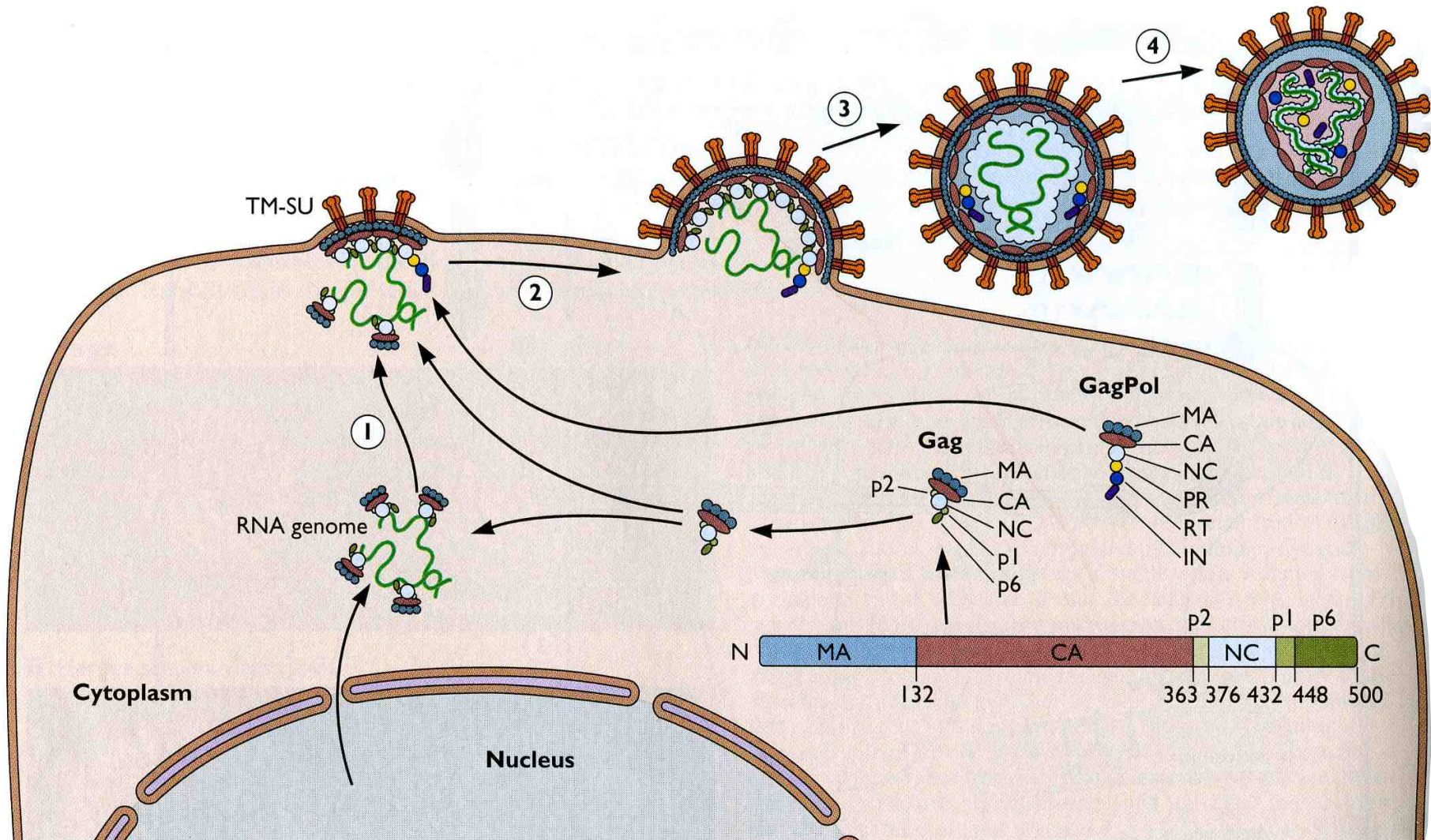


Polyprotein synthesis: the Gag polyprotein

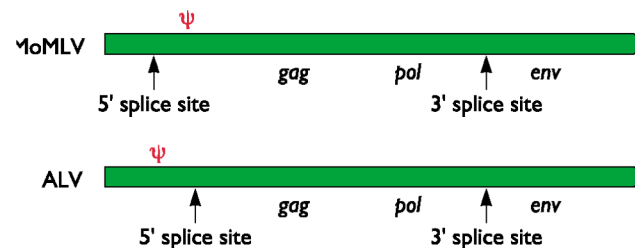
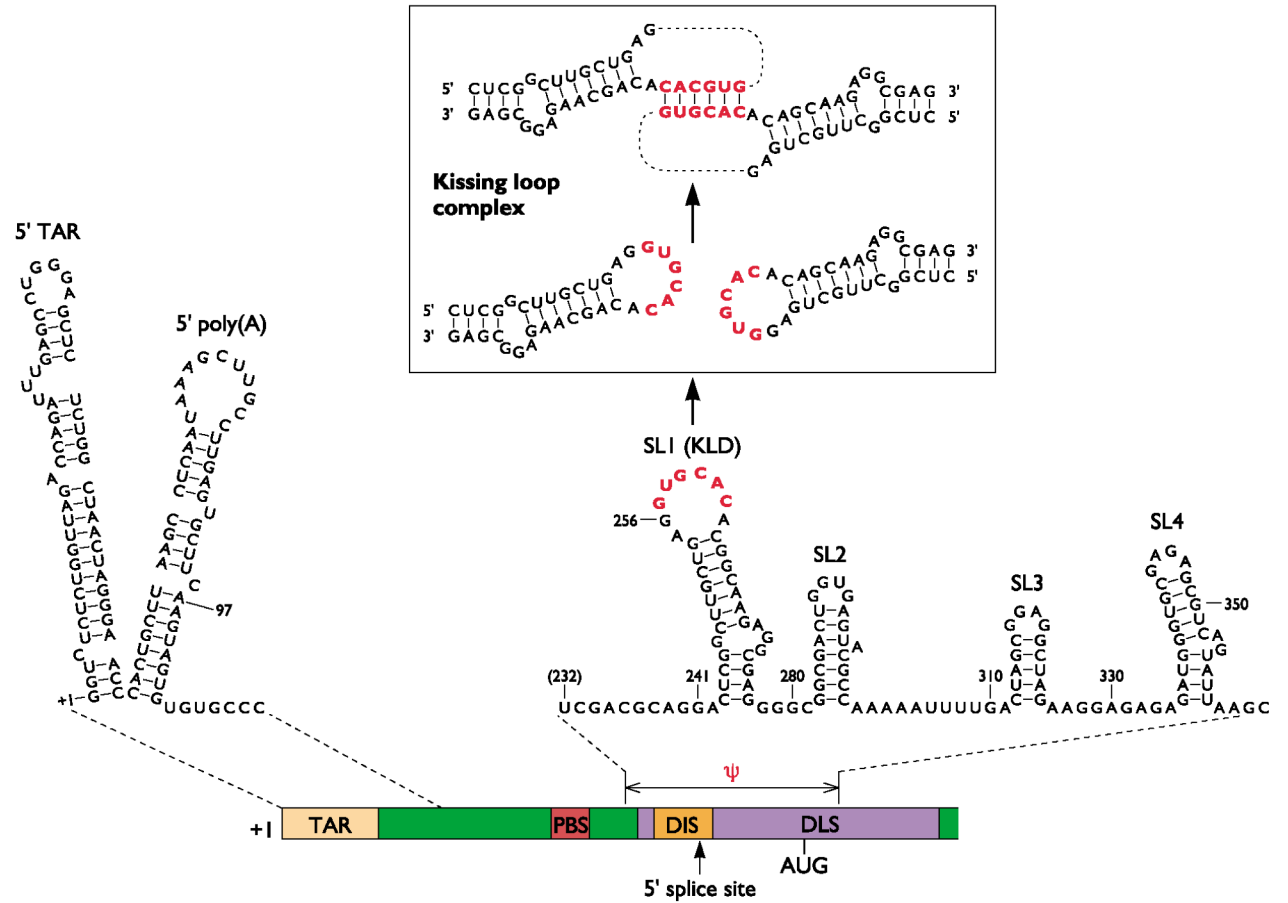
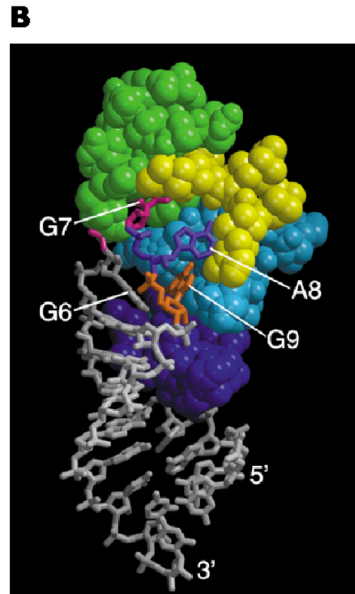
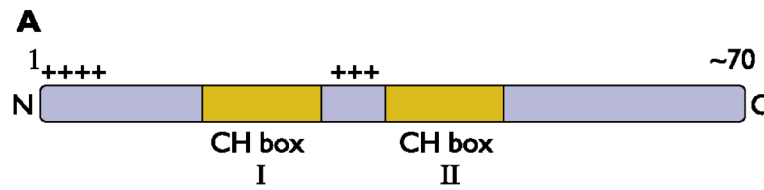


Ribosomal frameshifting: the Gag-Pol fusion

Assembly of a retrovirus from polyprotein precursors



Sequences important in packaging of retroviral genomes



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

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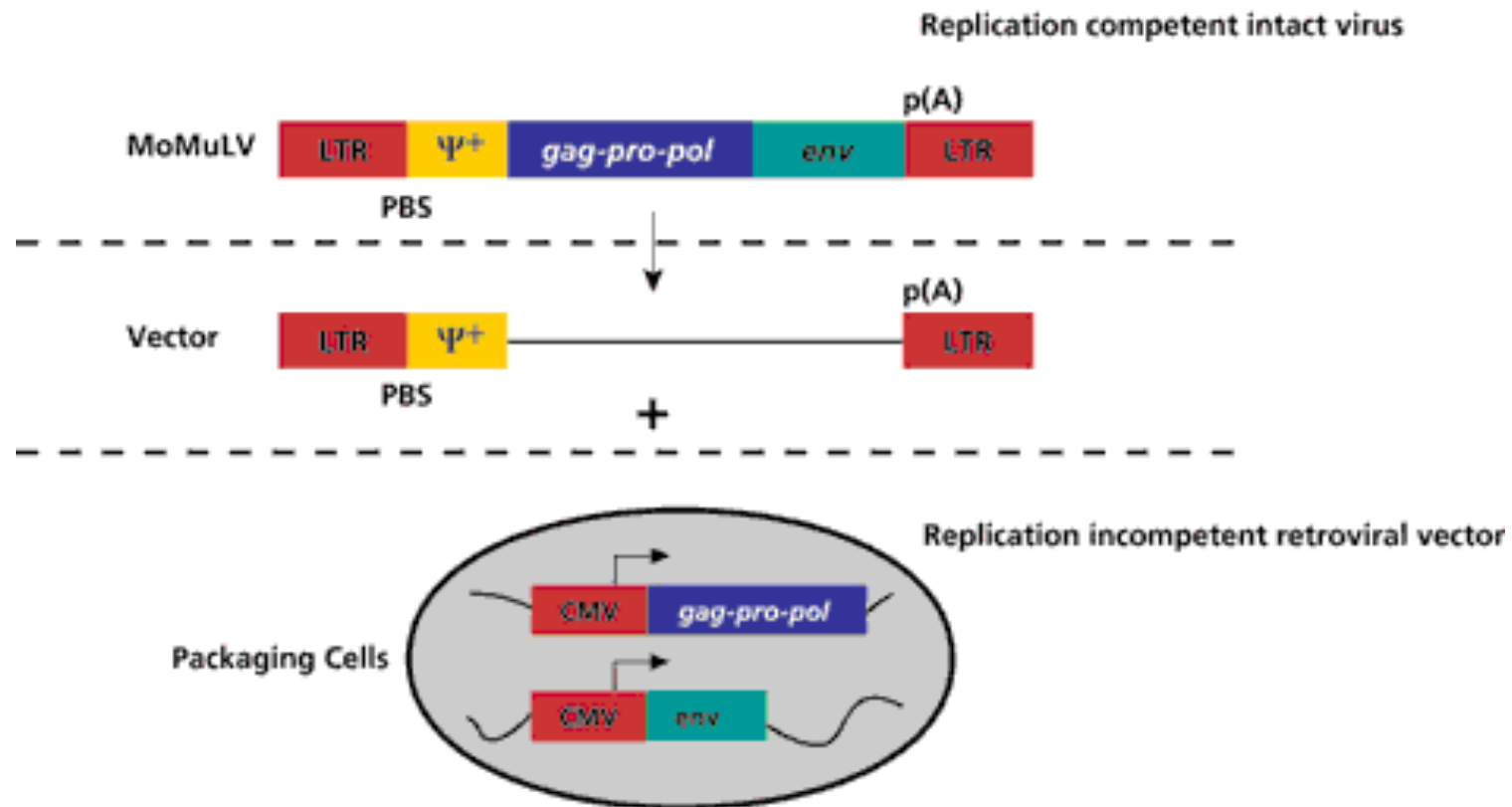
Favorable Features of Retroviruses as Vectors

- Well characterized
- Easily to manipulate (genomes 7-9 kb)
- They require 3 trans (gag, pol, env) and 7 major cis-active control elements (U3, R, U5, PBS, SD, Ψ , SA) in order to replicate
- Stability of recombinants vectors (plasmids)
- High efficiency of gene transfer
- Most are replication-defective
- Stable and precise integration of the transgene
- Low immunogenicity
- Can be pseudotyped to infect a broad range of cells

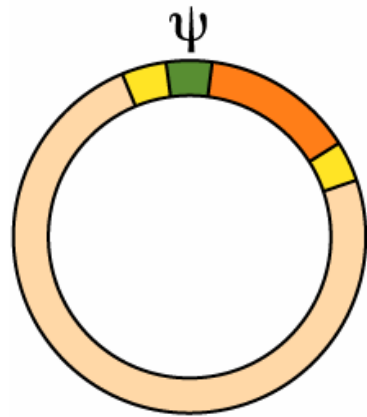
Retrovirus as vectors

- Replication-incompetent vectors
 - They bear deletions of some or all of the viral genes
 - They retain *cis*-acting viral sequences necessary for transmission (U3, R, U5, PBS, SD, Ψ , SA)
 - They need to be propagated in “packaging” cell lines that provide in *trans* gag, pol, env
- Replication-competent vectors (RCR)
 - Avian vectors (up to 2 kb inserts)

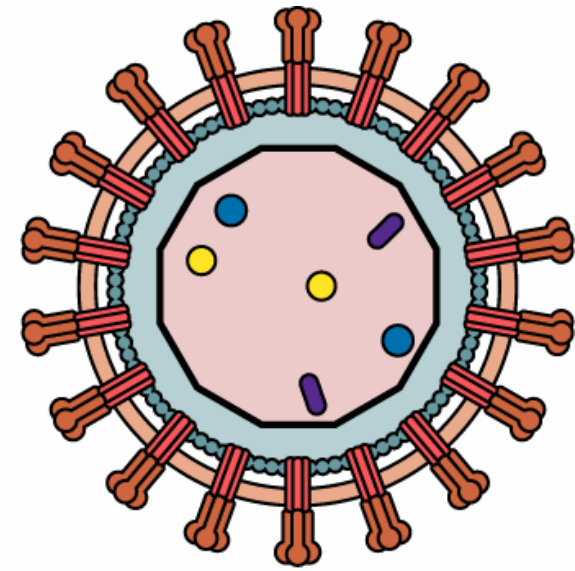
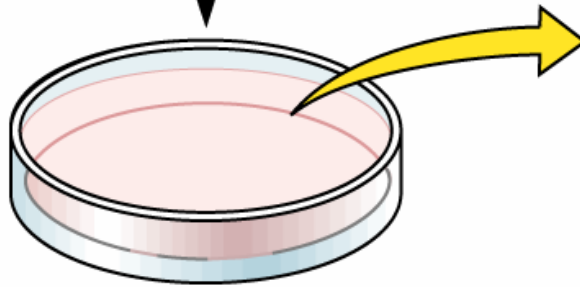
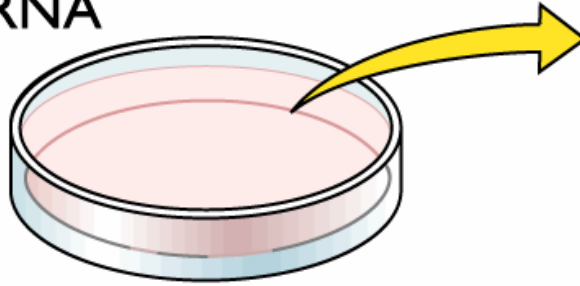
Replication-incompetent vectors



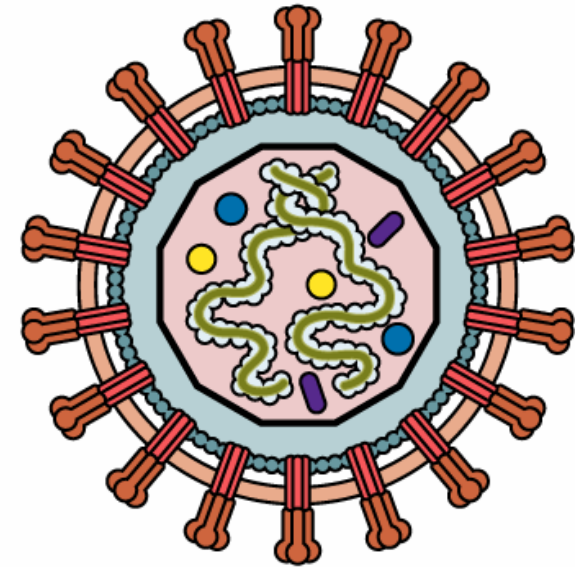
Packaging cell line that expresses viral structural proteins but no packageable viral RNA



Transfect vector plasmid containing foreign gene

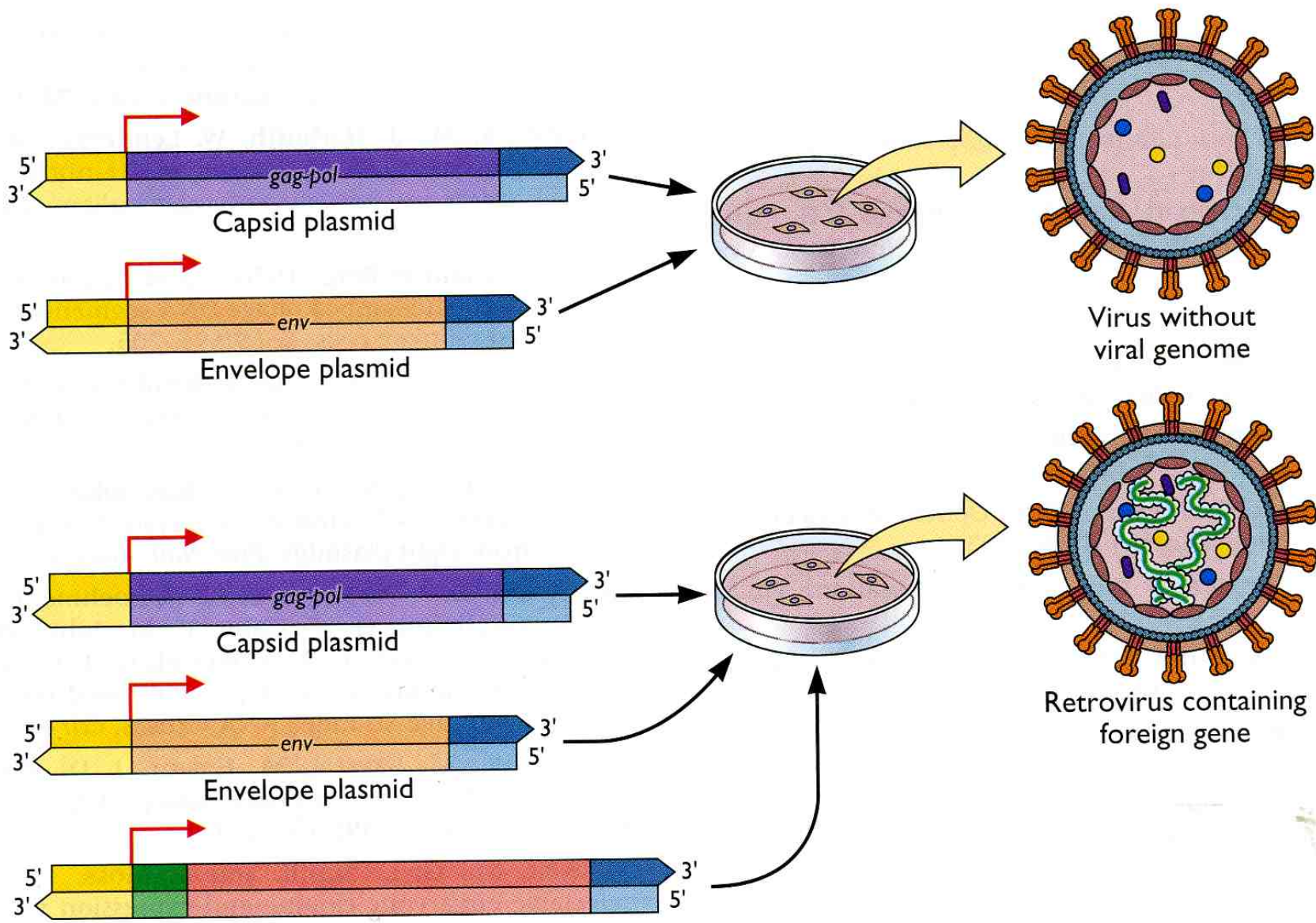


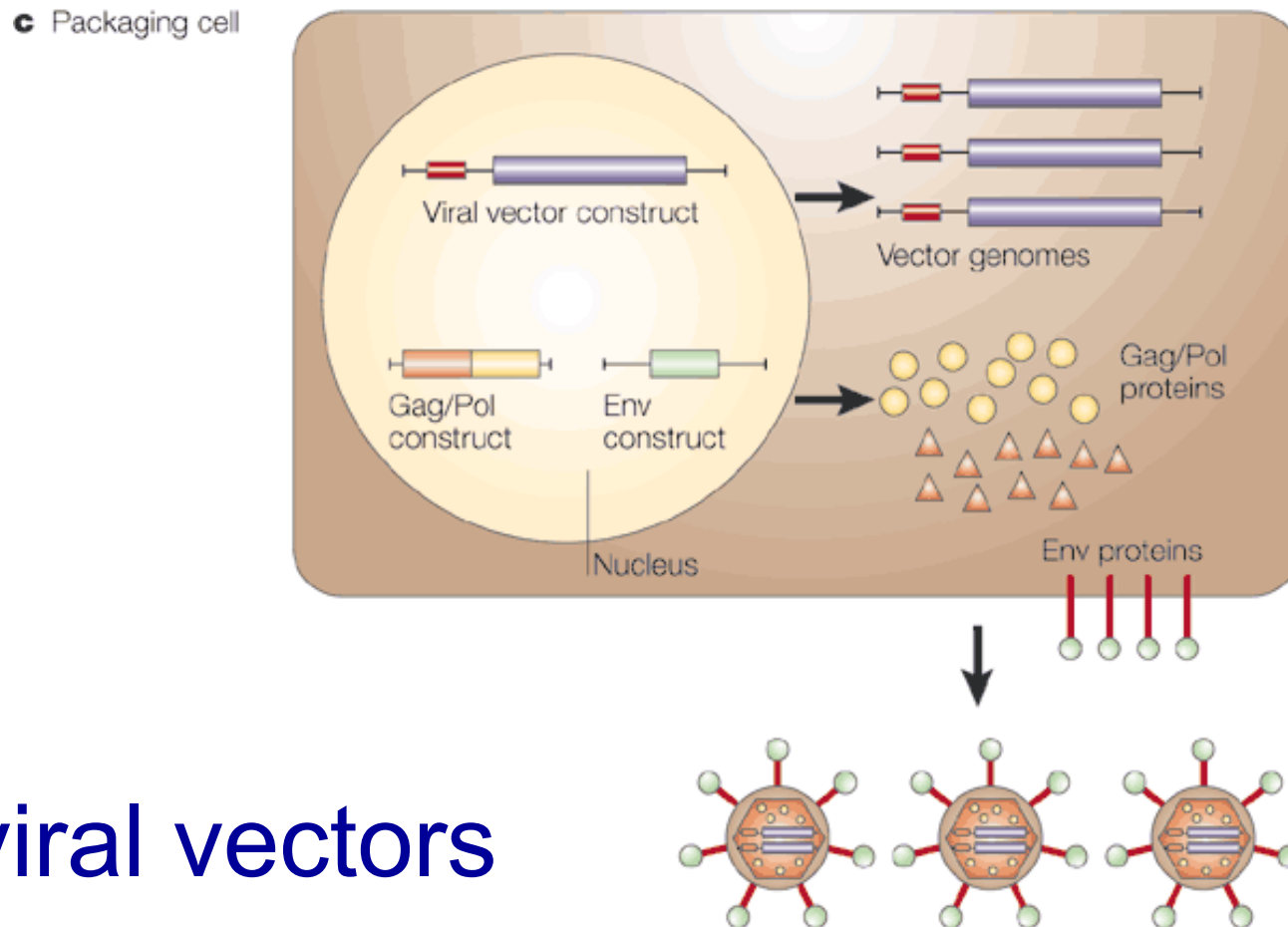
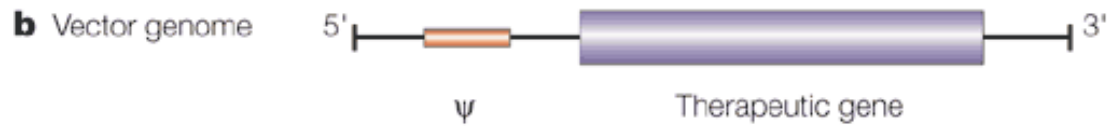
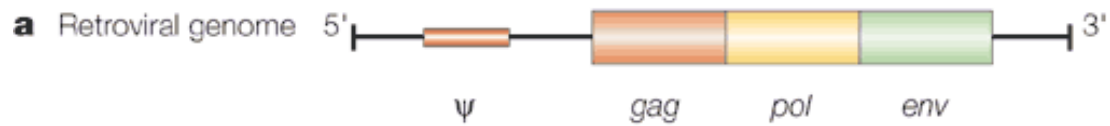
Virus without viral genome



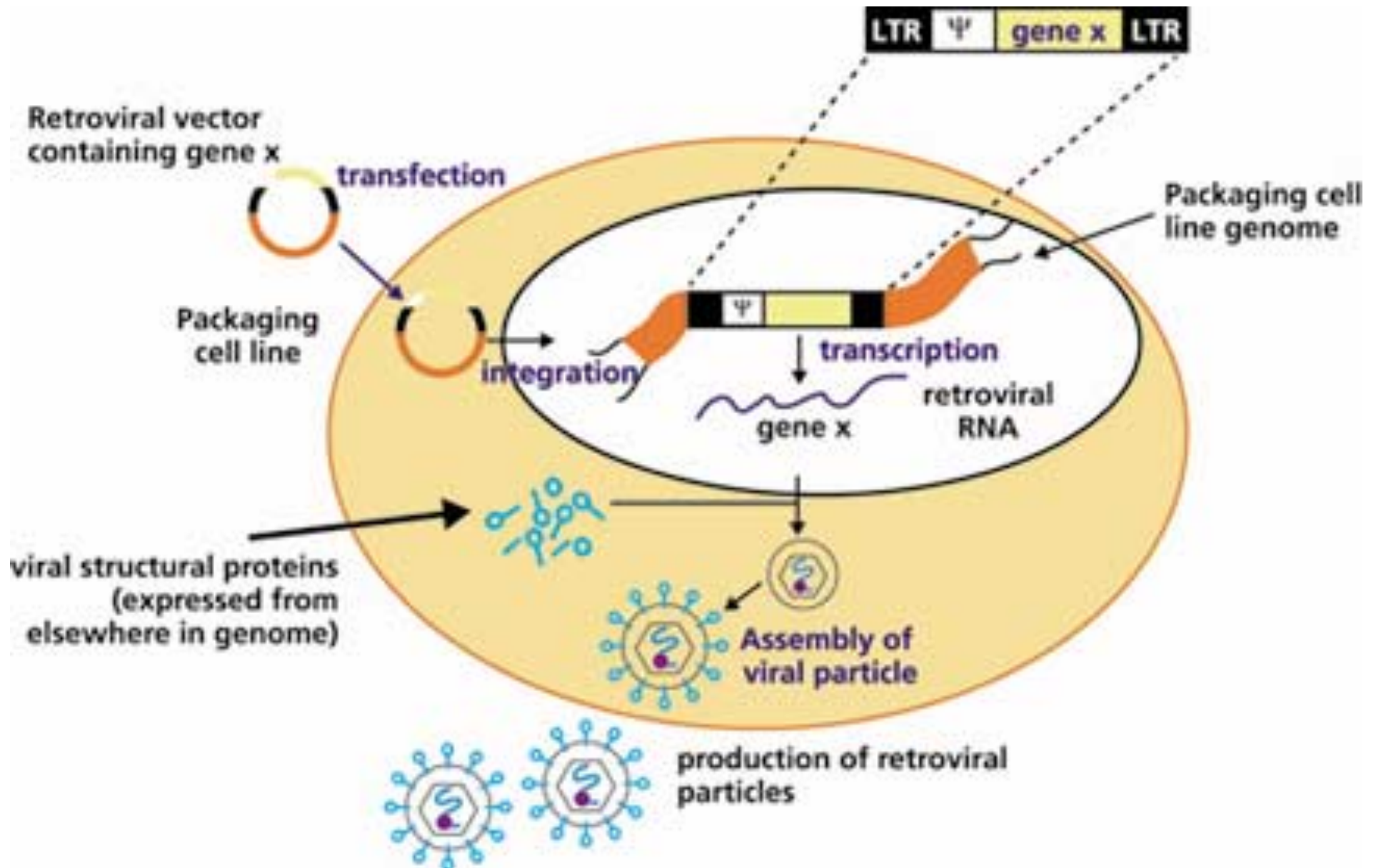
Retrovirus containing foreign gene

Retroviral vectors

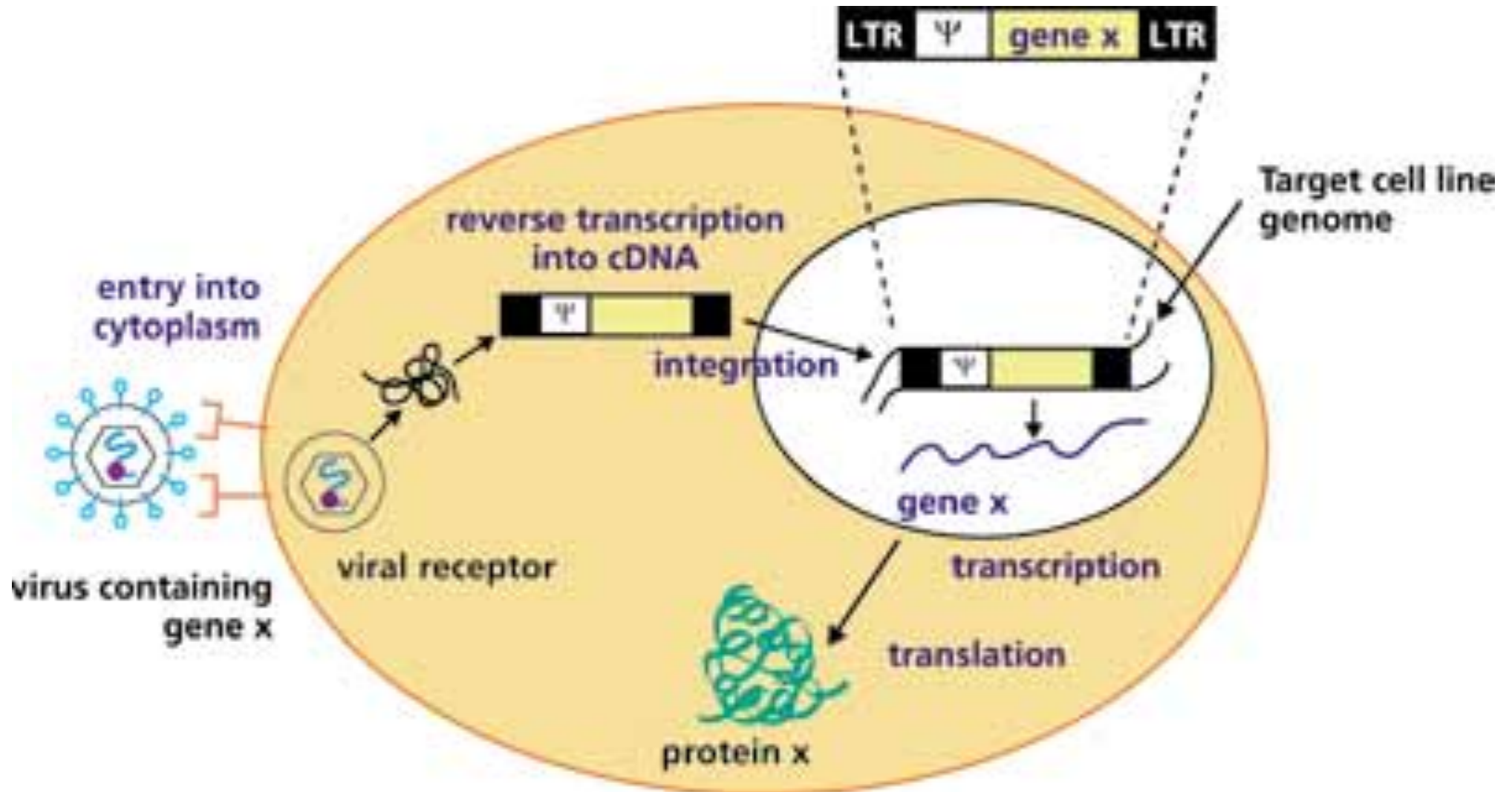




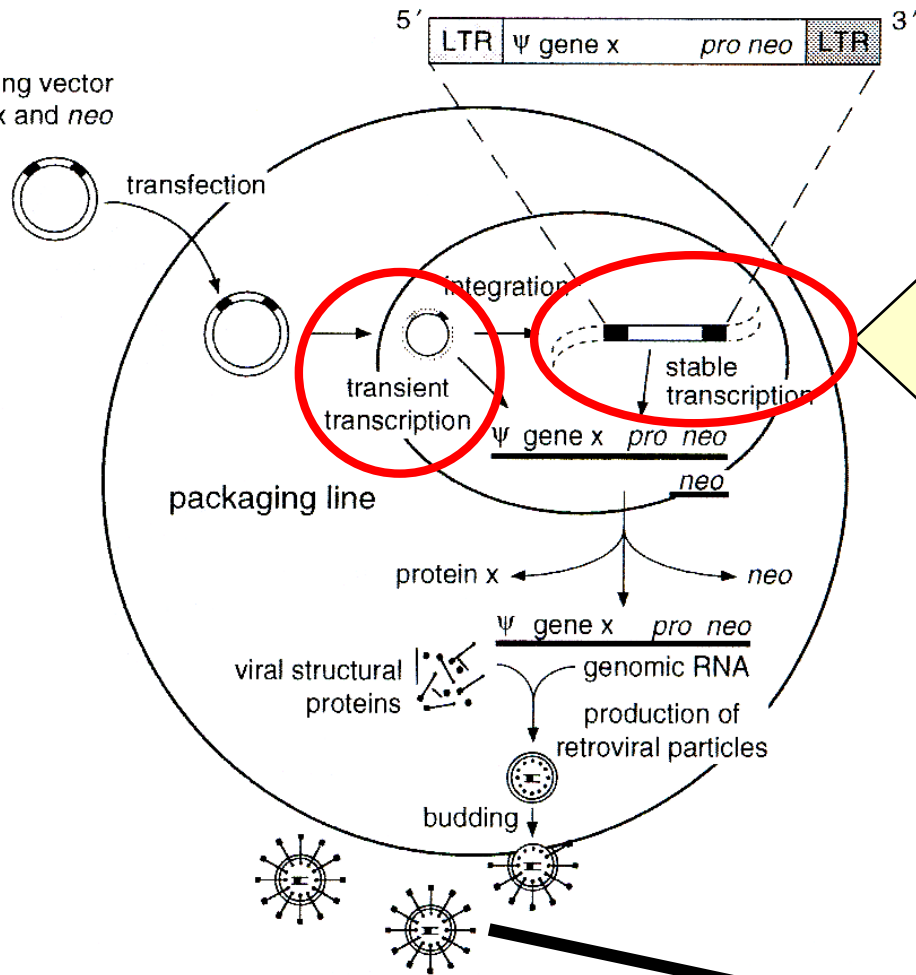
1. Production of Recombinant Retrovirus in the Packaging Cell



2. Infection of a Target cell and Expression by a Replication-Incompetent Retrovirus Vector

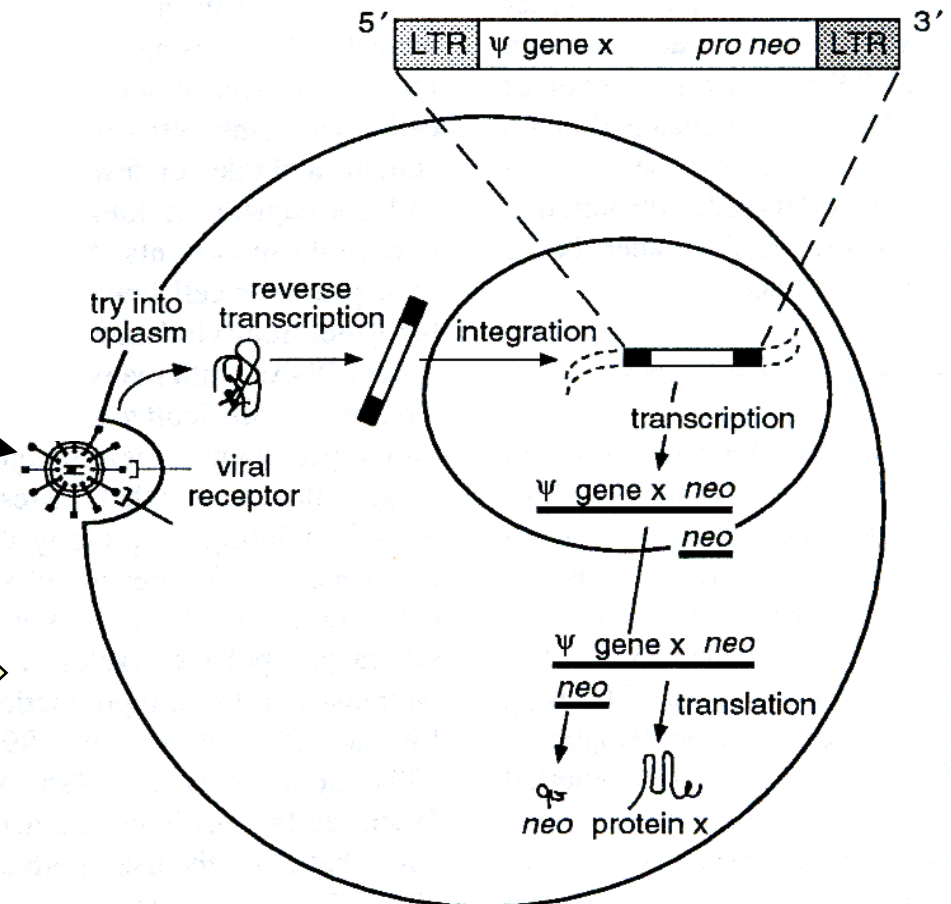


plasmid containing vector encoding gene x and *neo*

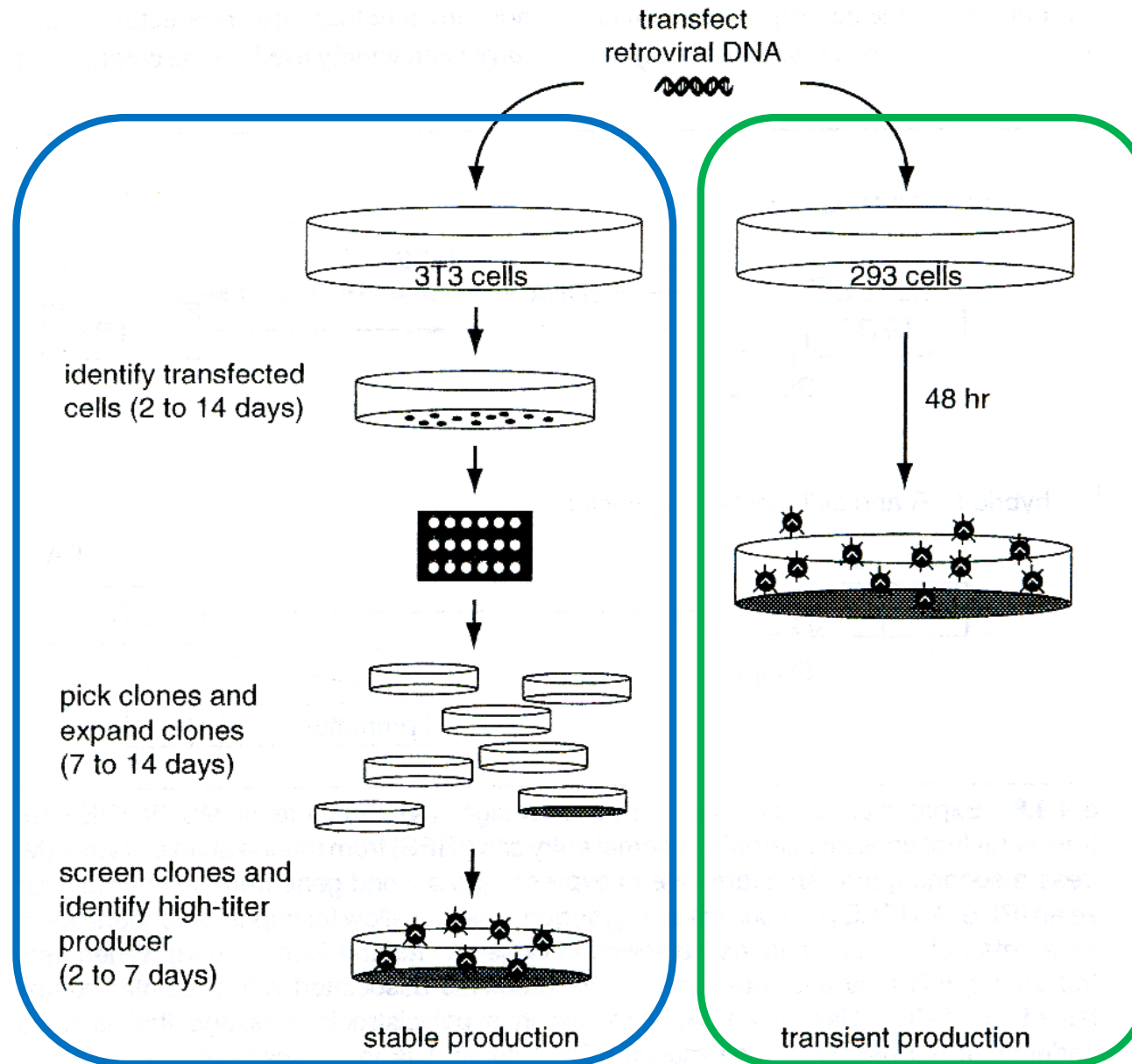


Production of infectious retroviral particles containing the gene of interest

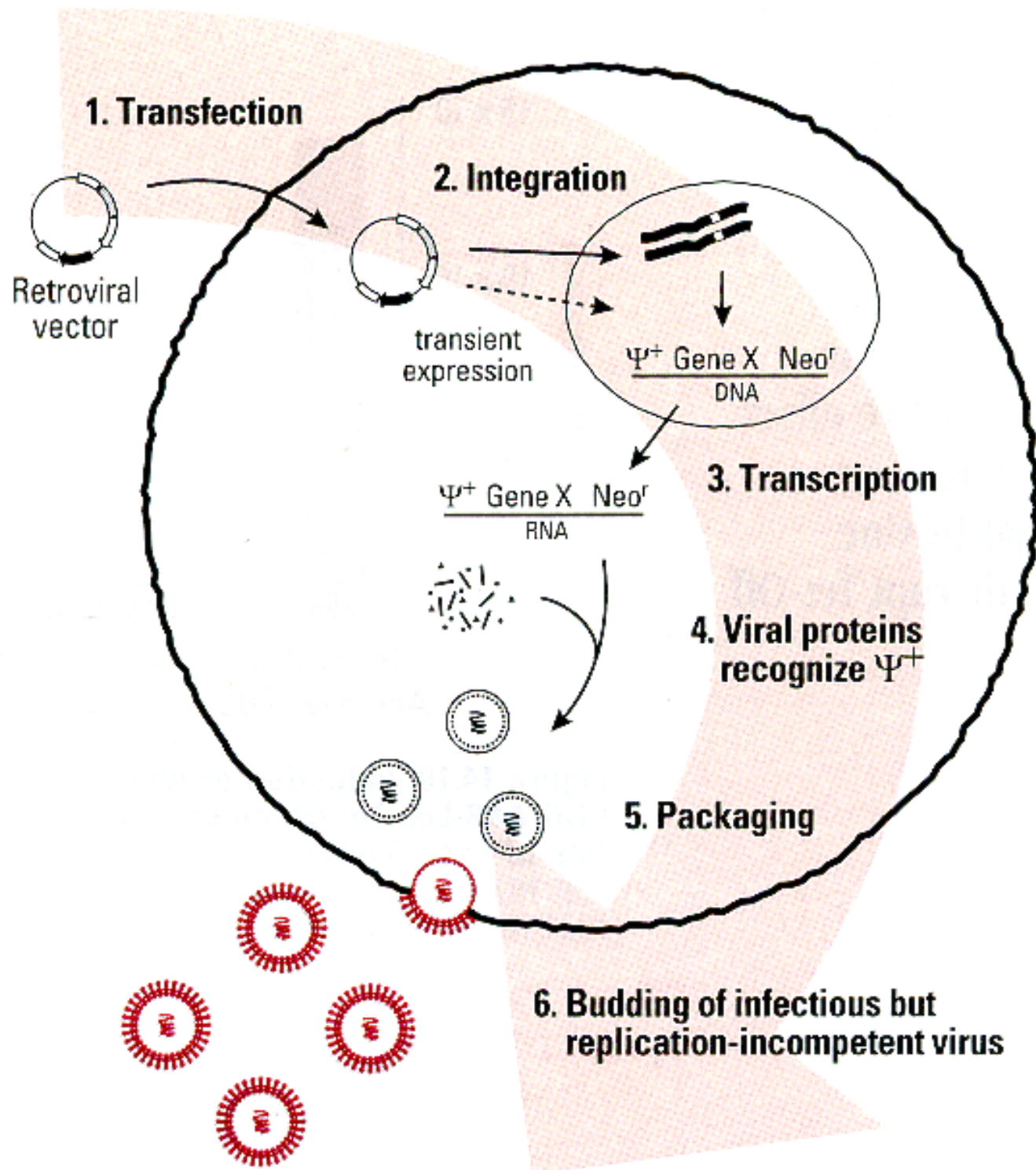
Infection of target cells and expression by a replication-incompetent retrovirus vector



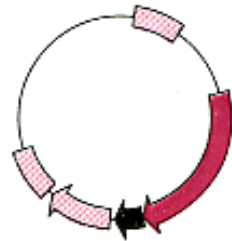
Production of recombinant retroviral stocks by stable and transient producer cell lines



Packaging cell:
produces viral
proteins from
stably integrated
genes



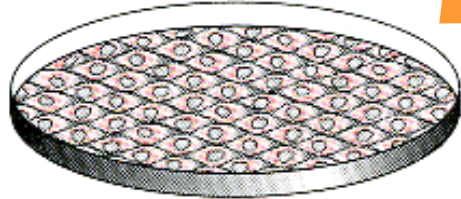
RetroXpress Vector
expressing gene
of interest



Transfection

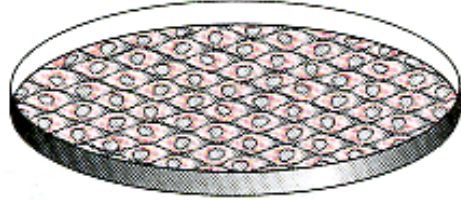
RetroPack PT67 cell line is
a NIH/3T3-based
packaging line expressing
the 10A1 viral envelop

- Transient virus production
(10^5 – 10^6 ffu/ml)



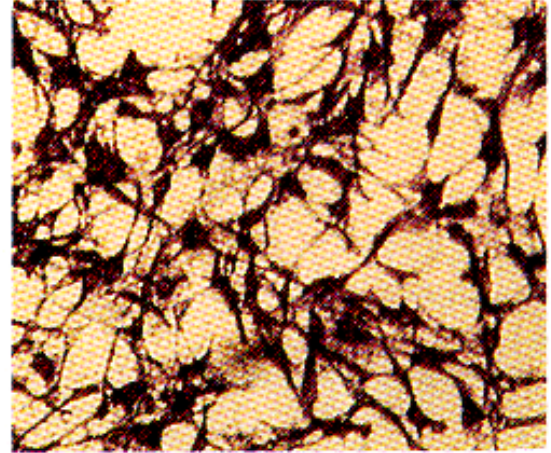
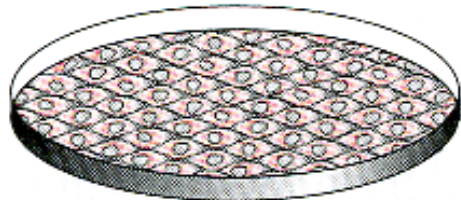
Antibiotic
selection

- Stable
virus-producing
population
(10^5 – 10^6 ffu/ml)

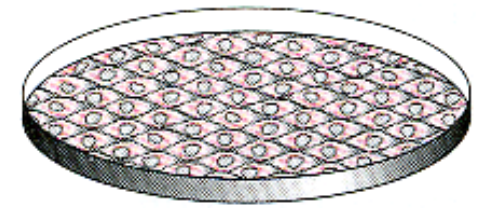


Isolation
of clones

- High-titer clone
(10^6 – 10^7 ffu/ml)

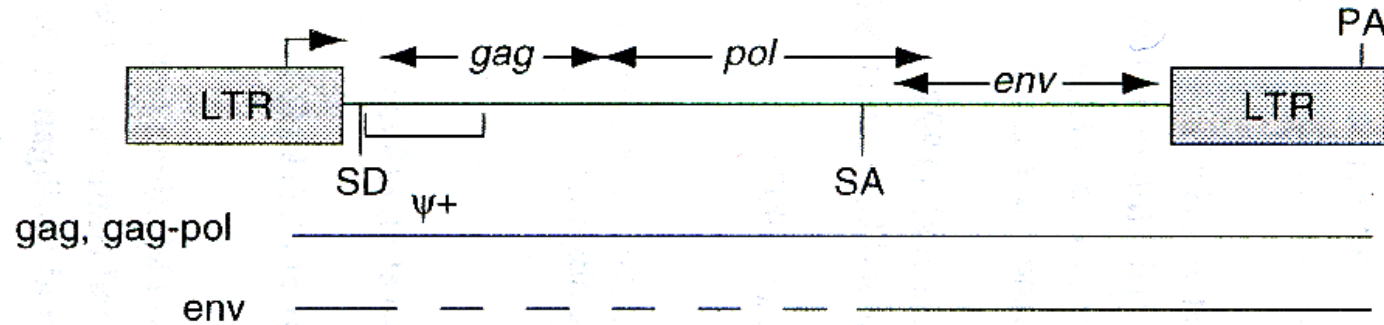


Collection of
virus and infection
of target cells

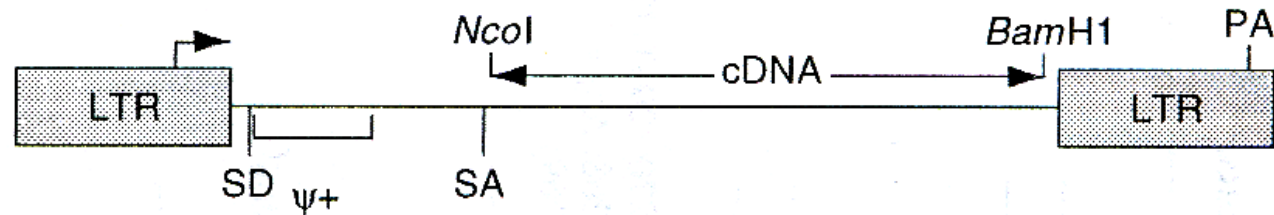


Development of retroviral vector design

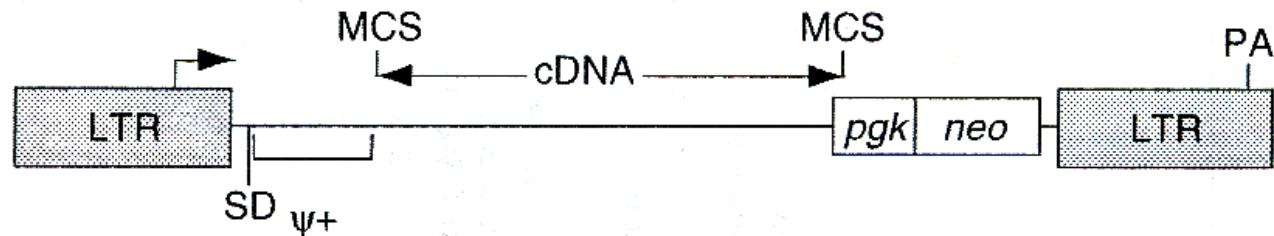
A MoMuLV (wild-type retrovirus)



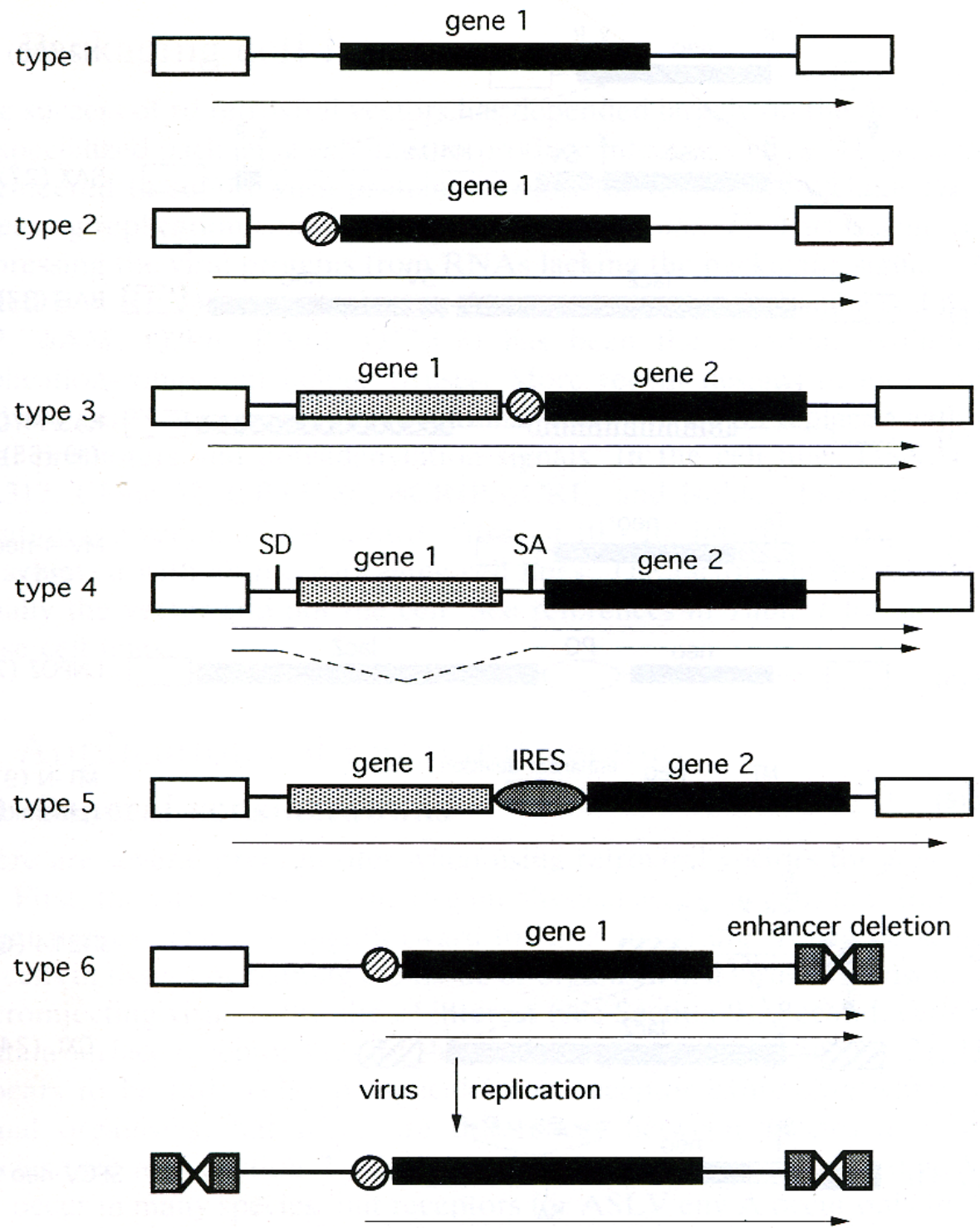
B splicing retroviral vector



C nonsplicing retroviral vector with internal promoter



Replication-defective retroviral vectors

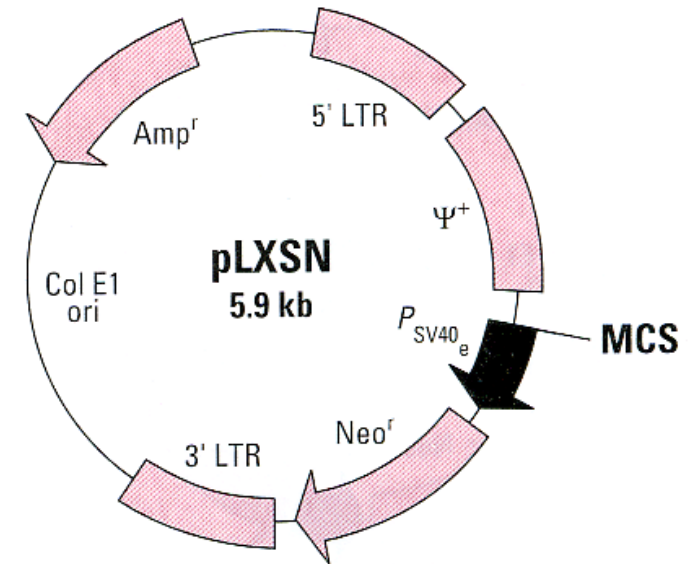
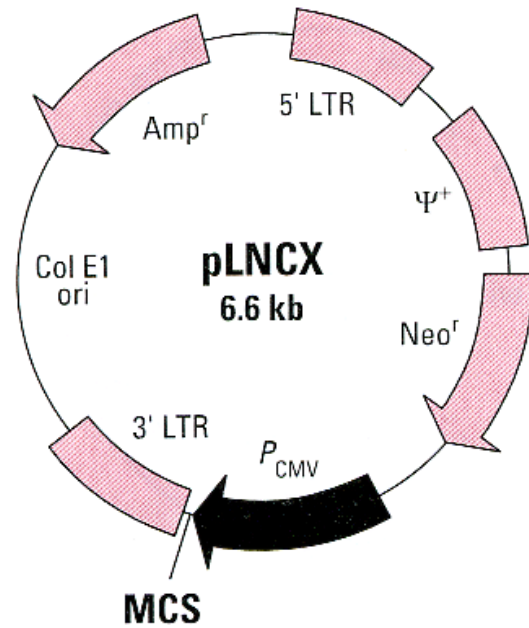


□ LTR

● Internal promoter

● IRES

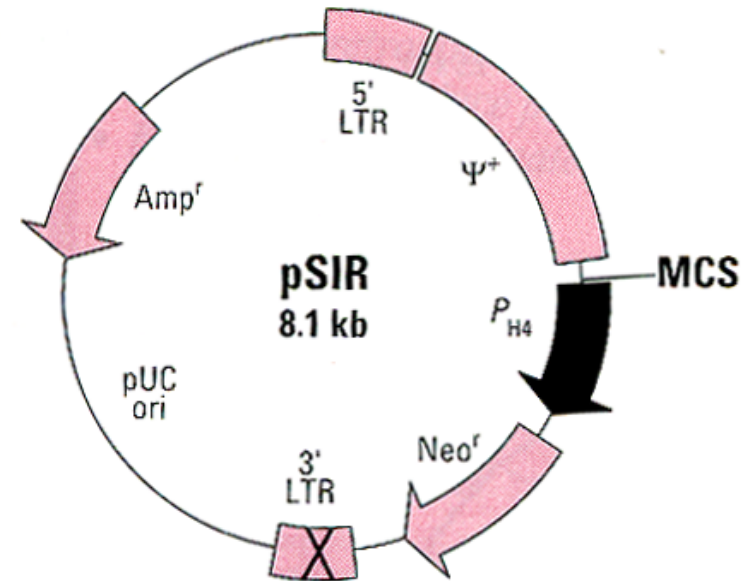
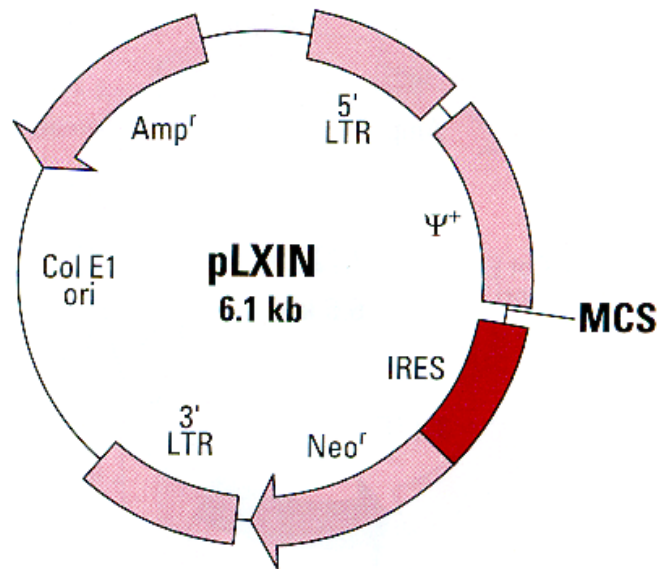
Retroviral expression vectors



GCGGCCCAAGCTTGTAAACATCGATAAAATA
Hind III Hpa I Cla I

GCGCCGGAATTCGTTAACTCGAGGATCCGGCTGTG
EcoRI Hpa I Xho I BamHI

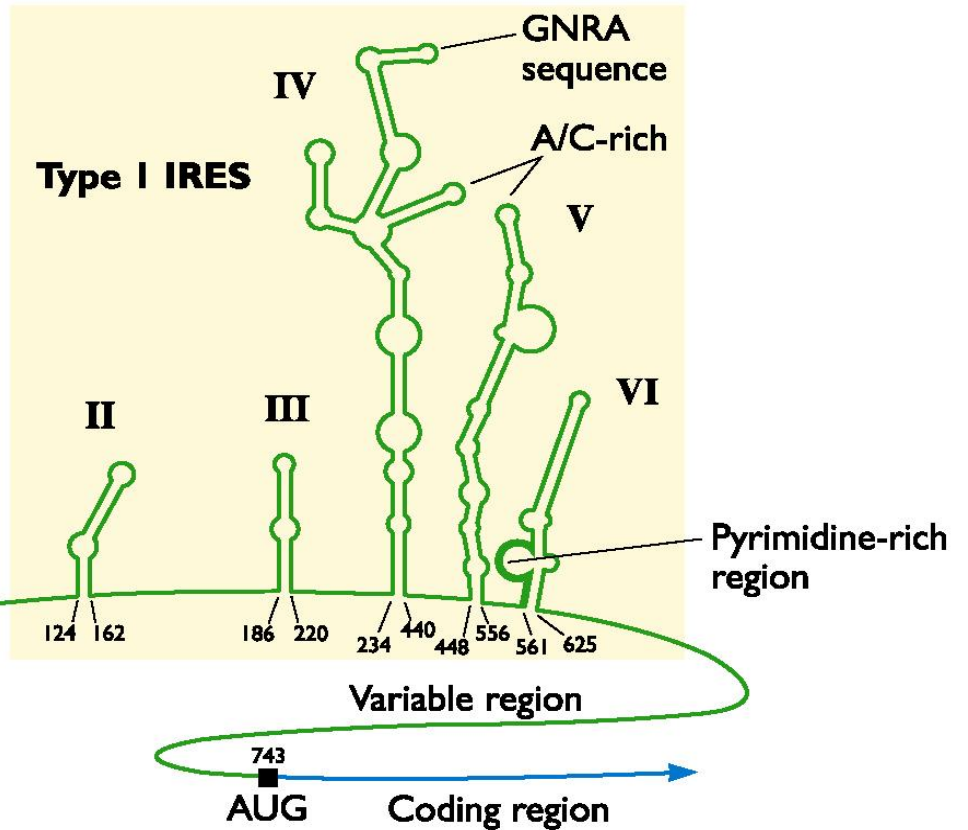
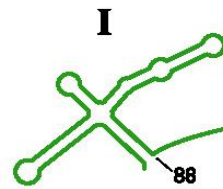
Retroviral expression vectors



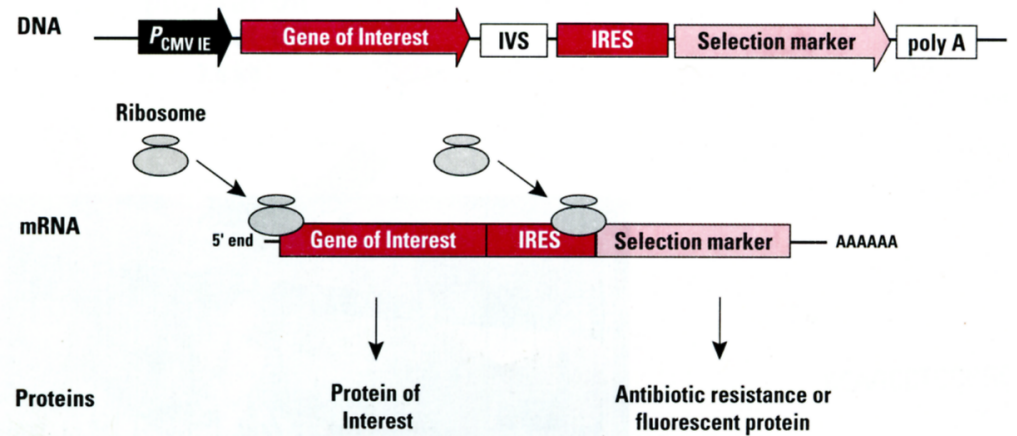
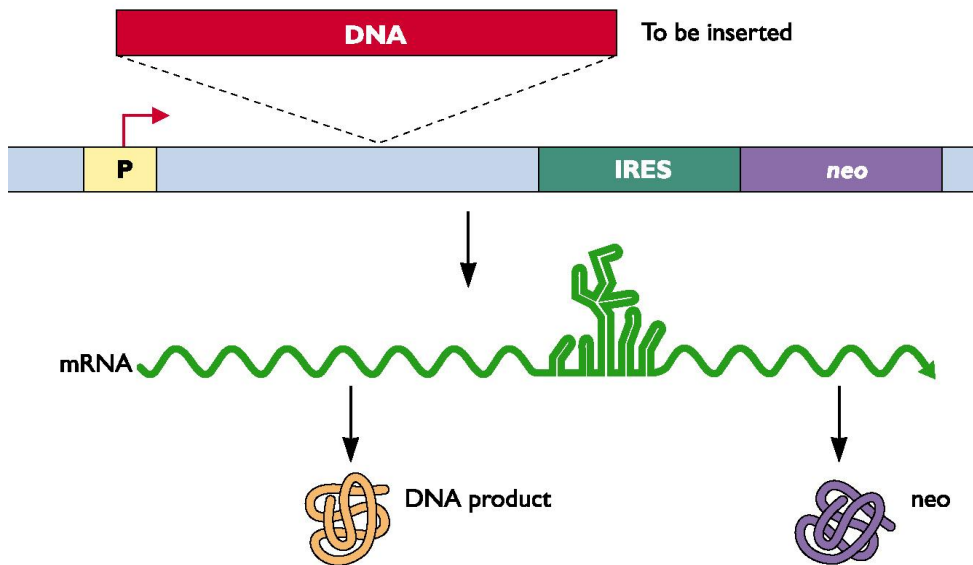
GGAATTCGTTAACTCGAGGATCCACTAGTAACGGCCGCCAGAATTCG
*EcoR*I *Hpa*I *Xho*I *Bam*H I *EcoR*I

CCCCTCGAGAAGCTTGTCTGACGGATCCGAATTC
*Xho*I *Hind* III *Bam*H I *Eco*R I

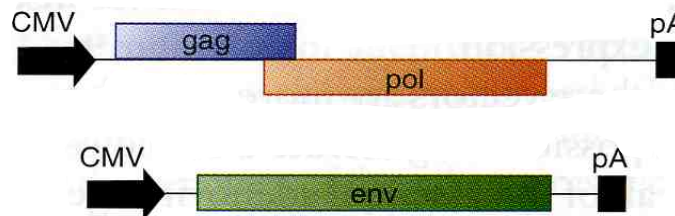
Picornaviruses IRES and their use in molecular biology



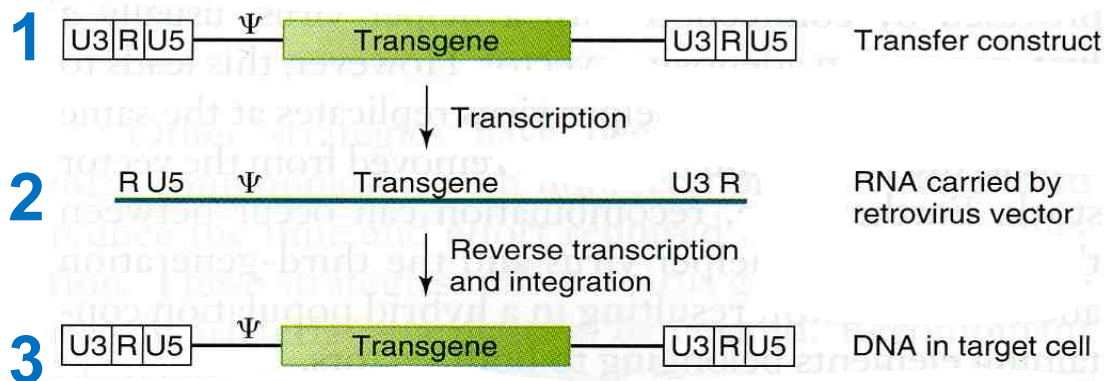
Expression cloning vector



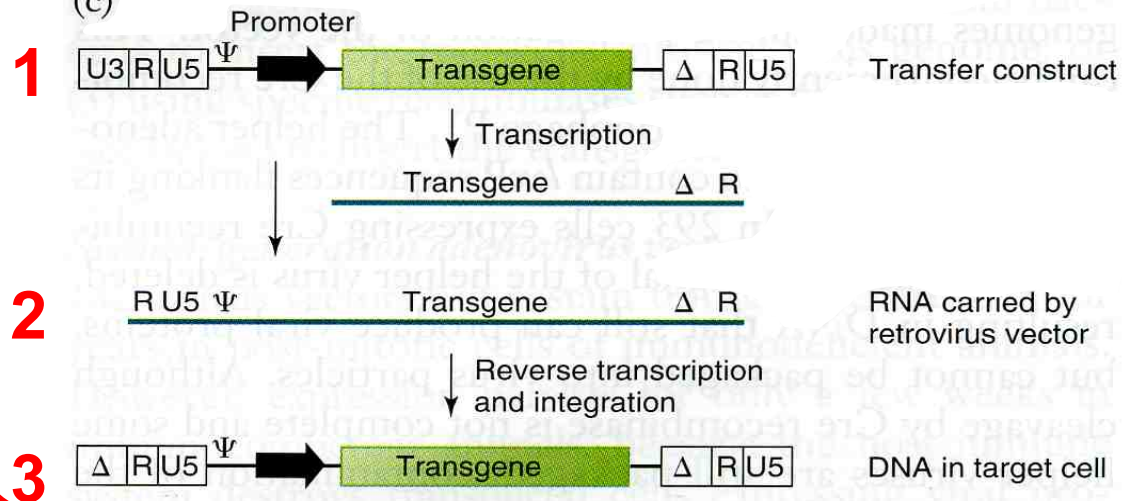
(a)



(b)



(c)



Safety-modified retrovirus vectors (SIN vectors)

Tropism of Retrovirus Vectors

Retrovirus tropism is determined at three levels:

- 1) Viral envelope proteins (gpSU);
- 2) Nuclear translocation and integration
-defined by structural features of p30^{CA}
- 3) Transcriptional activity of the LTR in the transfected cell
- MLV LTR is active in most mammalian cell types

Retroviridae host-cell receptors and co-receptors

Human immunodeficiency virus type 1	CD4	Ig-like	Chemokine receptors (Ccr5, Cxcr4, Ccr3)
Human immunodeficiency virus type 2	CD4	Glycolipid	Chemokine receptors
Simian immunodeficiency virus	CD4	Ig-like	Chemokine receptors
Gibbon ape leukemia virus	Glvrl	Sodium-dependent phosphate transport protein	← A
Feline leukemia virus B	Glvrl	Sodium-dependent phosphate transport protein	← A
Amphotropic murine leukemia virus	Ram-1	Sodium-dependent phosphate transport protein	← A
Ecotropic murine leukemia virus	Cat	Cationic amino acid transport protein	← E
Subgroup A avian leukosis and sarcoma virus	Tva	Low-density lipoprotein receptor protein family	
Subgroup B and D avian leukosis and sarcoma viruses	Car1	Tnf receptor family protein superfamily	
Bovine leukemia virus	BLVRcp 1	Unknown	
Feline immunodeficiency virus	Cxcr4	7-transmembrane superfamily	
Visna virus	Major histocompatibility complex class II molecule	Ig-like	

The envelope determines which cells the retrovirus enter

Host-range of MoMuLV-derived Vectors

- **Ecotropic** glycoprotein, gp70, allows infection of rat and mouse cells
- **Amphotropic** glycoprotein gp70 endows a murine virus with a very broad host range (mouse, human, chicken, dog, cat, mink cells)
- **Polytropic** receptors can be utilized for retrovirus entry. Pseudotyping the retroviral envelope with the VSV G protein confers a host range capable to infect mammalian, fish, frogs and insect cells

Host-range of Retroviral Vectors

Vector pseudotype	Cells that can be transduced	
	Mouse	Human
Ecotropic	Yes	No
Amphotropic	Yes	Yes
GALV	No	Yes
VSV G	Yes	Yes
RD114	No	Yes
10A1	Yes	Yes

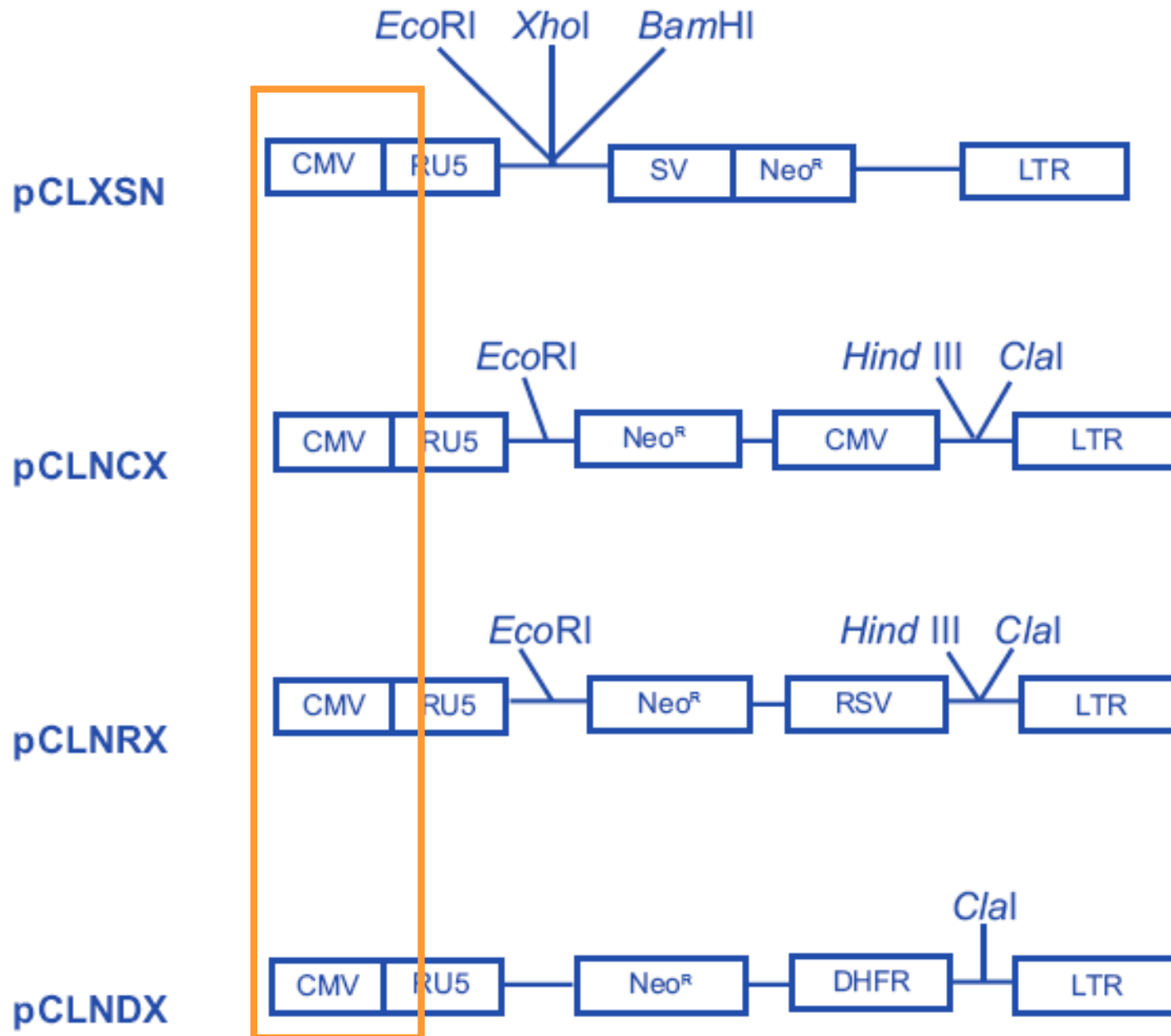
Disadvantages of Retrovirus Transduction System

- Post-mitotic cells cannot be transduced
- Unable to transduce large (>11kb) DNA fragments
- Random integration and genome rearrangement (risk of insertional activation of cellular genes)

**Retrovirus vectors - a research lab
application:**

**generation of a GFP-expressing retroviral
vector by using the Retromax system**

Retromax system: choice of vectors



Retromax system: choice of packaging vectors

Ecotropic
(usually (MoMuLV))

mouse and rat cells only
(not human)

Amphotropic
(from 4070 MuLV)

most mammalian cells
(no hamster)

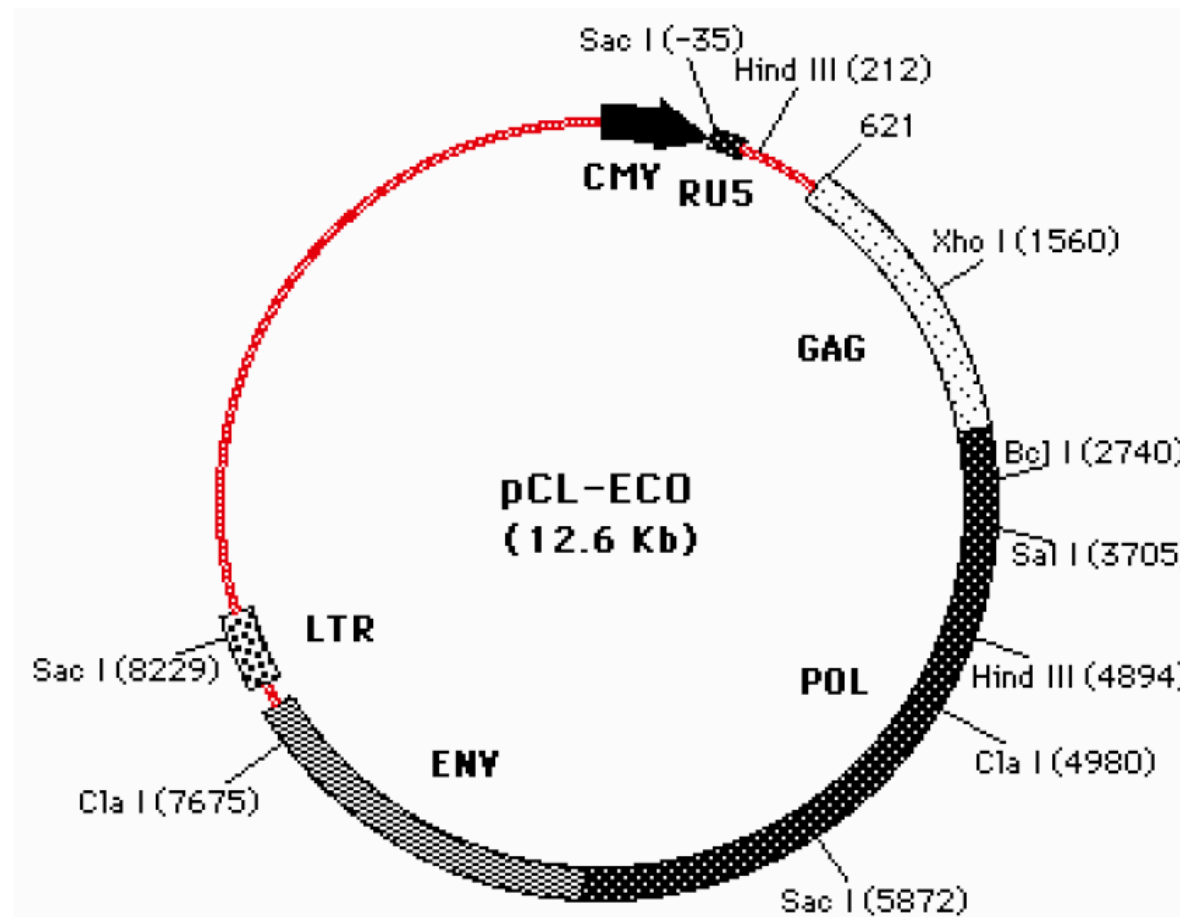
Gibbon Ape leukemia
virus (GALV)

many mammalian cells
(including hamster)

10A1 (MuLV)

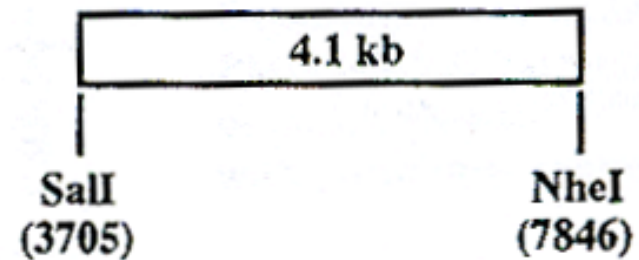
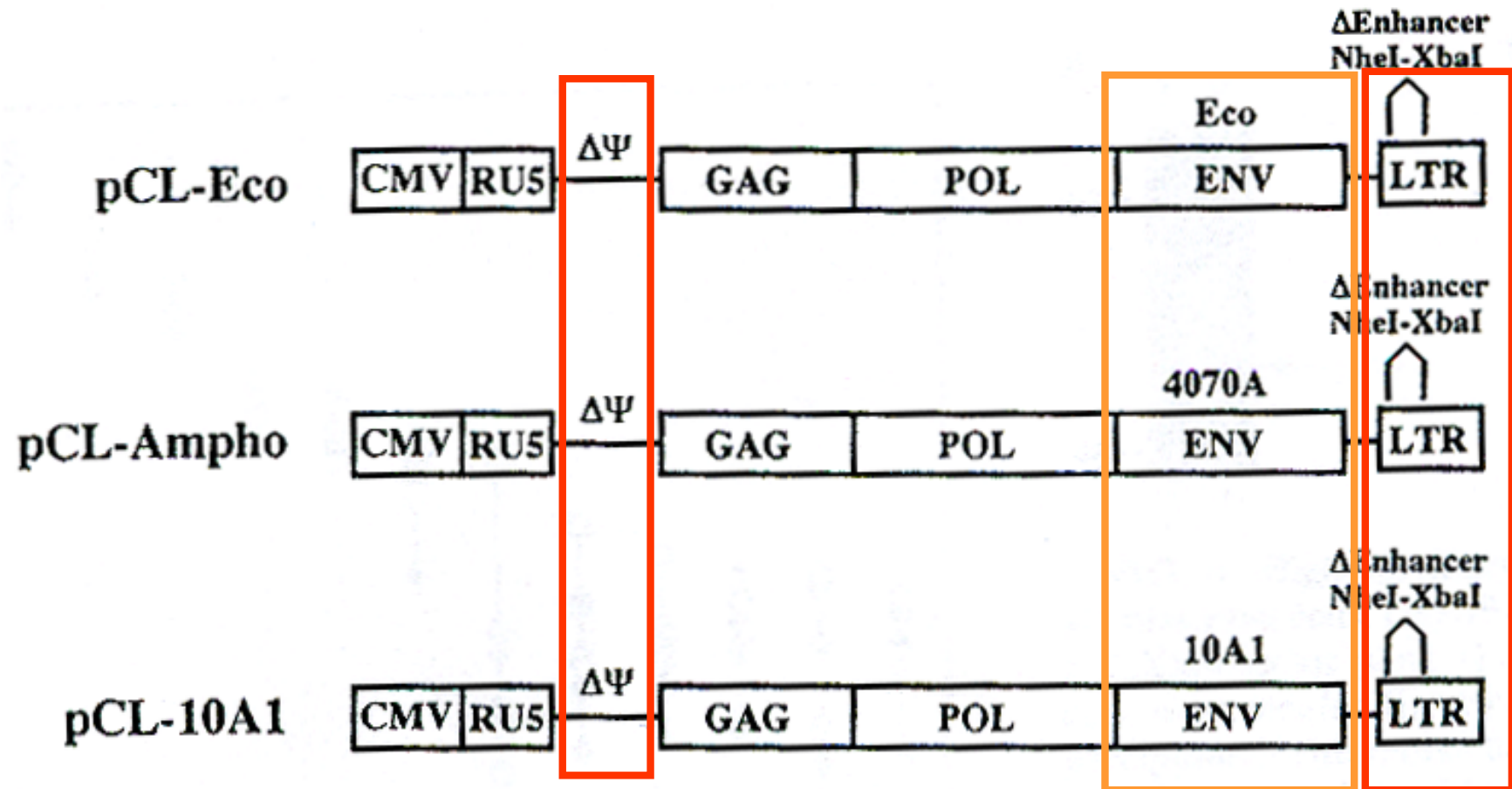
most mammalian cells
(including hamster)

Retromax system: choice of packaging vectors

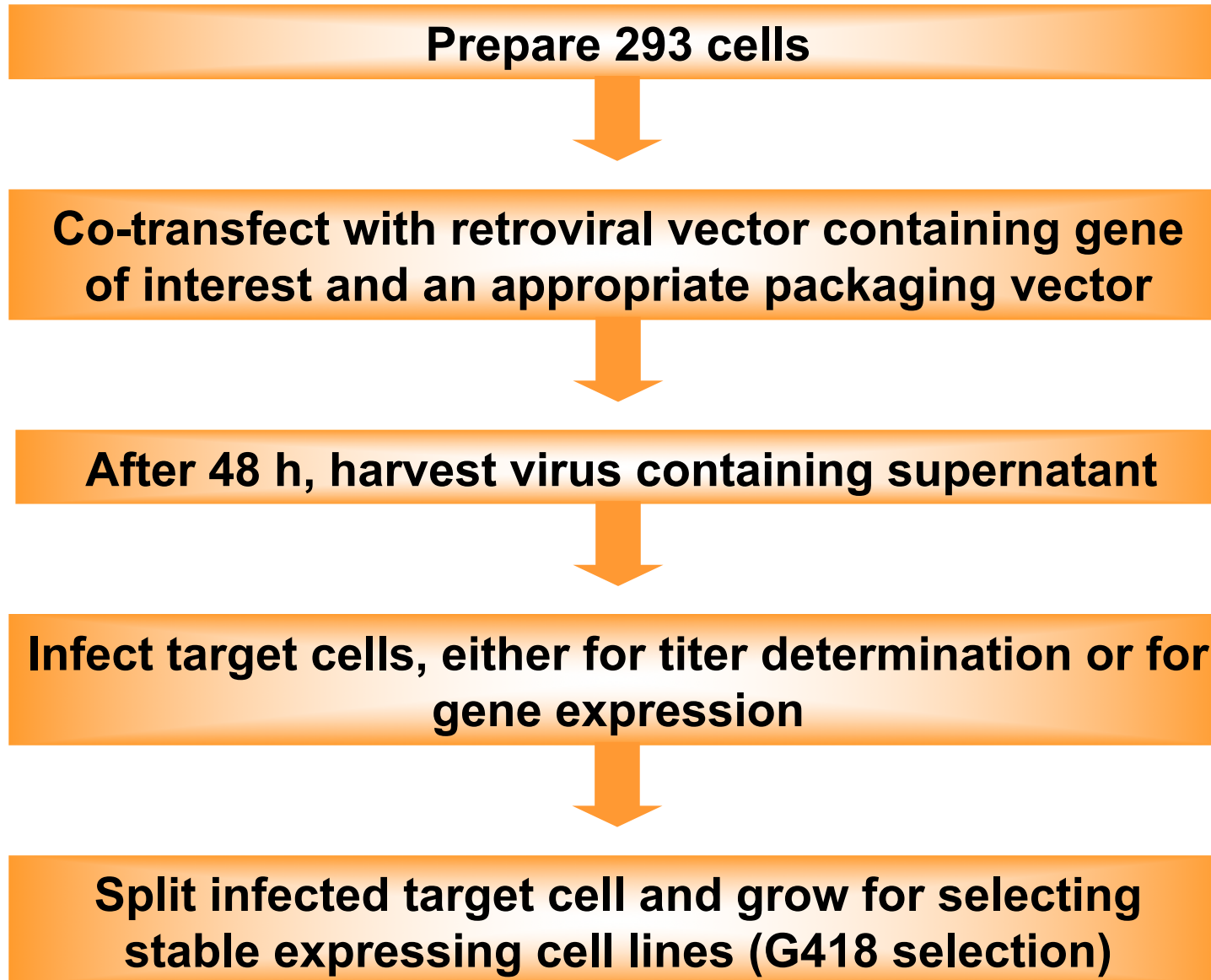


The **pCL-Eco** packaging vector. The gene coding for **env** was replaced with env gene from different MULV strains (4070A and 10A1) to create **pCL-Ampho** and **pCL-10A1** packaging vectors

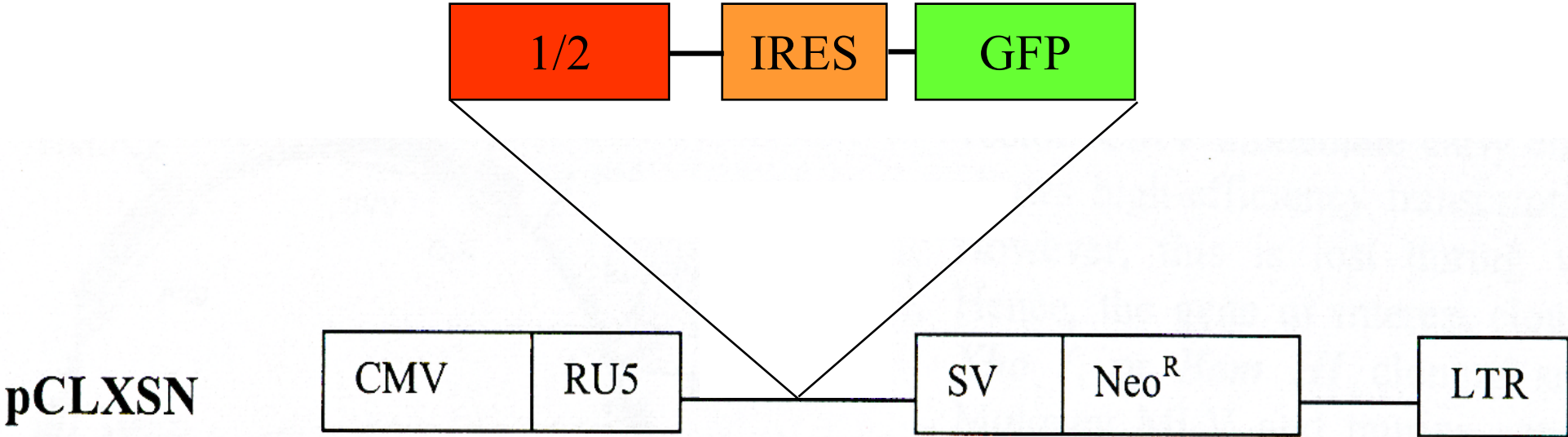
Retromax system: pCL packaging vectors



Retromax system: outline of the procedure

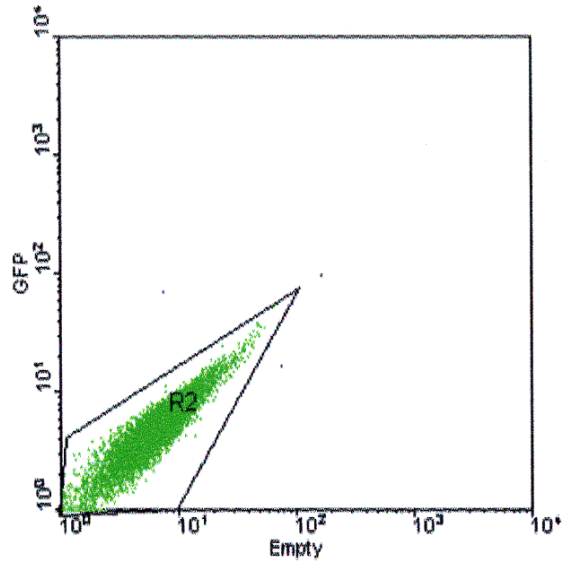


Retromax system: construction of pCLXSN-GFP vectors

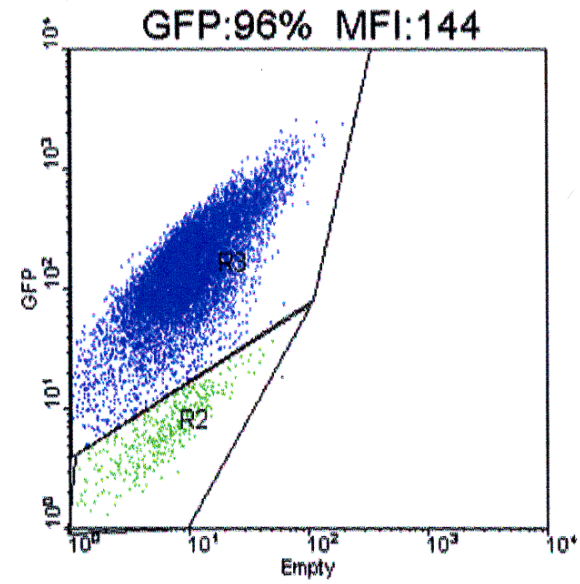


GFP exp in infected HUVECs after 1 wk of G418 selection

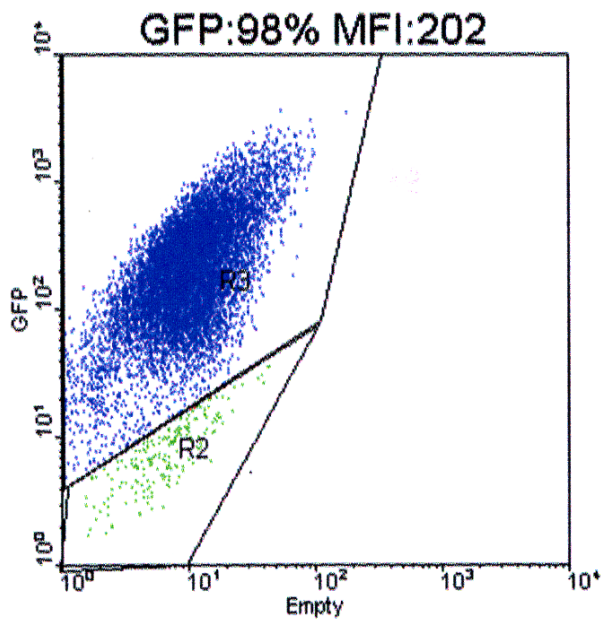
Mock



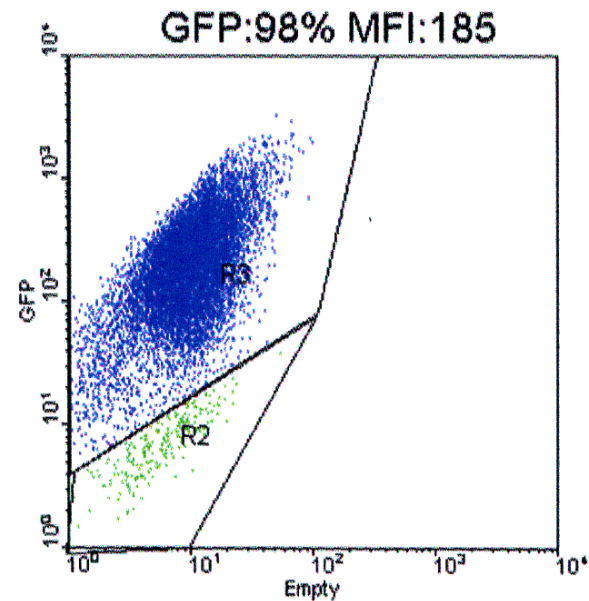
P1



P2



PE (GFP)



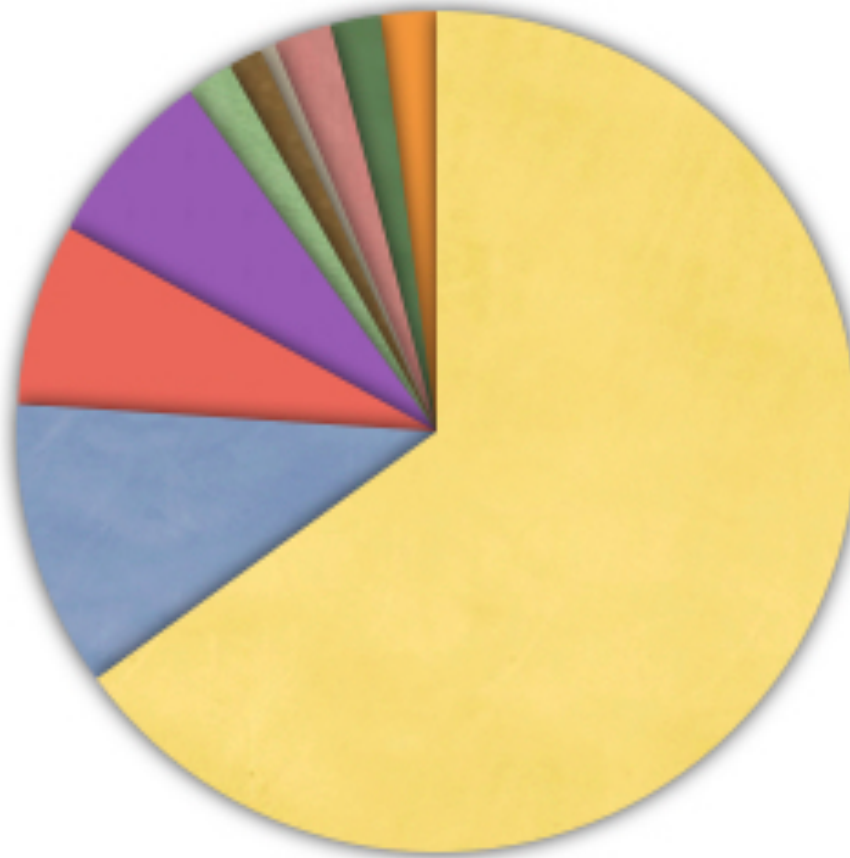
Retrovirus Vectors and Gene Therapy

Gene therapy: for what?

Indications Addressed by Gene Therapy Clinical Trials



2017 - n. 2597

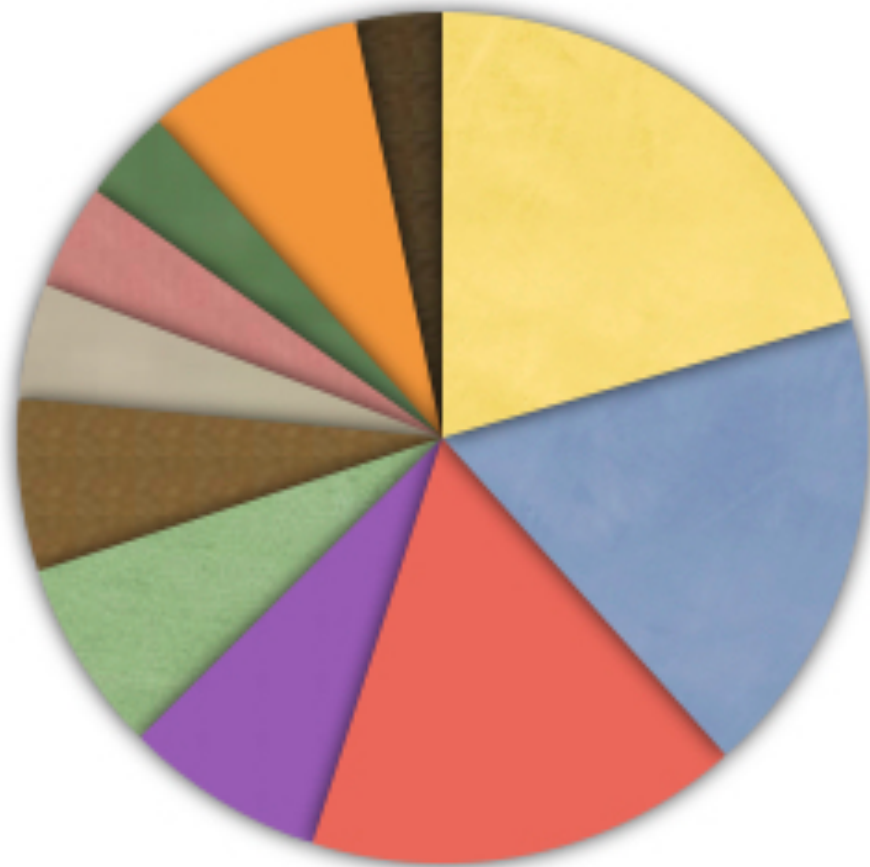


- Cancer diseases 65% (n=1688)
- Monogenic diseases 11.1% (n=287)
- Infectious diseases 7% (n=182)
- Cardiovascular diseases 6.9% (n=180)
- Neurological diseases 1.8% (n=47)
- Ocular diseases 1.3% (n=34)
- Inflammatory diseases 0.6% (n=15)
- Other diseases 2.2% (n=58)
- Gene marking 1.9% (n=50)
- Healthy volunteers 2.2% (n=56)

Gene therapy : main viral vectors systems

Viral vector	Description	Advantages	Limitations	Applications
Adenovirus (Ad)	Icosahedric, non- enveloped, genome of 36 kb, non-integrative	Easy propagation in high titers, infection of most cell types; insertion of large DNA fragments	High immunogenicity, inducing important cellular and humoral immune responses that can be fatal	Therapies that require transient gene expression: cancer therapy, angiogenesis induction and DNA vaccine production (due to its inflammatory and immunogenic properties)
Retroviruses (Retrovirus and Lentivirus)	Integrative in proliferative (retrovirus and lentivirus) and quiescent (lentivirus) cells	Low immunogenicity, possibility of insertion of large DNA fragments (up to 8 kb)	Insertional mutagenesis	Genetic diseases of T cells and hematological diseases (Retrovirus), HIV/AIDS
Adeno-associated virus (AAV)	Icosahedric, non- enveloped, single-stranded DNA, genome of 4.7 kb, integrative	Low immunogenicity, easy propagation in high titers, infection of most of cell types, long-term gene expression	Limited capacity for insertion of DNA fragments	Genetic diseases, tumors, neurological, ocular and cardiovascular diseases, others

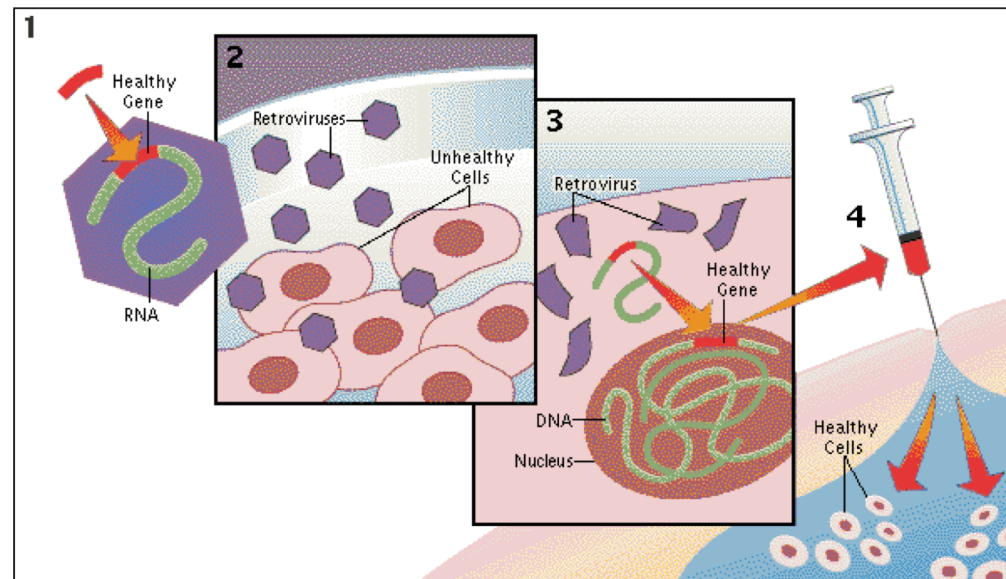
Vectors Used in Gene Therapy Clinical Trials



- Adenovirus 20.5% (n=547)
- Retrovirus 17.9% (n=478)
- Naked/Plasmid DNA 16.6% (n=442)
- Adeno-associated virus 7.6% (n=204)
- Lentivirus 7.3% (n=196)
- Vaccinia virus 6.6% (n=175)
- Lipofection 4.4% (n=117)
- Poxvirus 4% (n=107)
- Herpes simplex virus 3.5% (n=93)
- Other vectors 8.4% (n=223)
- Unknown 3.3% (n=88)

Retroviruses as vectors for delivering gene therapy

Disease	Defect	Incidence	Viral vector
Severe combined immunodeficiency	Adenosine deaminase (25% of patients)	Rare, <1 in 10 ⁵ live births	Gammaretrovirus
Lipoprotein lipase deficiency	Common cytokine receptor γ chain (X-linked)	1 in 50,000–100,000 live births	Self-inactivating gammaretrovirus
Hemophilia B	Lipoprotein lipase	Rare, 1–2 in 10 ⁶ live births	AAV ^{a,b}
Hemoglobinopathies and thalassemias	Factor IX deficiency	1 in 30,000 males	AAV
α_1 -Antitrypsin deficiency (inherited emphysema, liver disease)	Defects in α - or β -globin gene	1 in 600 in specific ethnic groups	Self-inactivating lentivirus
Retinal degenerative disease, Leber's congenital amaurosis (LCA)	α_1 -Antitrypsin not produced	1 in 3,500	AAV
X-linked adrenoleukodystrophy	Retinal pigment epithelium-specific 65-kDa protein	<10% of LCA cases (LCA, ~1 in 80,000 live births)	AAV
Wiskott-Aldrich syndrome (eczema-thrombocytopenia-immunodeficiency syndrome)	ABCD1 transporter	1 in 20,000–50,000 live births	Self-inactivating lentivirus
	Was protein	1–10 in 10 ⁶ males	Self-inactivating lentivirus

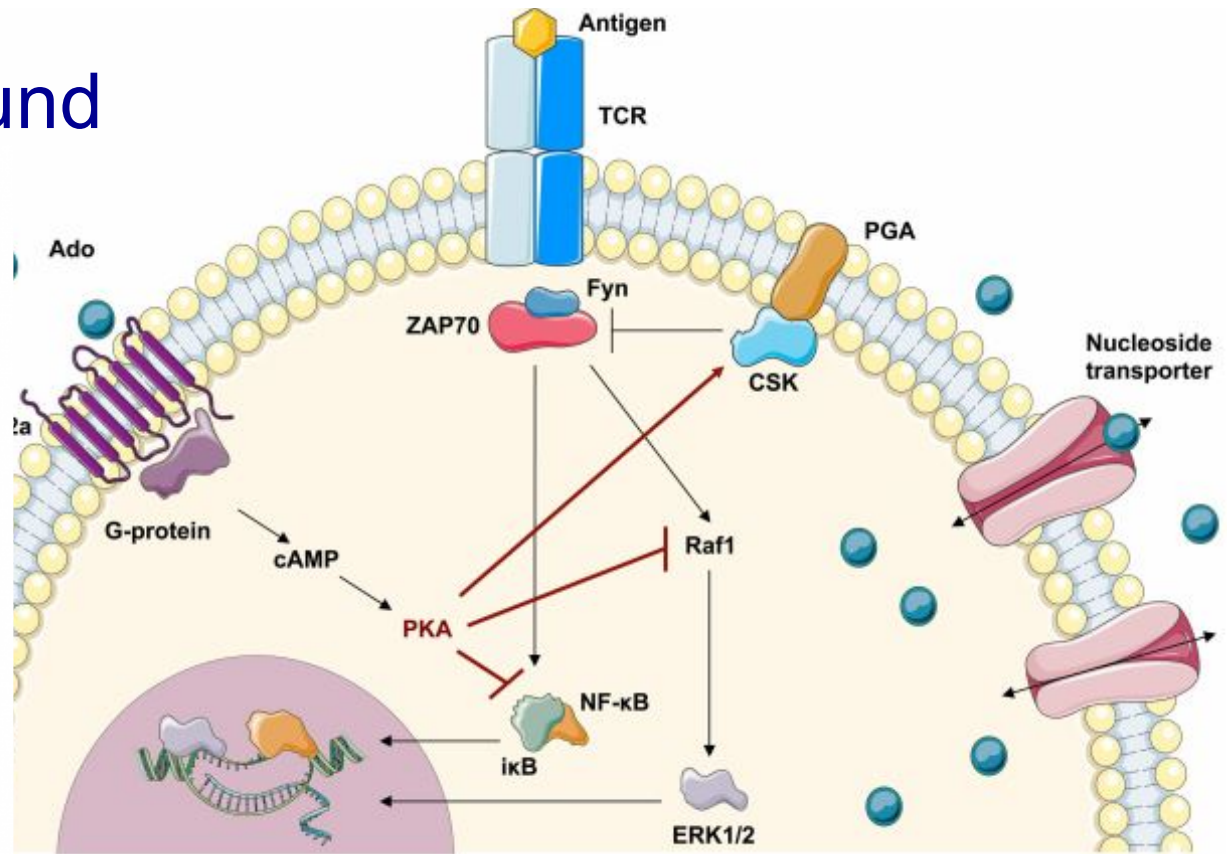


Retrovirus for gene therapy of ADA-SCID



David Vetter: the first «bubble boy», 1971-1983

ADA-SCID: background



- Deficiency of **adenosine deaminase (ADA)**, an enzyme of purine metabolism.
- Mutations of the ADA gene cause profound lymphopenia (T, B, and NK cells).
- Untreated ADA-SCID is a fatal disorder.
- To restore ADA activity and reconstitute protective immunity: enzyme replacement therapy (ERT); allogeneic hematopoietic stem cell transplantation (HSCT), and **gene therapy (GT)** with autologous gene-corrected hematopoietic stem cells (HSC).

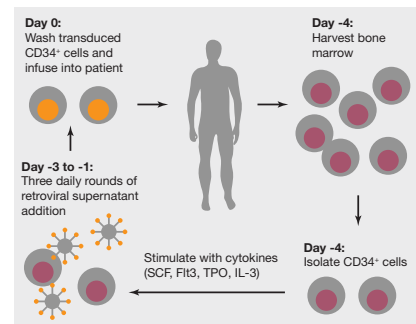
Retrovirus as a therapeutic agent for ADA- SCID

Leading Edge
Bench to Bedside



Gene Therapy for “Bubble Boy” Disease

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<http://dx.doi.org/10.1016/j.cell.2016.06.049>



NAME
Strimvelis (GSK2696273); international nonproprietary name: autologous CD34+ enriched cell fraction that contains CD34+ cells transduced with retroviral vector that encodes for the human ADA cDNA sequence

APPROVED FOR
Treatment of severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) in patients who cannot be treated by a bone-marrow transplant because they do not have a suitable, matched, related donor

TYPE
Gene therapy: autologous CD34+ enriched cell fraction that contains CD34+ cells transduced with retroviral vector (LXSN vector) that encodes for the human ADA cDNA sequence

CELLULAR TARGETS
CD34+ hematopoietic cells

EFFECTS ON TARGETS
Replaces defective adenosine deaminase in immune cells

DEVELOPED BY
GlaxoSmithKline, MolMed, San Raffaele Telethon Institute for Gene Therapy

Adenosine deaminase (ADA) deficiency results in the accumulation of toxic metabolites that destroy the immune system, causing severe combined immunodeficiency (ADA-SCID), often referred to as the “bubble boy” disease. Strimvelis is a European Medicines Agency approved gene therapy for ADA-SCID patients without a suitable bone marrow donor.

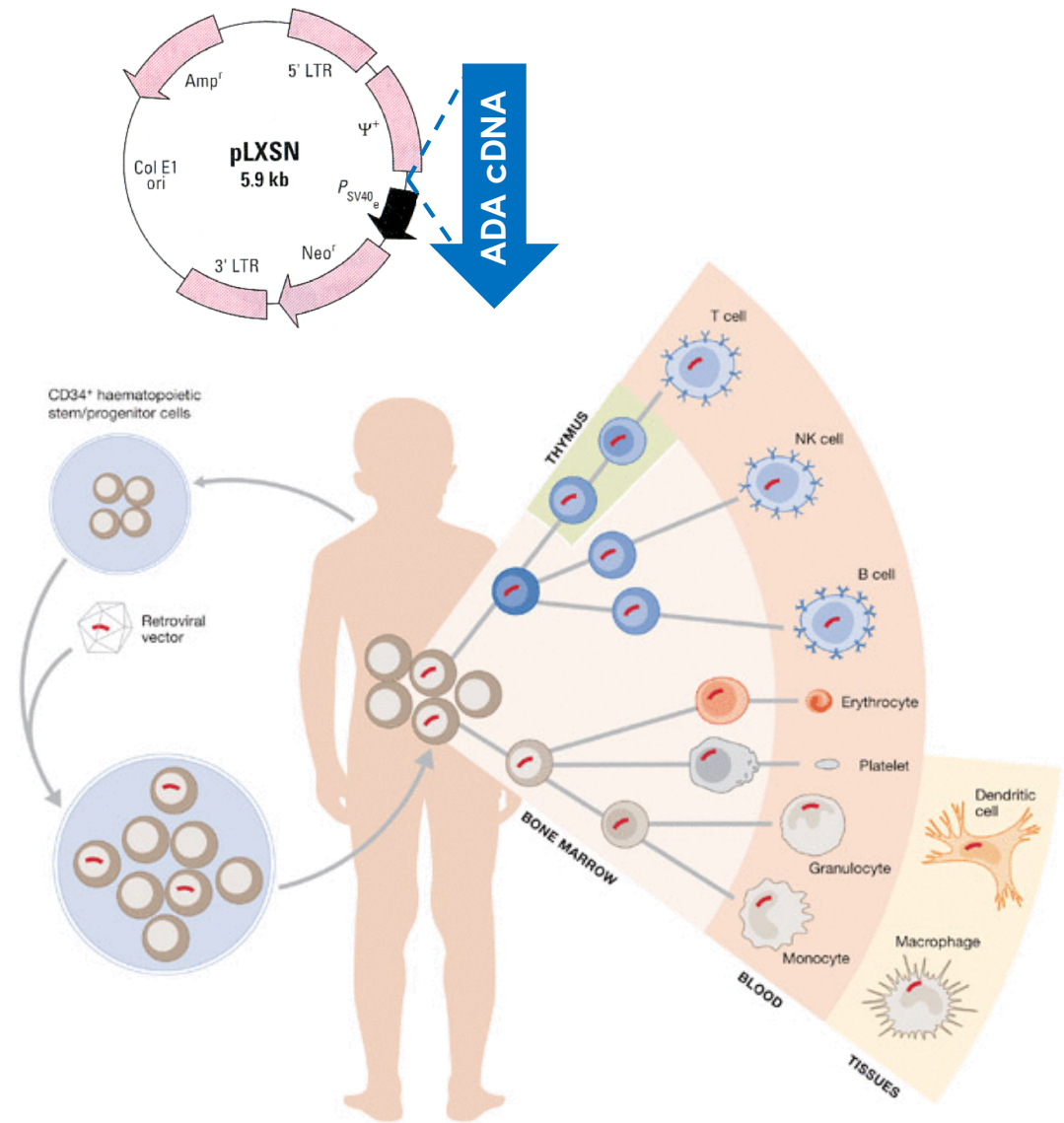
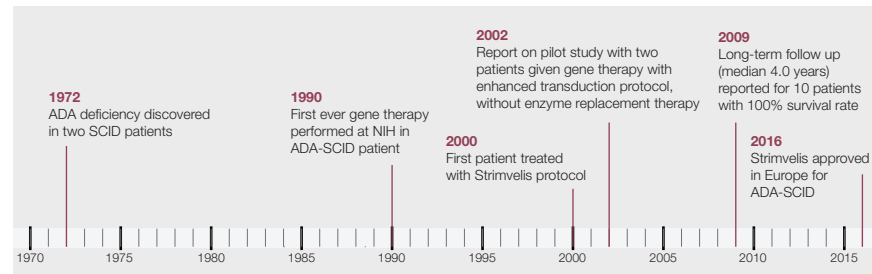
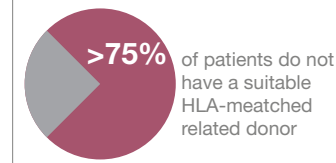
Disease frequency

1-9 OUT OF EVERY
1,000,000
LIVE BIRTHS

Treatments to date



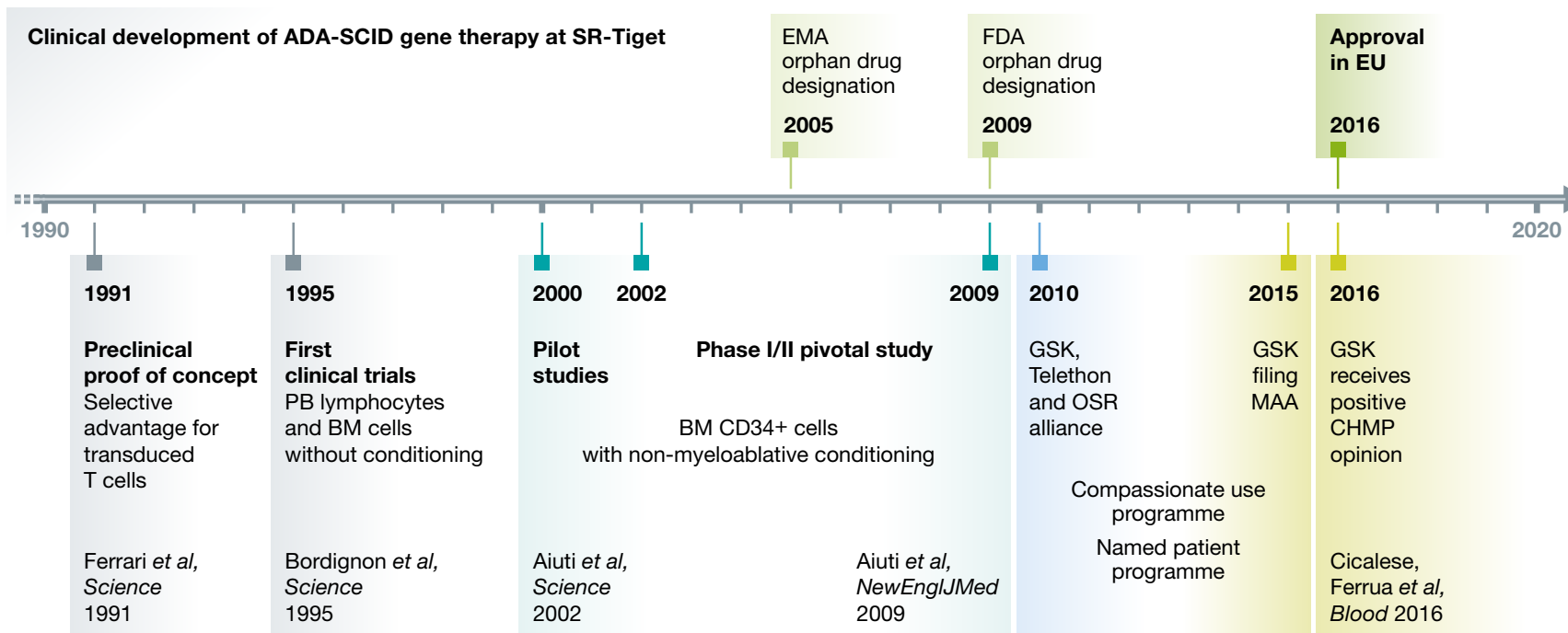
HLA-matched related donors



References for further reading are available with this article online: [www.cell.com/cell/fulltext/S0092-8674\(16\)30857-1](http://www.cell.com/cell/fulltext/S0092-8674(16)30857-1)



Retroviruses as vectors: development of Strimvelis



BM Bone marrow	EMA European Medical Agency	GSK GlaxoSmithKline plc	MAA Marketing Authorization Application
PB Peripheral blood	FDA U.S. Food and Drug Administration	OSR Ospedale San Raffaele	CHMP Committee for Medicinal Products for Human Use

Figure 1. Schematic representation of the key scientific and regulatory milestones in the clinical development of ADA-SCID gene therapy, leading to its approval in the EU.

EMA approved gene therapy for ADA-

Commentary



Gene therapy for ADA-SCID, the first marketing approval of an *ex vivo* gene therapy in Europe: paving the road for the next generation of advanced therapy medicinal products

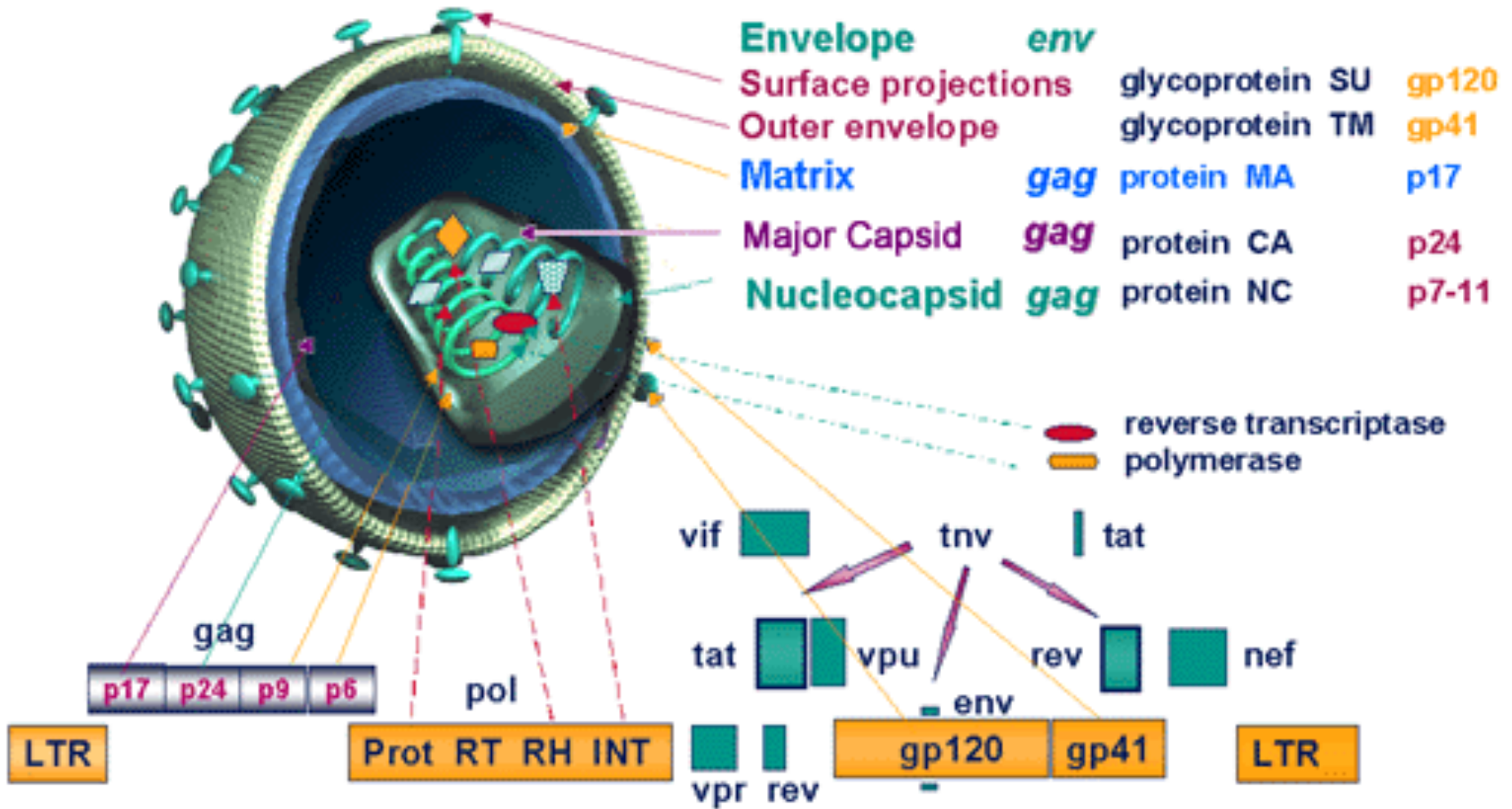
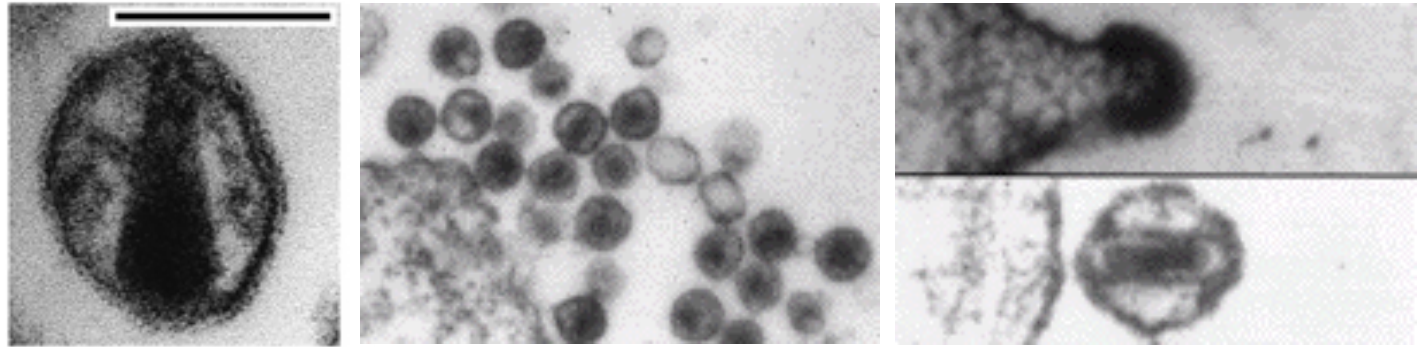
VIROLOGY

Engineering Viral Genomes: **Lentivirus Vectors**

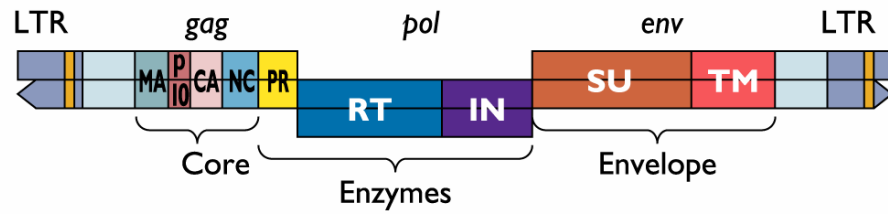
Viral vectors

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

HIV

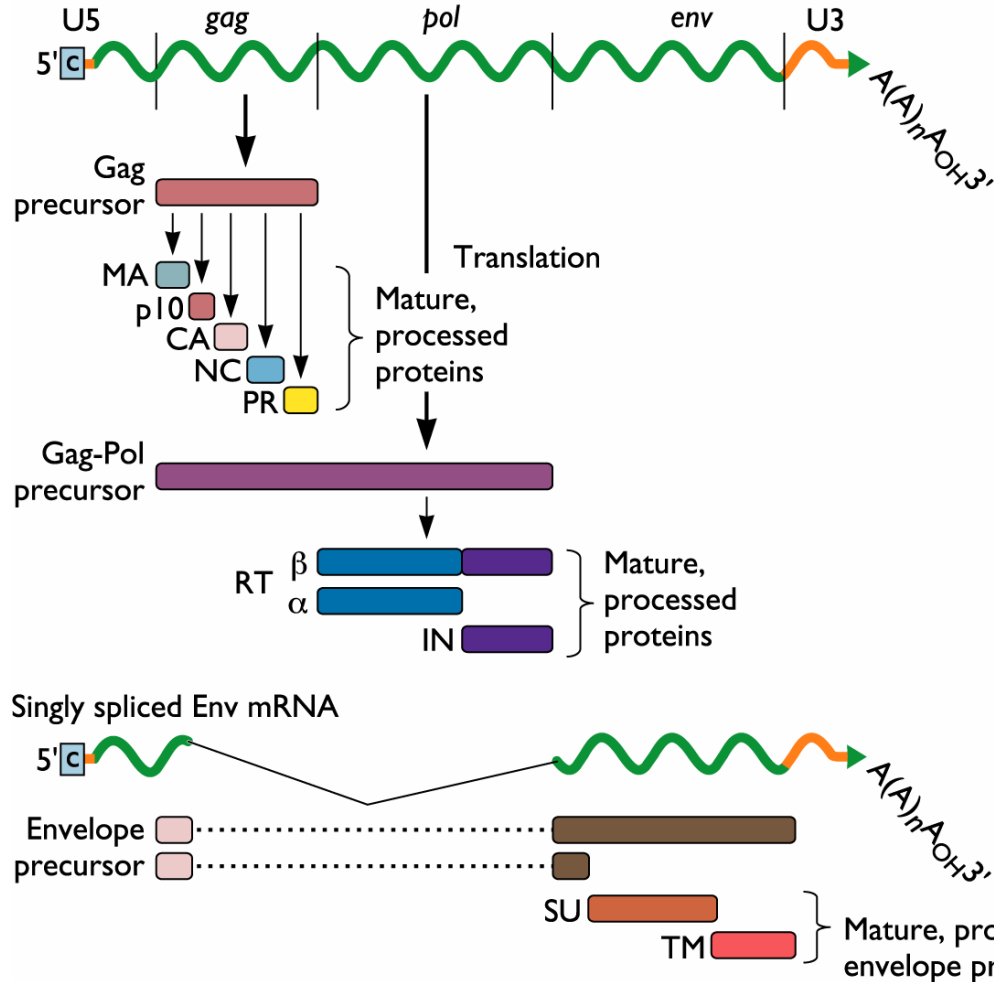


B Simple retrovirus (ALV)

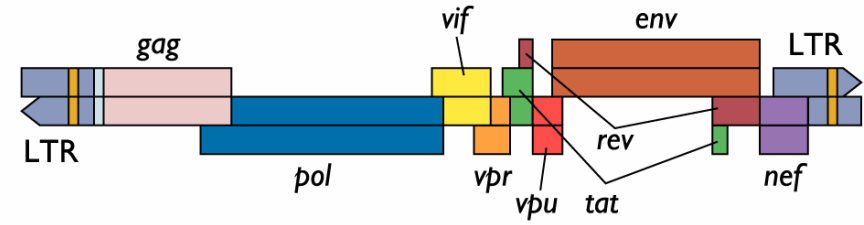


Genome expression

Genomic RNA, Gag-Pol mRNA, pre-mRNA

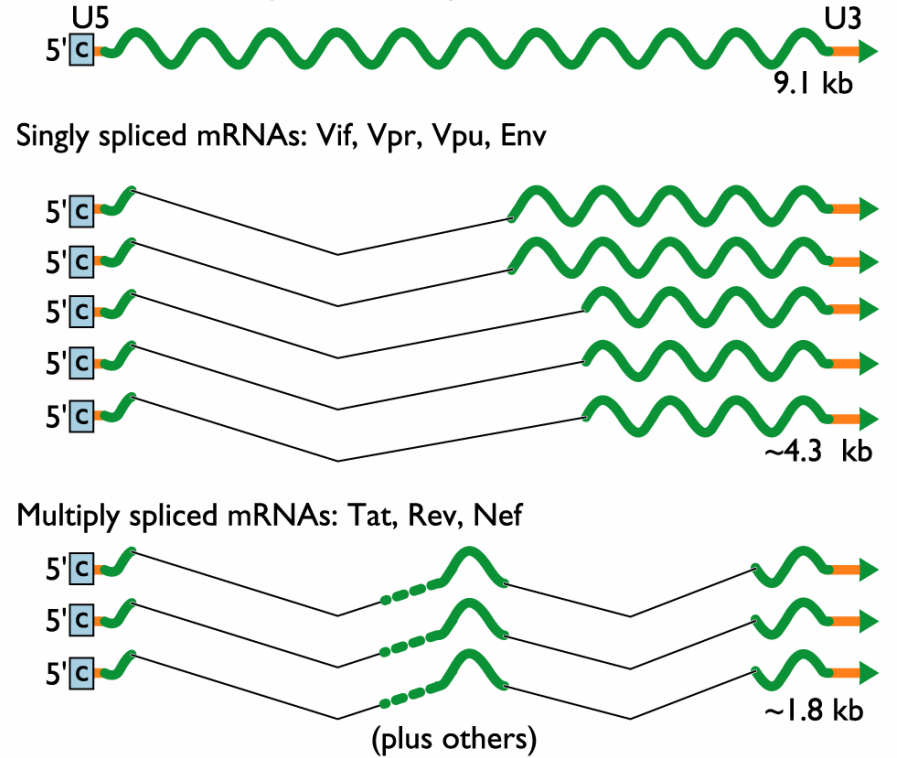


Complex retrovirus (HIV-1)



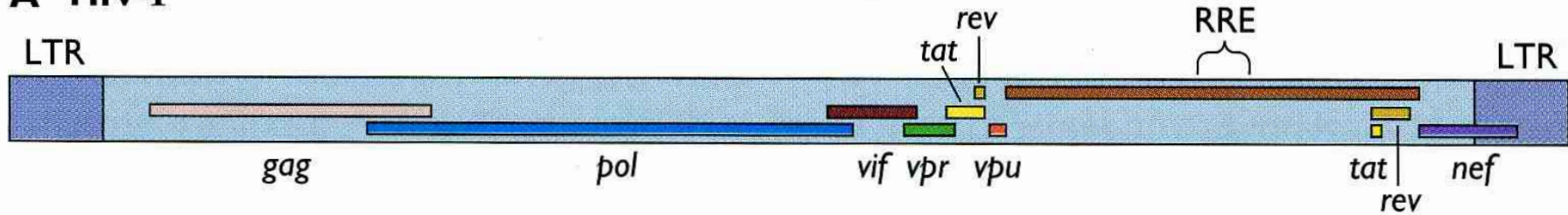
Genome expression

Genomic RNA, Gag-Pol mRNA, pre-mRNA

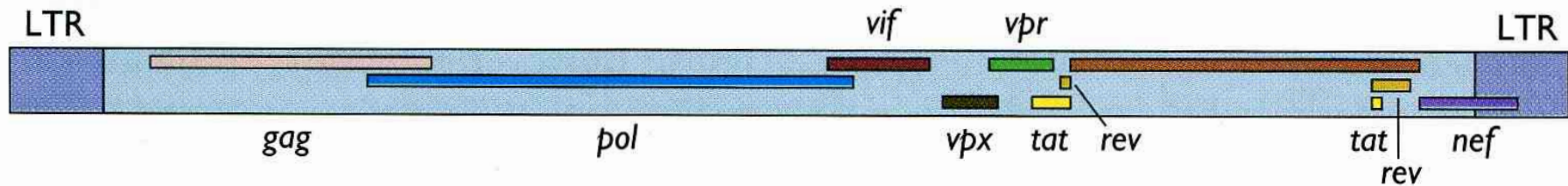


Genome organization of HIV-1 and HIV-2

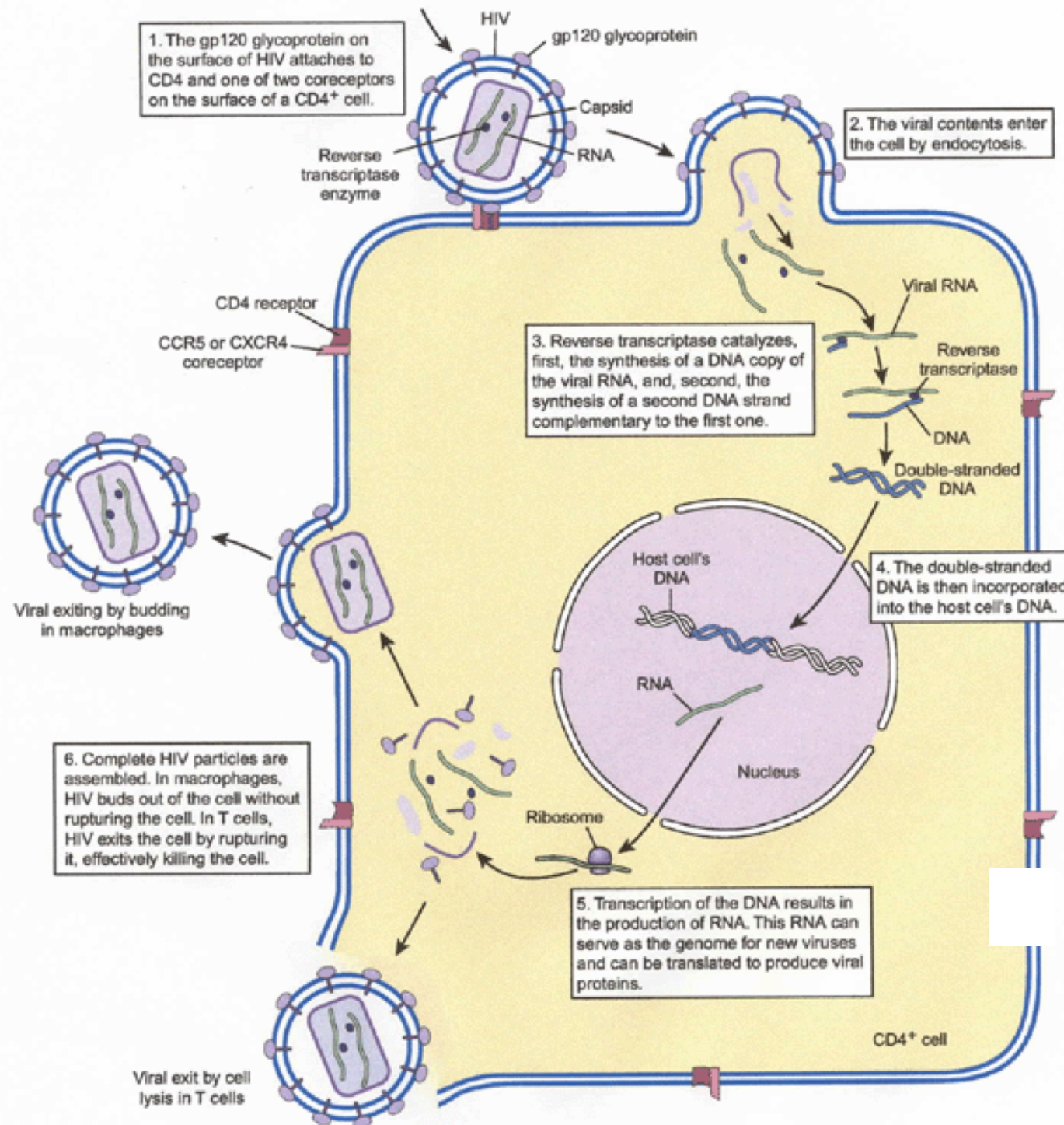
A HIV-1



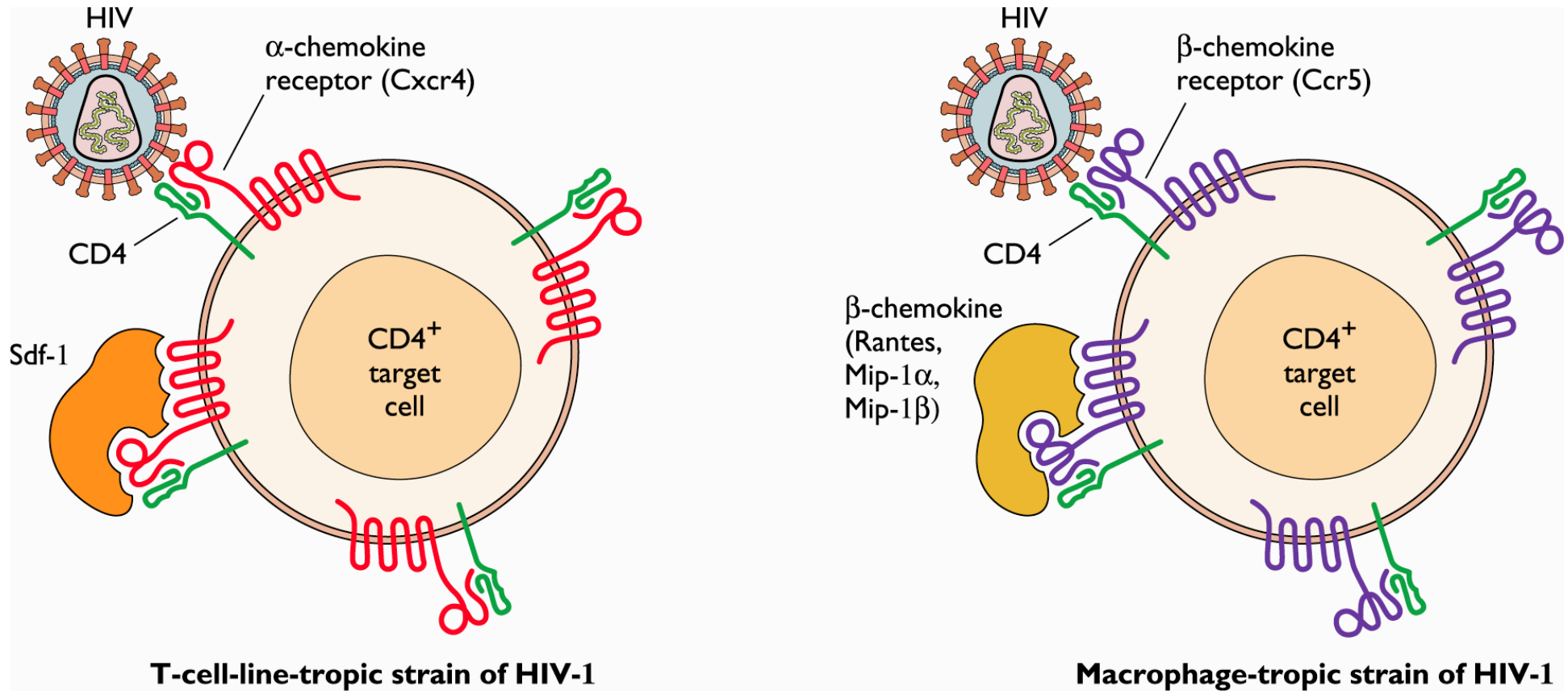
B HIV-2



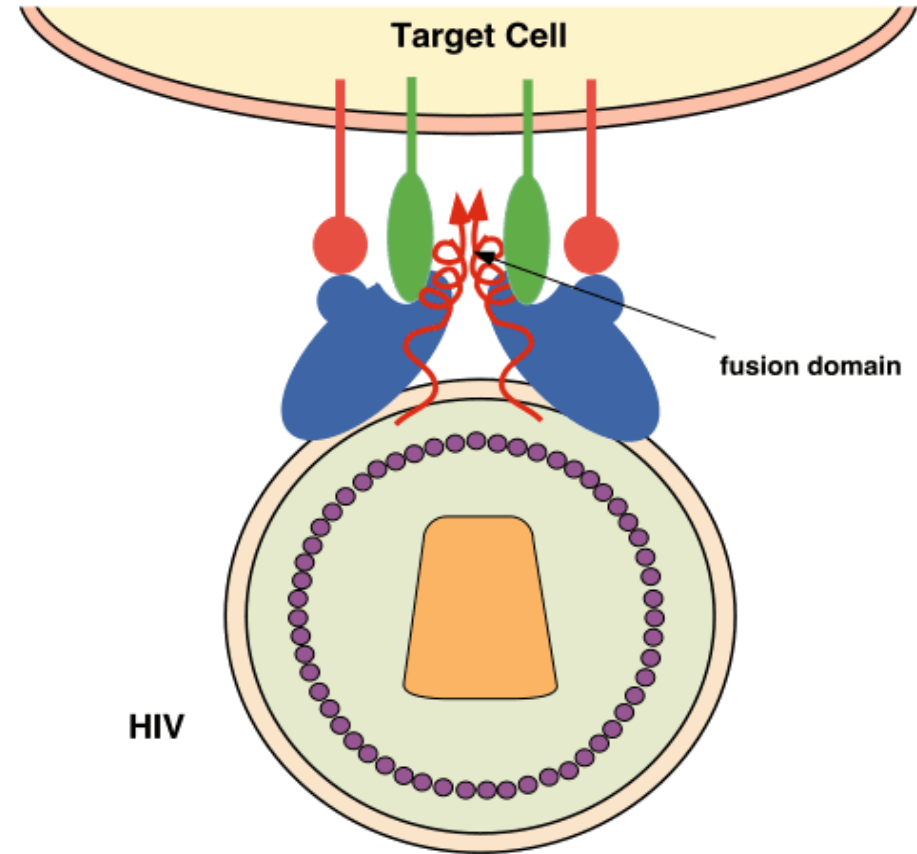
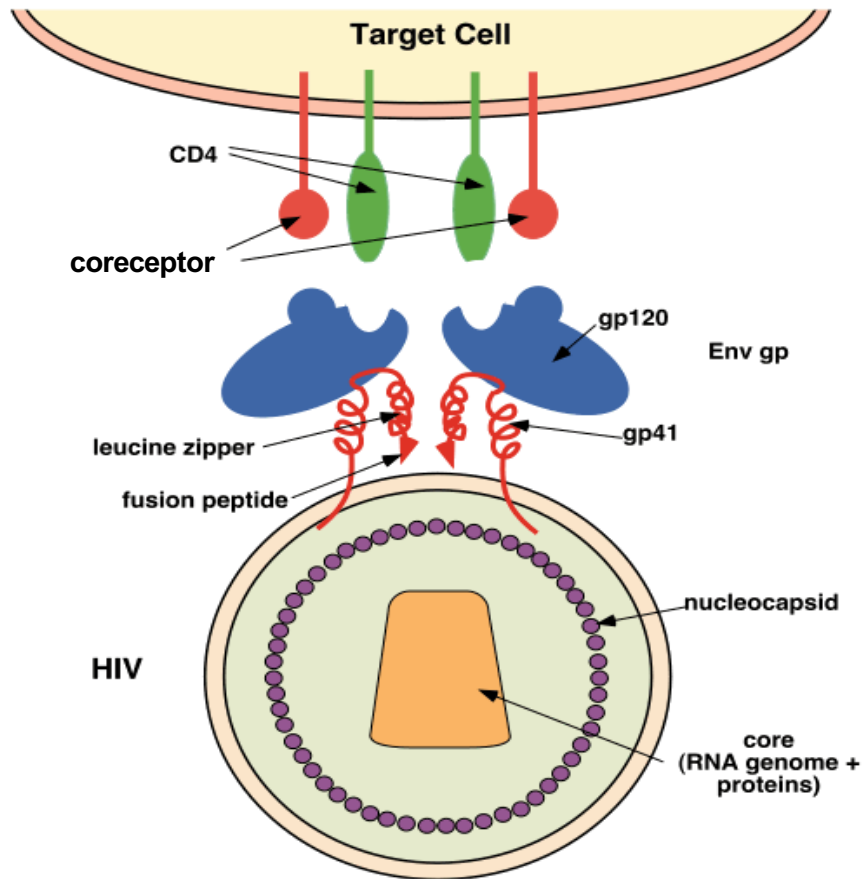
HIV life cycle



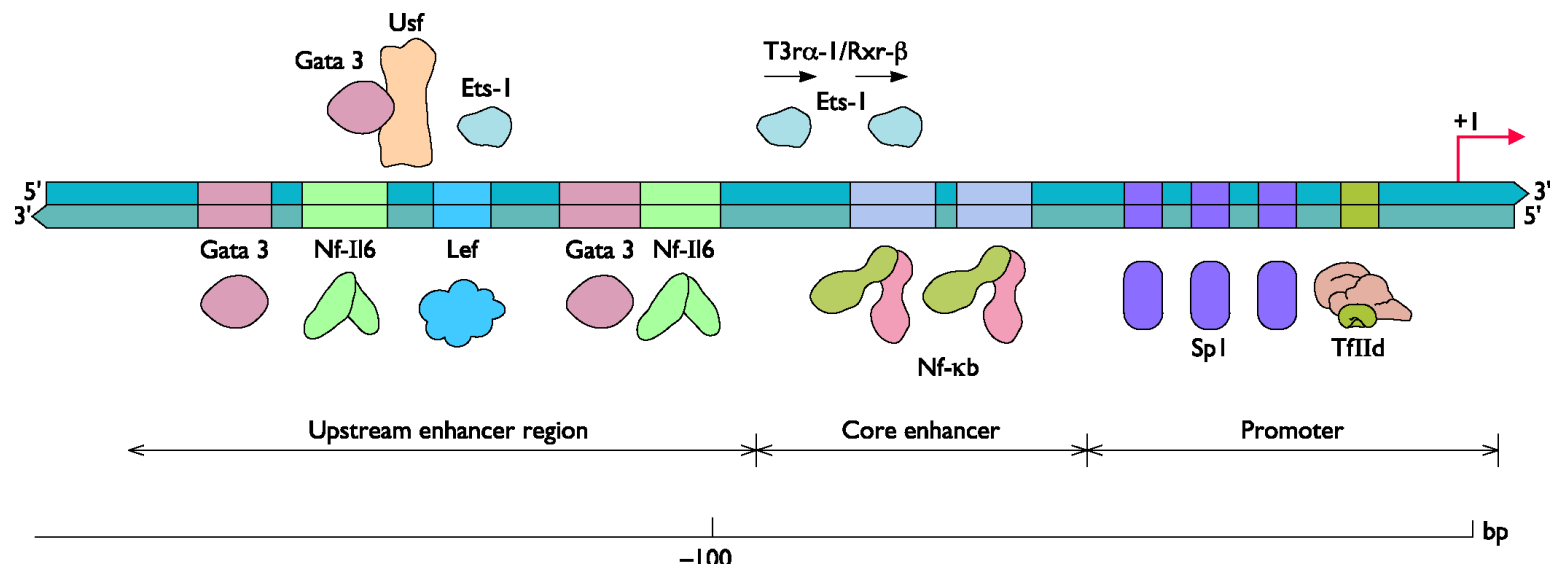
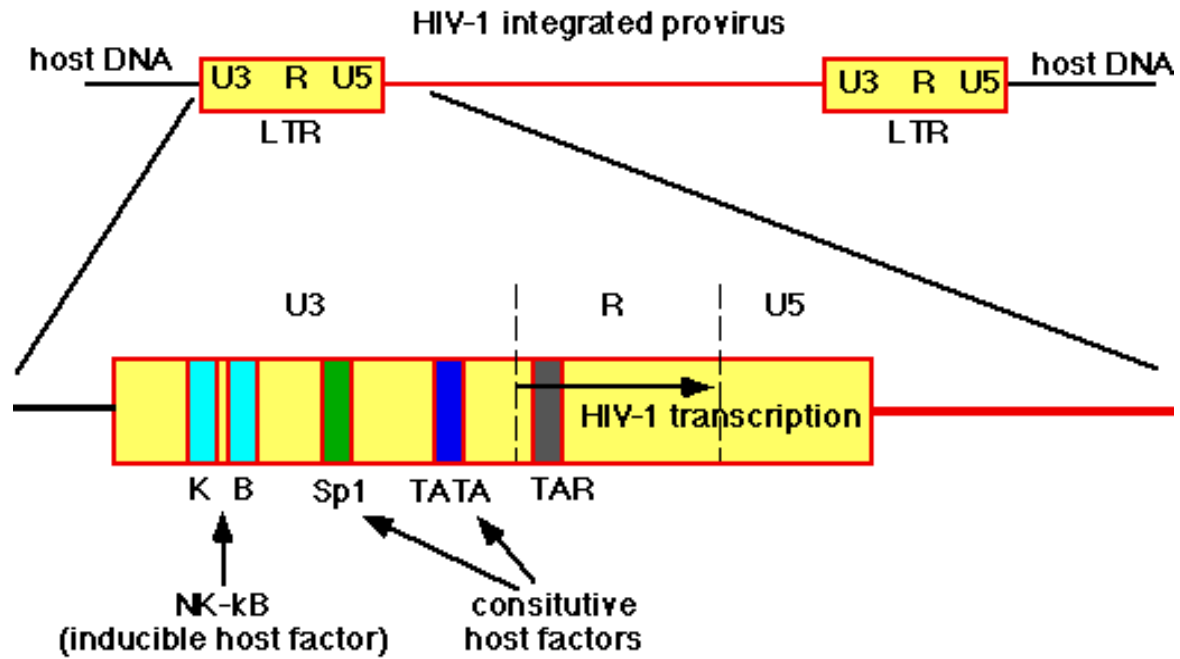
HIV receptor and coreceptors



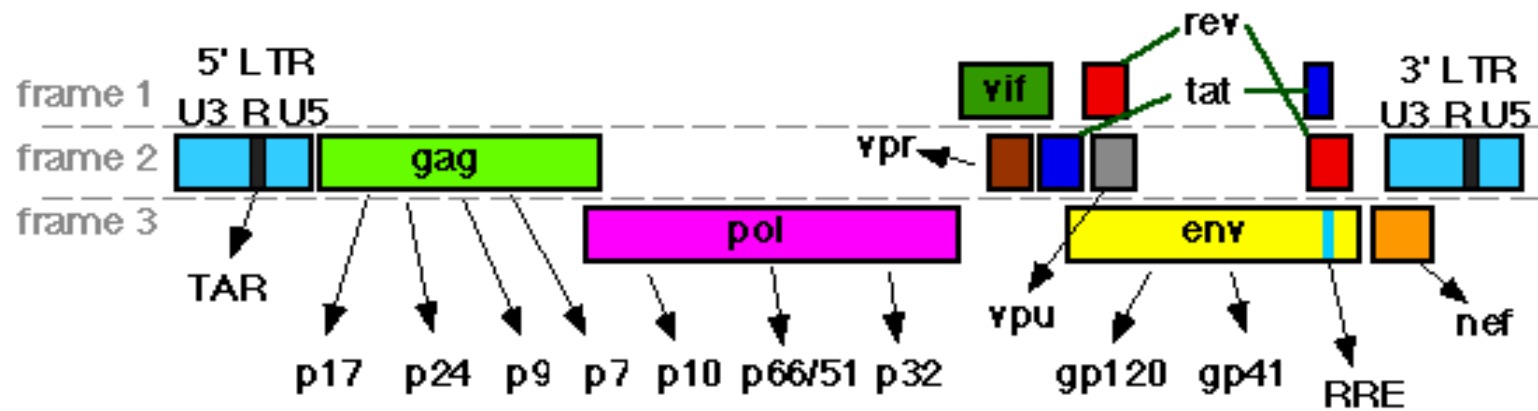
HIV attachment and entry into cells



HIV-1 LTR



HIV gene expression



gag:

p17 - myristylated capsid protein
 p24 - major capsid protein
 p9 - RNA-binding nucleocapsid protein
 p7 - RNA-binding nucleocapsid protein

pol:

p10 - protease
 p66/51 - reverse transcriptase
 (heterodimer)
 p32 - integrase

env:

gp120 - envelope glycoprotein (external)
 gp41 - envelope glycoprotein (transmembrane)

regulatory proteins:

tat: transactivating protein
 (transcription)
 rev: regulator of viral protein
 synthesis
 nef: "negative factor"

accessory proteins:

vif - virion infectivity factor
 vpr - viral protein R
 (transactivator?)
 vpu - viral protein U
 (virion release)

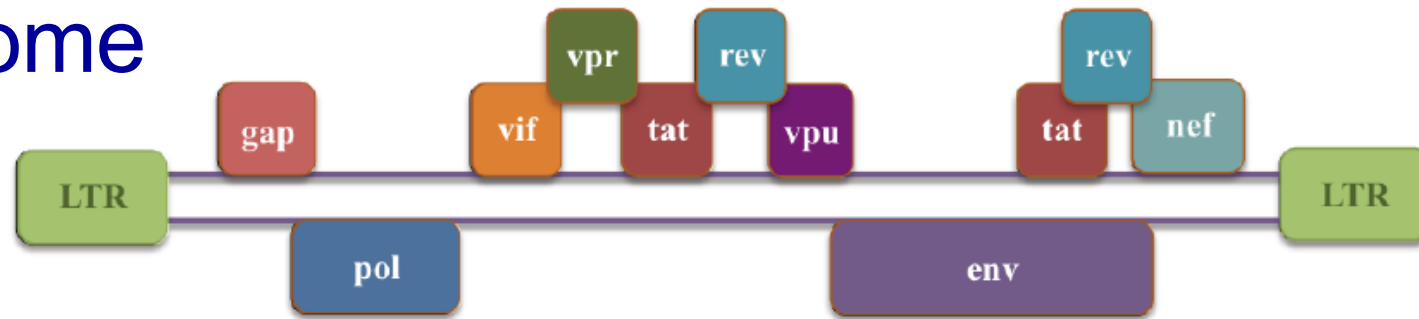
RNA sequence regions:

LTR - long terminal repeat
 U3 - unique 3' region
 R - terminal redundancy
 U5 - unique 5' region
 TAR - tat responsiveness
 RRE - rev response element

HIV auxiliary proteins

Protein ^b	Size (kDa)	Function	Location
Regulatory			
Tat	14	Transactivation; binds TAR to facilitate initiation and elongation of viral transcription	Primarily in cell nucleus
Rev	19	Regulation of viral mRNA expression; binds RRE and facilitates nuclear export of unspliced or singly spliced RNAs	Primarily in cell nucleus
Accessory			
Nef	27	Pleiotropic, can increase or decrease virus replication; down-regulates MHC-I and the CD4 receptor; influences T-cell activation; enhances virion infectivity	Cell cytoplasm, plasma membrane
Vif	23	Increases virus infectivity; helps in virion assembly and in viral DNA synthesis	Cell cytoplasm
Vpr	15	Helps in virus replication; causes G ₂ arrest; facilitates nuclear entry of preintegration complex	Virion
Vpu ^c	16	Helps in virus release; disrupts Env-CD4 complexes; causes CD4 degradation	Integral cell membrane protein
Vpx ^d	15	Nuclear entry of preintegration complexes	Virion

HIV genome

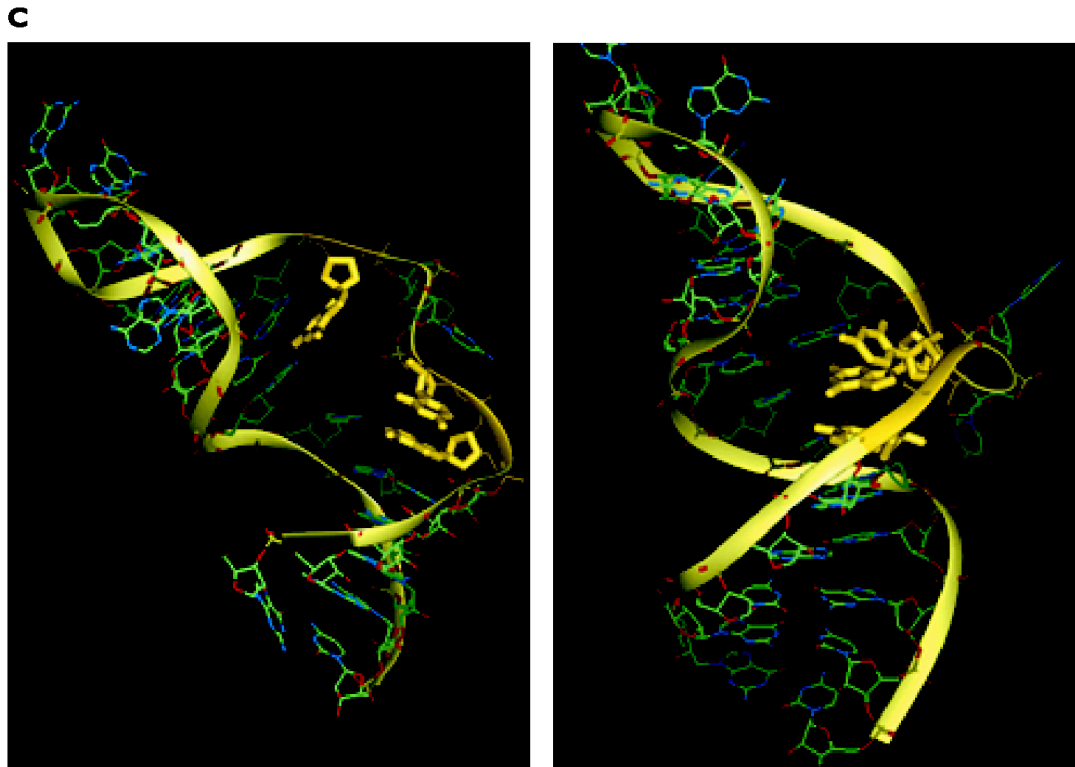
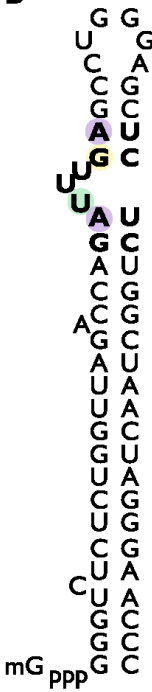
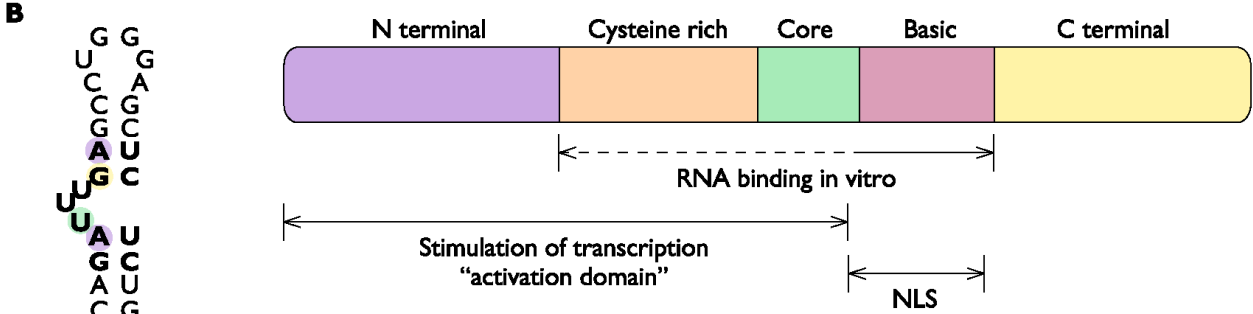
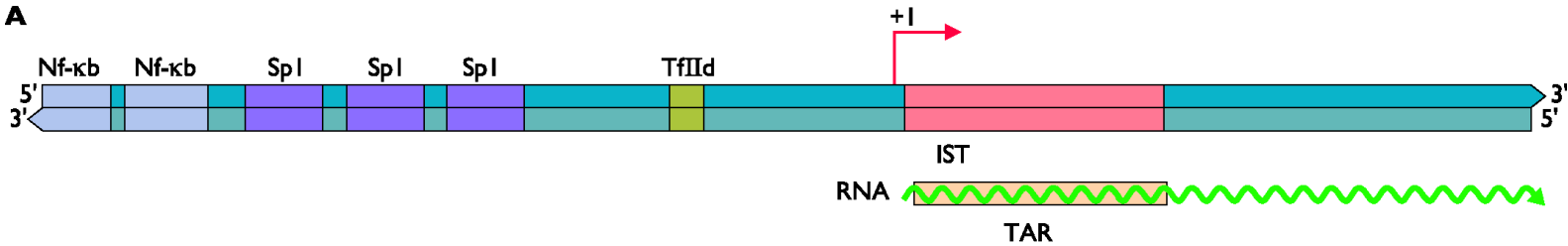


	GENE	PRECURSOR PROTEINS → PRODUCTS
Essential Genes & Regulatory Elements	gag	Group-specific antigen gag → MA, CA, SP1, NC, SP2, P6
	pol	Polymerase pol → RT, RNase H, IN, PR
	env	Envelope gp160 → gp120, gp41
	tat	HIV Transactivator Positive regulator of transcription
	rev	Regulator of expression of virion proteins Important for synthesis of major viral proteins and essential for viral replication
Accessory Genes	vif	Viral infectivity Required for infectivity in some cell types
	vpr	Virus protein R Nuclear import of pre-integration complex and host cell cycle arrest
	vpu	Virus protein U Proteasomal degradation of CD44 and release of virions from infected cells
	nef	Negative factor Role in apoptosis and key in increasing virus infectivity

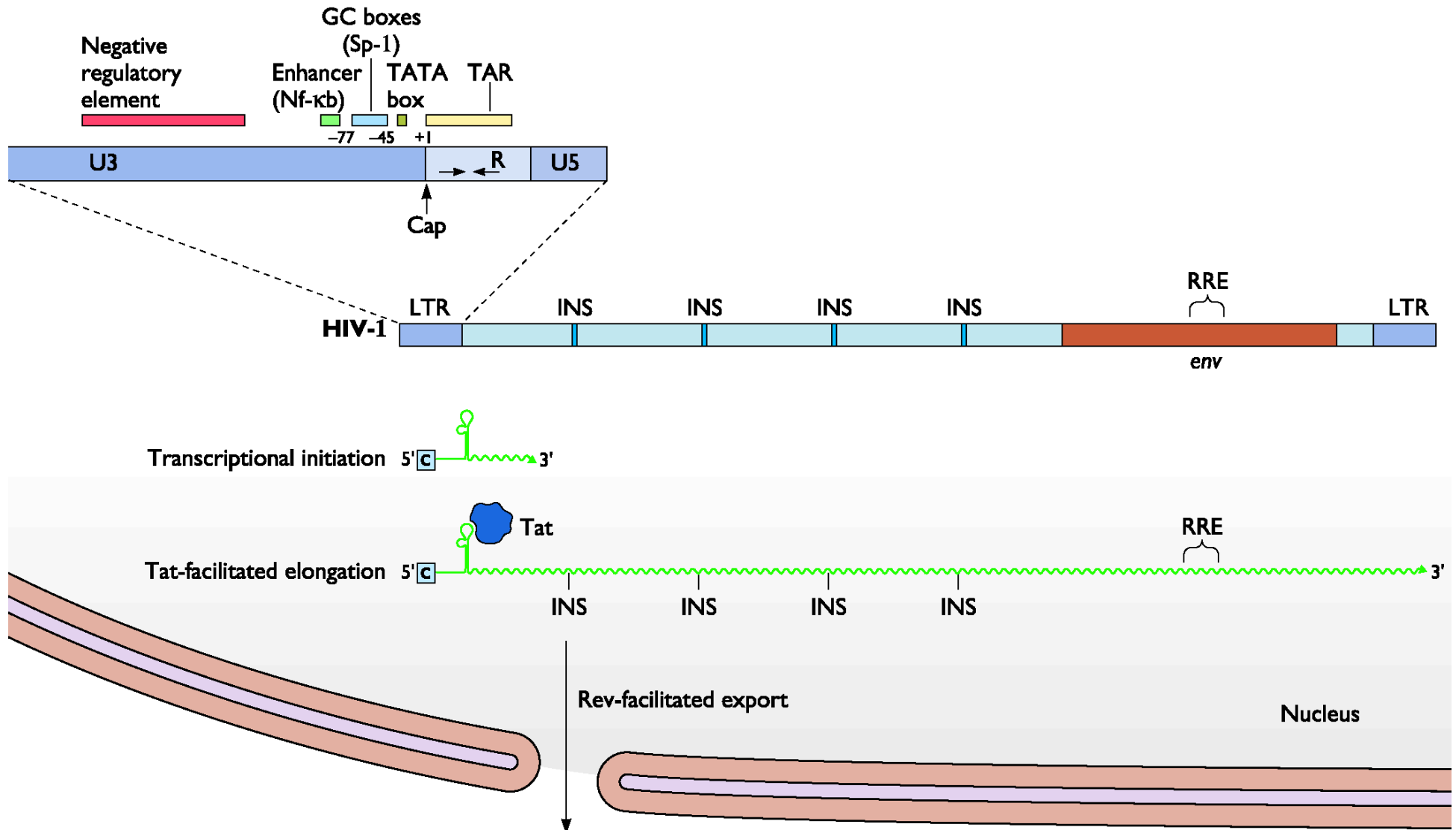
HIV Tat: features and functions

- 14 kDa protein encoded by a multisplliced mRNA
- Binds the TAR element and stabilizes mRNA conformation
- Binds cyclin T1+CDK9 and stimulates the kinase activity of TFIIH
- Stimulates phosphorylation of RNA pol II CTD and increase its processivity
- Allows the transcription of long mRNA (e.g., gag-pol full length mRNA)
- Stimulates production of viral RNA as much as 100 fold

HIV TRANSCRIPTION - Tat and TAR

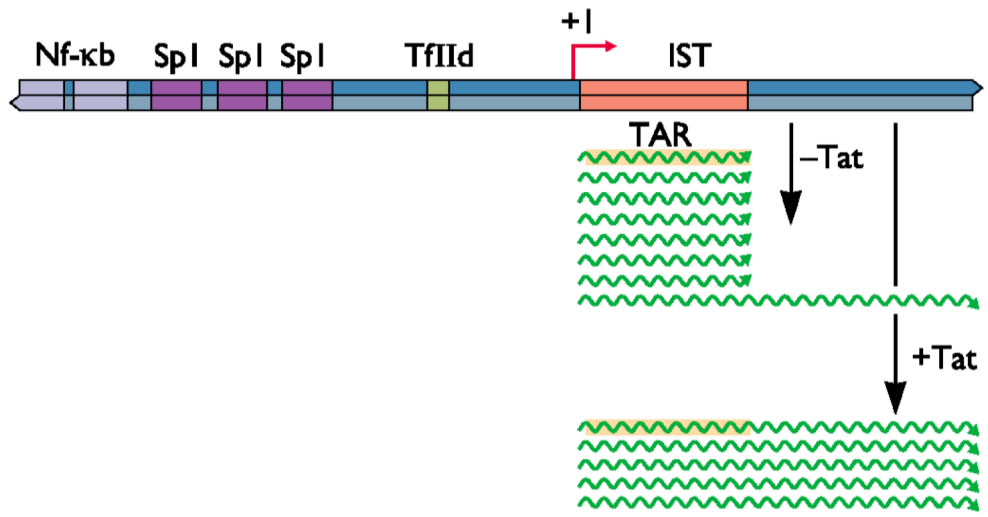


HIV TRANSCRIPTION - Tat and TAR

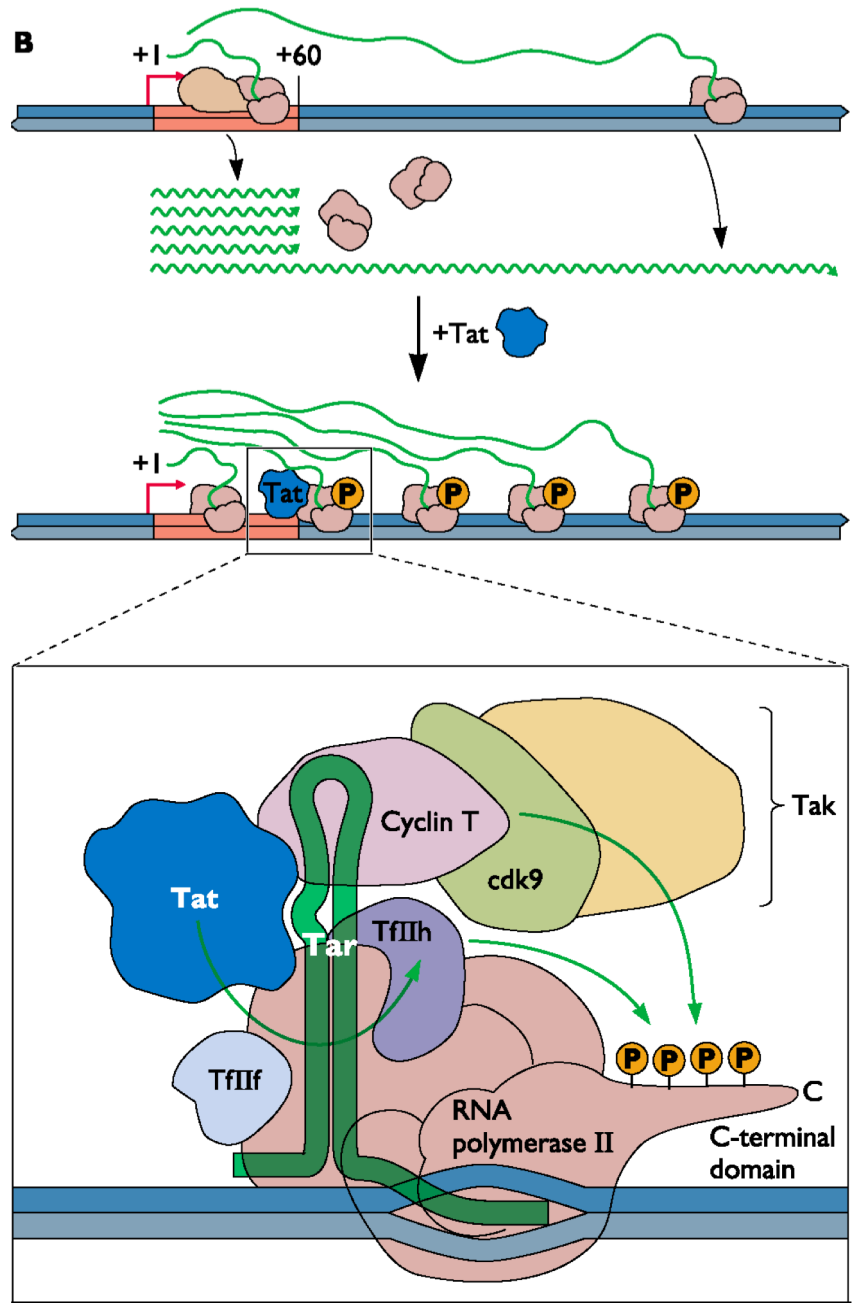


HIV TRANSCRIPTION

Tat and TAR



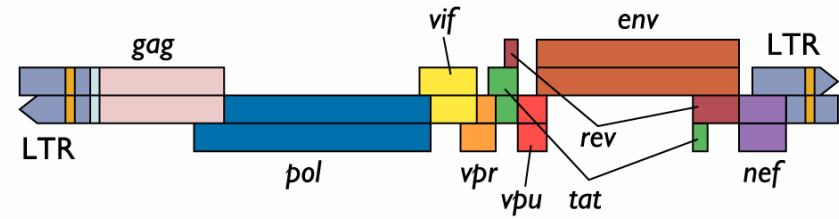
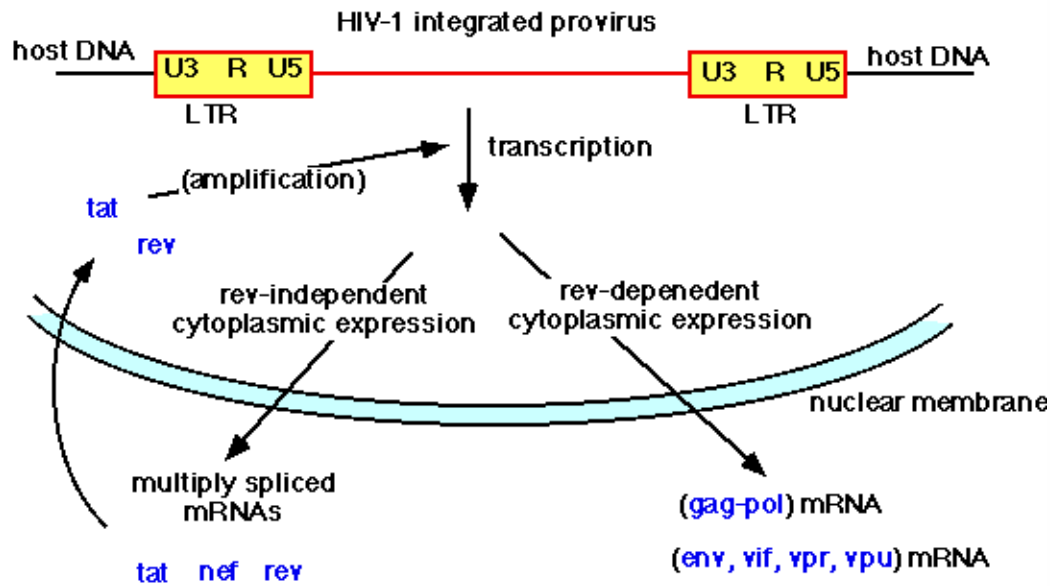
HIV-1 transcription in the presence or absence of Tat



Stimulation of transcription of HIV-1 proviral DNA by Tat

HIV TRANSCRIPTION

mRNA splicing

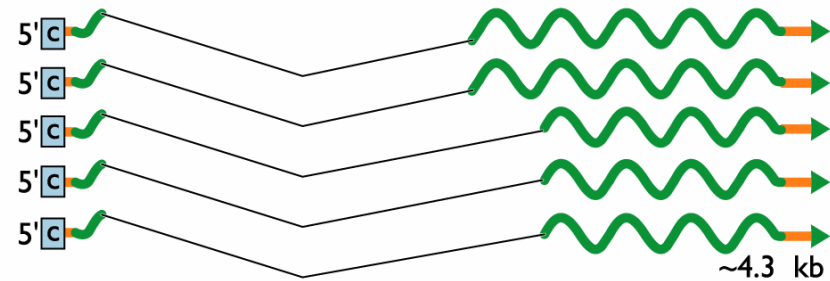


Genome expression

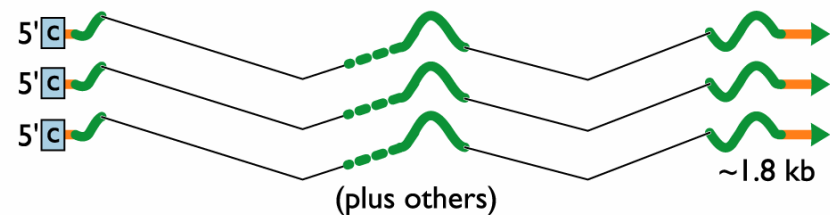
Genomic RNA, Gag-Pol mRNA, pre-mRNA



Singly spliced mRNAs: Vif, Vpr, Vpu, Env



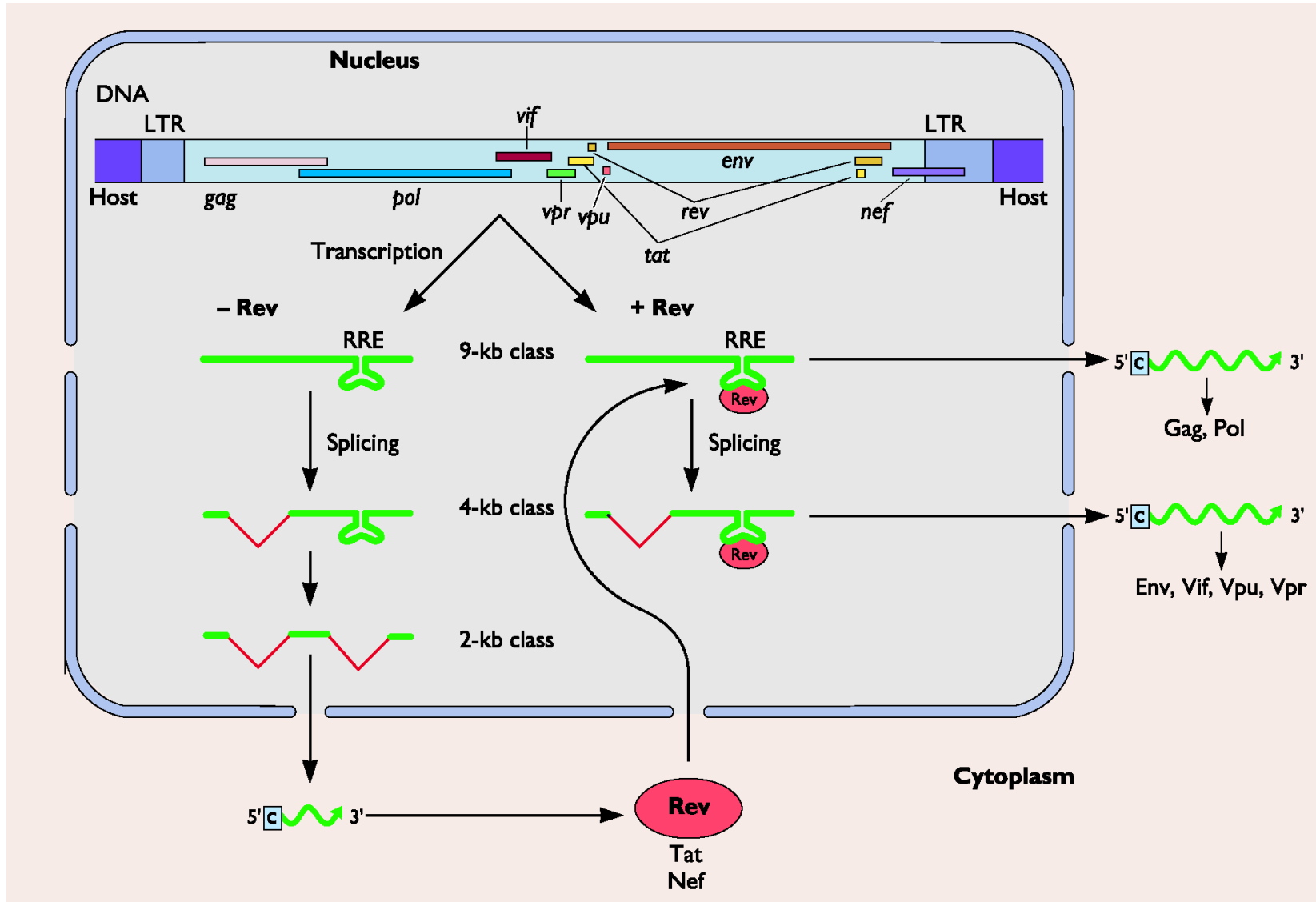
Multiply spliced mRNAs: Tat, Rev, Nef



HIV Rev: features and functions

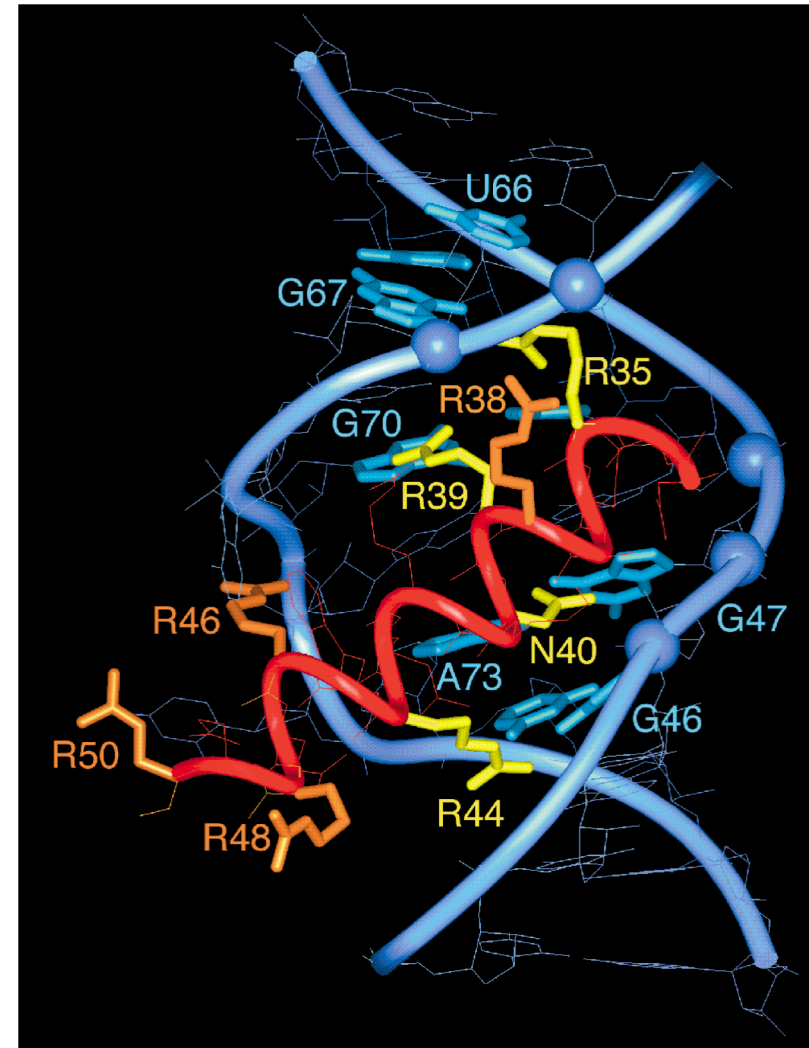
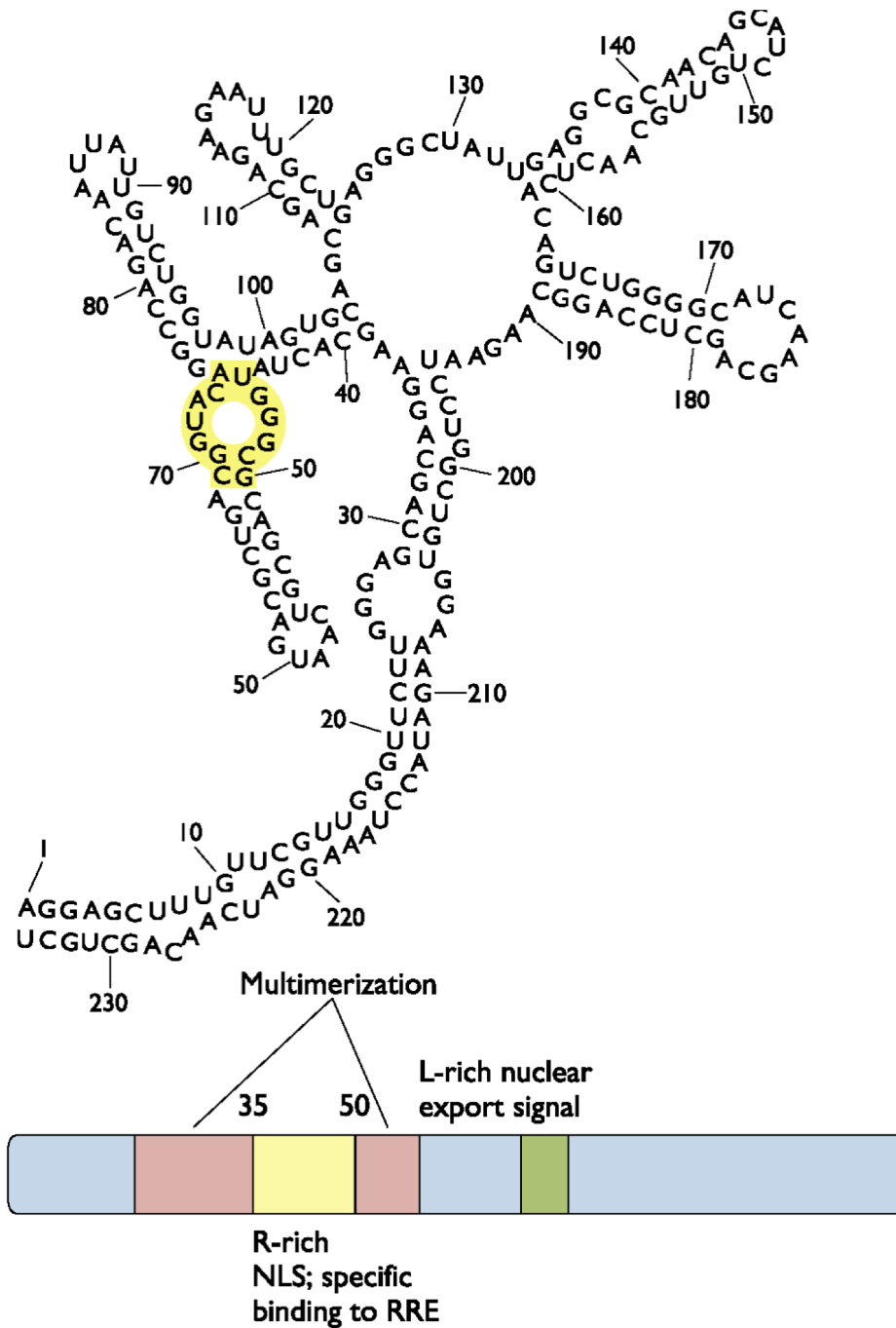
- 19 kDa protein encoded by a multispliced mRNA
- Binds the RRE element of HIV mRNAs
- Allows export to cytoplasm of RRE-containing mRNAs from which virion structural proteins and enzymes are made. It interacts with exportin-1 and Ran-GTP
- Allows expression of proteins encoded by unspliced mRNAs (gag-pol) or single-spliced (Vif, Vpr, Vpu, Env)
- Determines a shift in HIV gene expression (regulatory protein -----> structural proteins)
- Absent in simple Retroviruses in which full-length mRNAs contain a constitutive export sequences

Regulation of export of HIV-1 mRNAs by the viral Rev protein

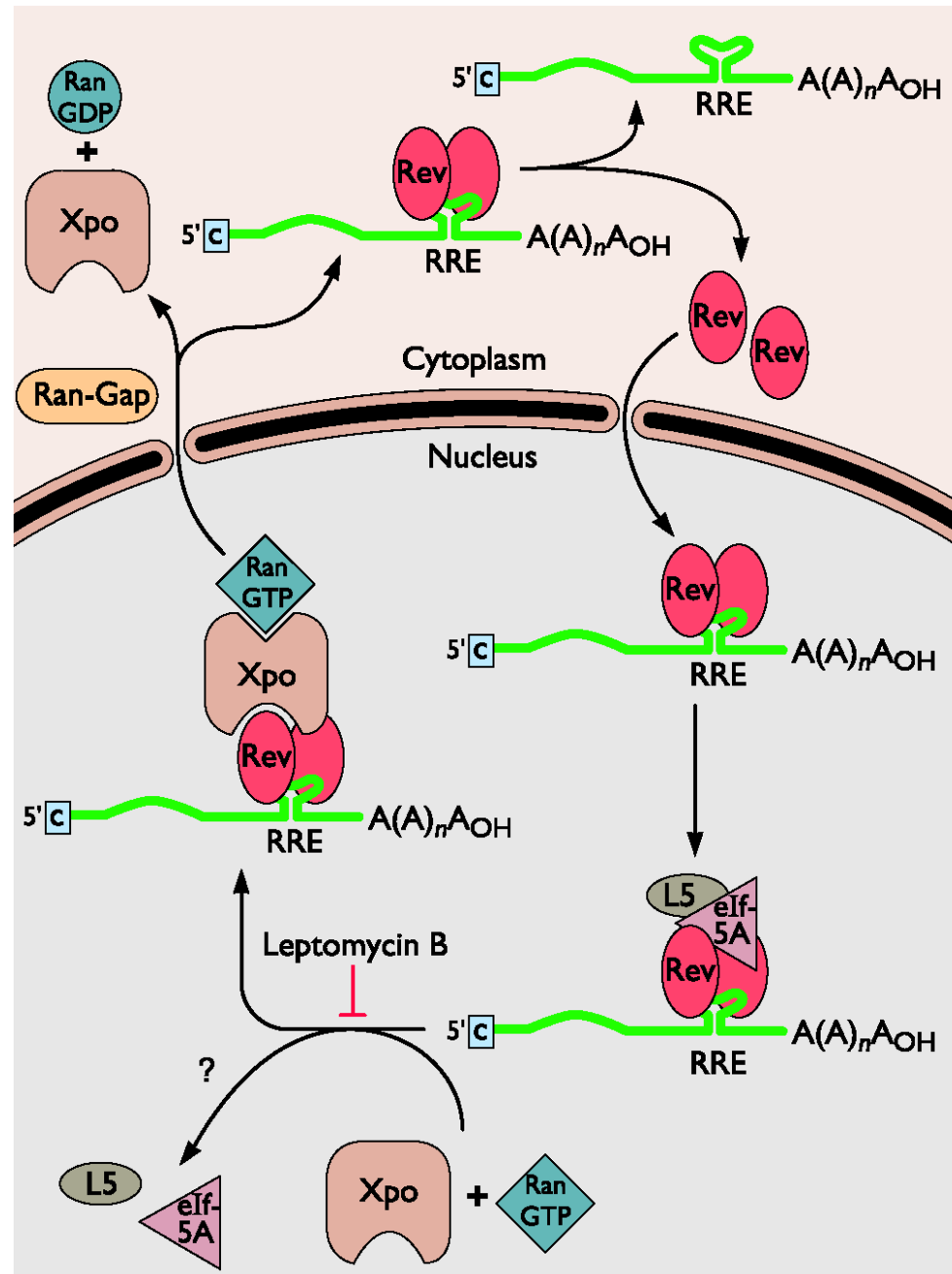


Prior to Rev synthesis only fully spliced mRNA (2-kb class) are exported in the cytoplasm. When Rev is made it enters the nucleus and binds the RRE in unspliced (9-kb class) and singly-spliced (4-kb class) viral mRNAs. This interaction induces export to cytoplasm of RRE-containing mRNAs, from which virion structural proteins and enzymes are made.

Binding of Rev protein to the rev-responsive element (RRE)

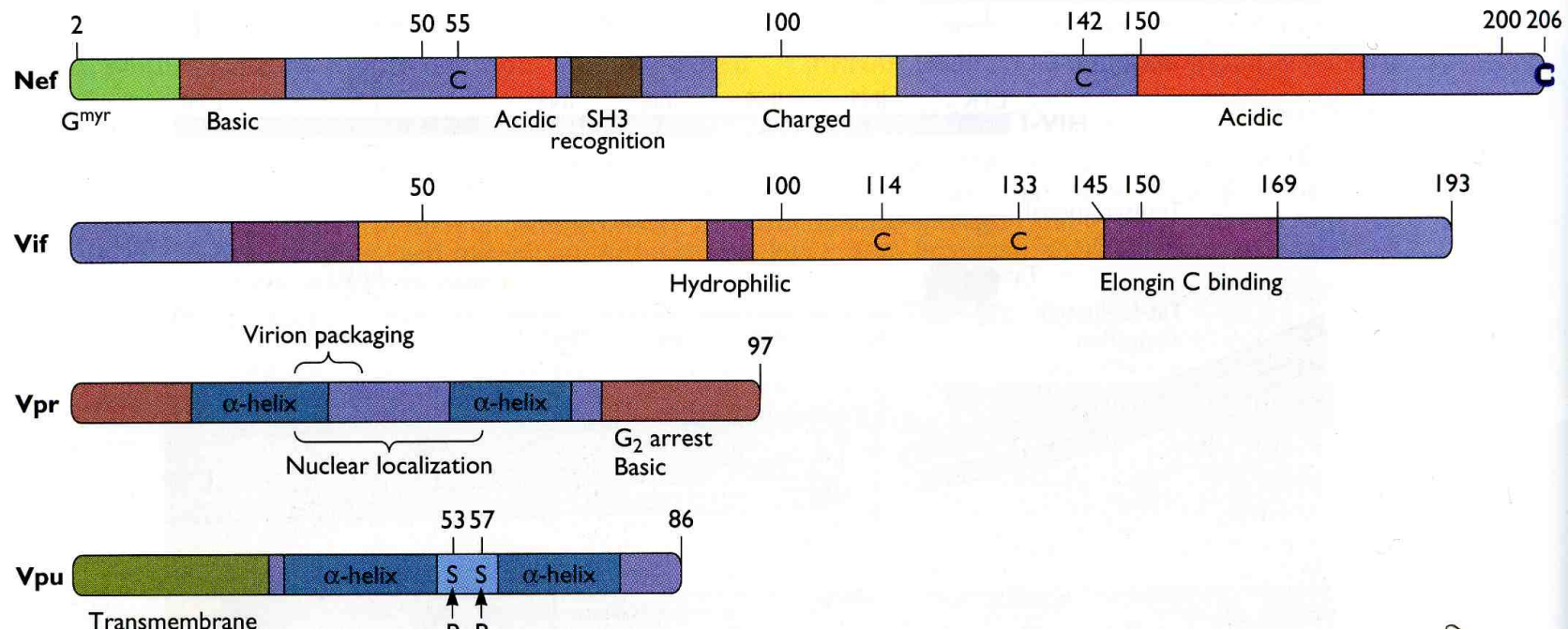


Model of export HIV-1 mRNAs containing introns by the viral Rev protein

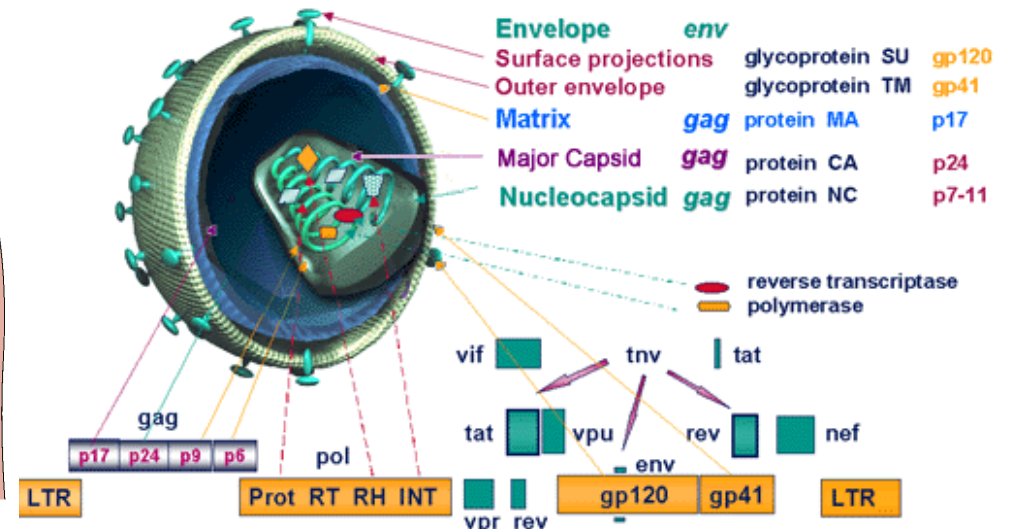
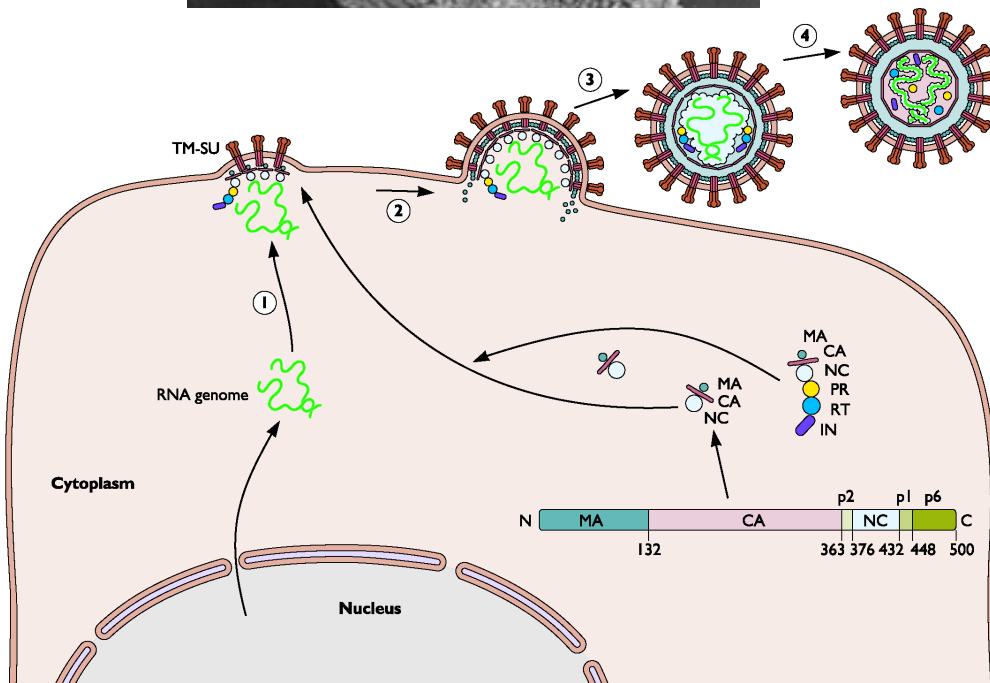
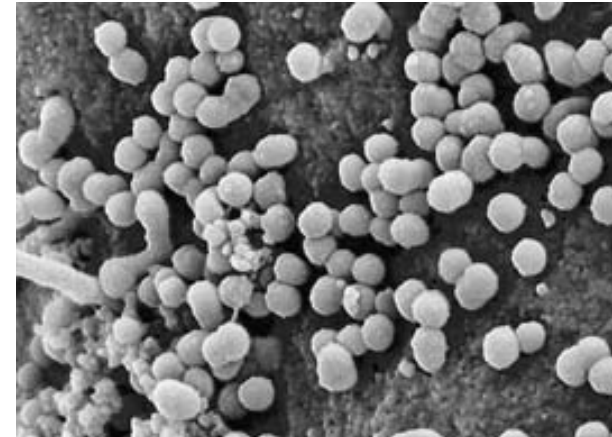
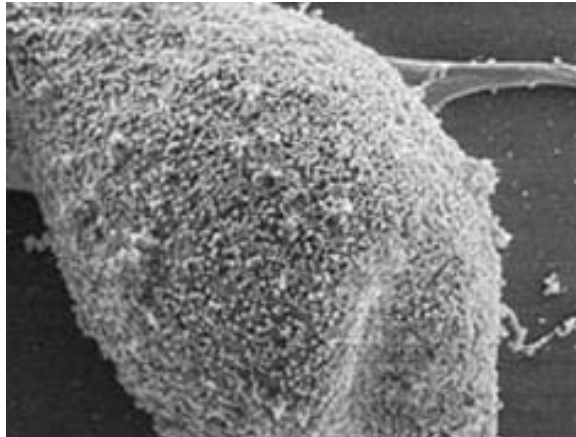


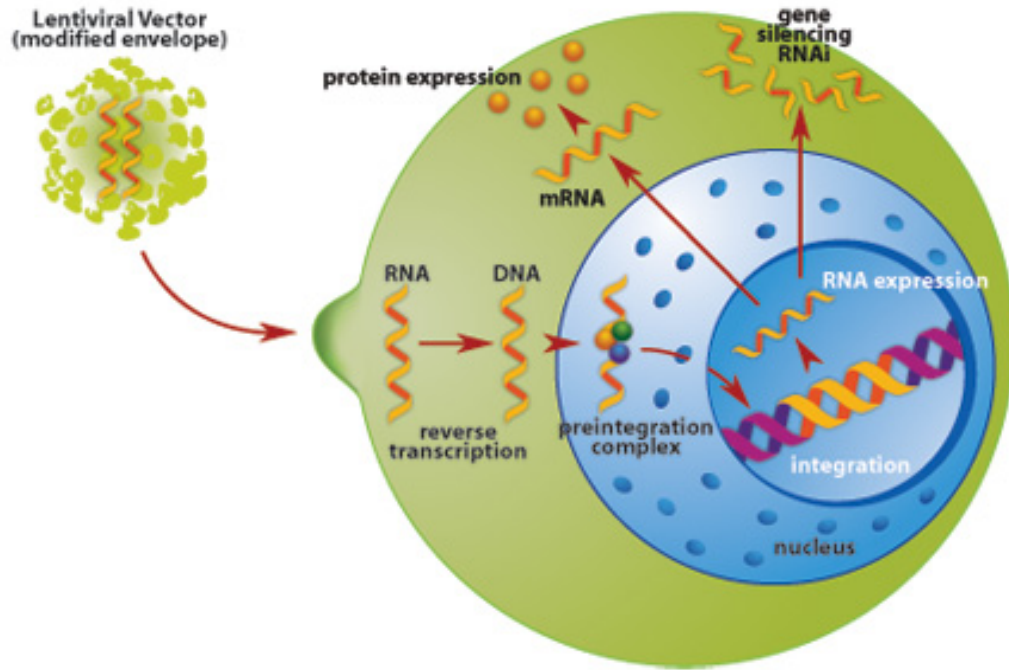
HIV auxiliary proteins

Protein ^b	Size (kDa)	Function	Location
Regulatory			
Tat	14	Stimulation of transcription; binds TAR to facilitate initiation and elongation of viral transcription	Primarily in cell nucleus
Rev	19	Regulation of viral mRNA production; binds RRE and facilitates nuclear export of unspliced or singly spliced RNAs	Primarily in cell nucleus
Accessory			
Nef	27	Pleiotropic, can increase or decrease virus replication; reduces expression of MHC class I and the CD4 receptor; affects T-cell activation; enhances virion infectivity	Cell cytoplasm, plasma membrane
Vif	23	Increases virus infectivity; affects virion assembly and/or viral DNA synthesis	Cell cytoplasm
Vpr	15	Causes G ₂ arrest; facilitates nuclear entry of preintegration complex	Virion
Vpu ^c	16	Affects virus release; disrupts Env-CD4 complexes; CD4 degradation	Integral cell membrane protein
Vpx ^d	15	Nuclear entry of preintegration complexes	Virion

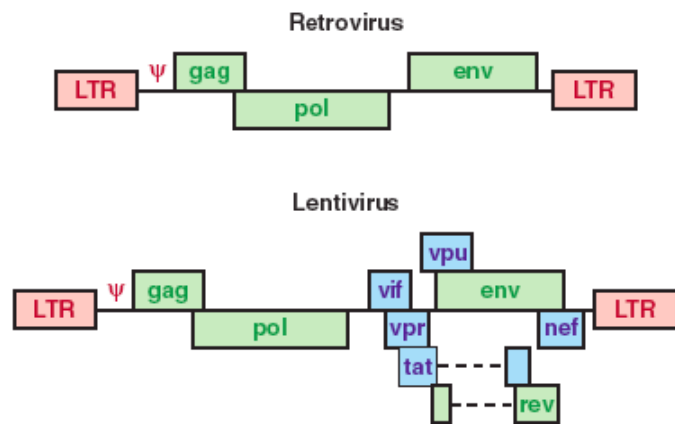


HIV egress by budding at the plasma membrane





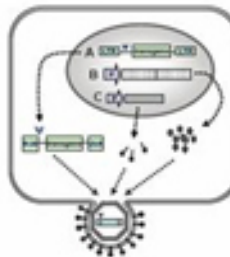
Lentiviral vectors



Lentivector Design



Virus Particle Production



Ex vivo Gene transfer
Cell lines and Primary cells



In vivo Gene transfer



Favorable Features of Lentivirus Vectors

- HIV-1 integrates its DNA and completes a replication cycle in fully differentiated, non dividing cells (macrophages).
- So, this virus must have a mechanism for the active transport of preintegration complexes into the nucleus.
- Vpr and a minor, phosphorylated, form of the matrix (MA/p17) protein direct nuclear import of the HIV-1 preintegration complex.
- Nuclear localization signals have been found in the IN protein of HIV-1

Retrovirus and Lentivirus vectors

Viral System	Transient expression		Stable expression			
	Dividing Cells	Non Dividing Cells	Dividing Cells	Neuronal Cells	Drug or Growth Arrested Cells	Contact Inhibited Cells
Adenovirus	•	•				
Retrovirus	•		•			
Lentivirus	•	•	•	•	•	•

Lentiviral Vector Construction

Several components are essential to generate a lentiviral vector, including:

1. A lentiviral construct: with LTRs and the Packaging Signal Psi (Ψ);
2. The transgene of interest: e.g., a cDNA, miRNA, or shRNA cloned into the lentiviral construct;
3. Helper plasmids: packaging and envelope plasmids;
4. A packaging cell line: the “factory” in which the viral vector production takes place. The lentiviral construct with the transgene and helper plasmids are transiently transfected into a packaging cell line such as HEK-293T cells, where they get assembled.

Since Lentiviruses have high mutation and recombination rates, so the likelihood that HIV could self-replicate and be produced during vector manufacturing by recombination is a serious safety concern. To reduce that probability, in a safety-modified lentiviral vector:

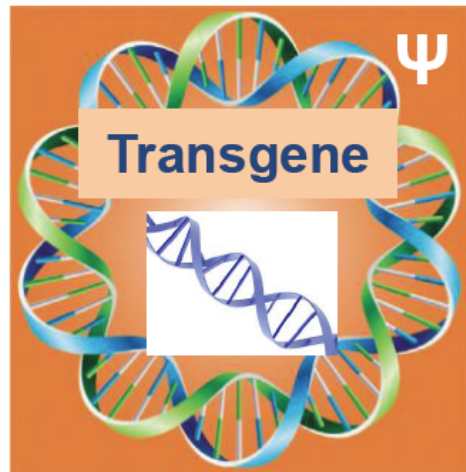
Essential genes must be separated into different plasmids, and the four viral accessory genes (*vif*, *vpr*, *vpu* and *nef*) deleted.

Thus, multiple recombination events would be necessary to reconstitute a **Replication-Competent Lentivirus (RCL)**.

Lentiviral Vector Generations Summary Table

	First Generation	Second Generation	Third Generation
Plasmids	3	3	4
Deletion in 3' LTR - SIN	No	No	Yes
Packaging plasmids with HIV genes	1	1	2
Accessory genes: vif, vpr, vpu, nef	All present	All absent	All absent
tat and rev genes	On a single packaging plasmid	On a single packaging plasmid	tat is absent; rev on a separate plasmid
gag and pol genes	Same plasmid	Same plasmid	Same plasmid
Recombination events needed to generate Replication Competent Lentiviruses (RCL)*	2 recombinations	3 recombinations	4 recombinations between plasmids without homology & must pick a promoter to complement SIN deletion

First-generation: includes a packaging system with all HIV genes except for the env gene (usually heterologous) that is included in another vector.



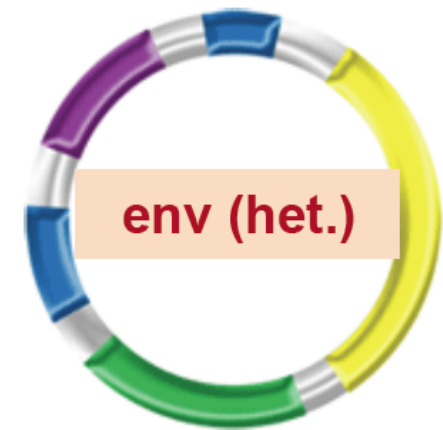
Transfer vector

+



Packaging plasmid

+



Envelope plasmid

* The risk of formation of RCLs exists not only during lentiviral vector production, but also during experiments involving materials infected with wild-type HIV. Recombination between the lentiviral vector and HIV can lead to the generation of new viruses with unknown safety consequences. For that reason, experiments involving human materials not screened for HIV pose an enhanced risk for laboratory workers.

Second-generation: Researchers discovered that the four HIV accessory genes - vif, vpr, vpu and nef - were not required for HIV replication in immortalized cell lines. This led to the engineering of second-generation vectors. In this system, the four accessory genes were eliminated leaving the gag and pol reading frames and the tat and rev genes. In general, lentiviral vectors with a wild-type 5' LTR need the 2nd generation packaging system because they need tat for activation



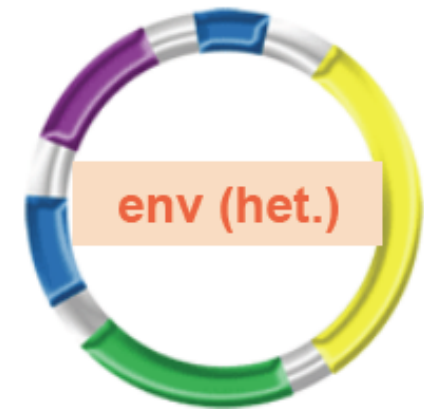
Transfer vector

+



Packaging plasmid

+



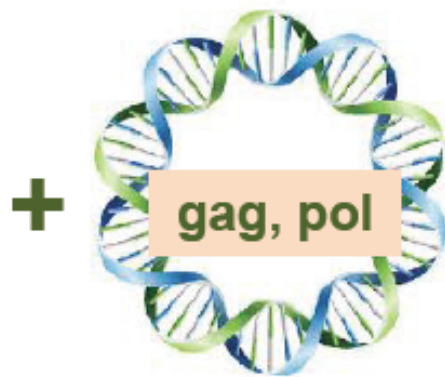
Envelope plasmid

* The risk of formation of RCLs exists not only during lentiviral vector production, but also during experiments involving materials infected with wild-type HIV. Recombination between the lentiviral vector and HIV can lead to the generation of new viruses with unknown safety consequences. For that reason, experiments involving human materials not screened for HIV pose an enhanced risk for laboratory workers.

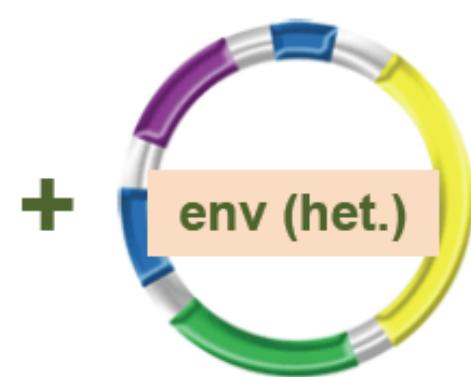
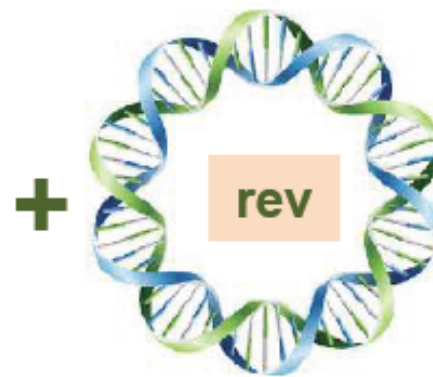
Third-generation / Self-Inactivating (SIN): In a third-generation vector, the 3' LTR is modified, with *tat* being eliminated and *rev* provided in a separate plasmid. Since the HIV promoter in the 5' LTR depends on *tat*, a vector without *tat* needs to have its wild-type promoter replaced with a heterologous enhancer/promoter to ensure transcription. Such promoter could be either viral (like CMV) or cellular (like EF1- α).



Transfer vector



Packaging plasmids

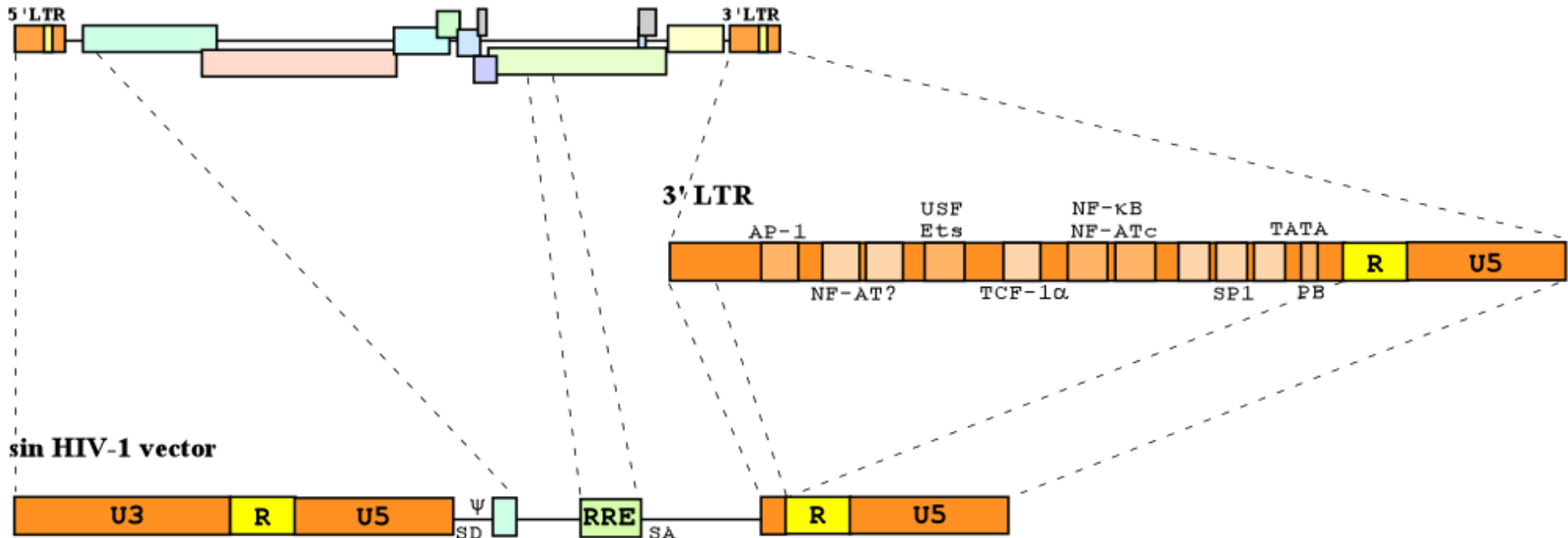


Envelope plasmid

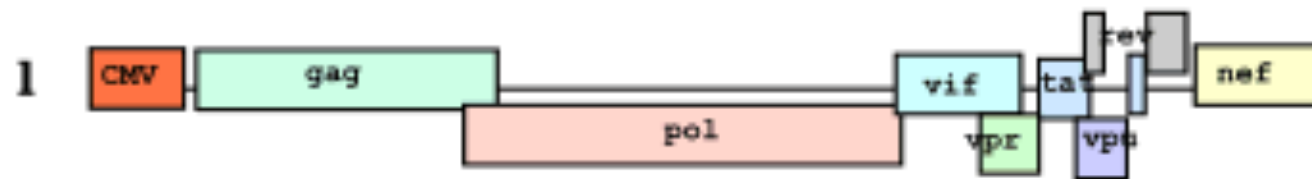
* The risk of formation of RCLs exists not only during lentiviral vector production, but also during experiments involving materials infected with wild-type HIV. Recombination between the lentiviral vector and HIV can lead to the generation of new viruses with unknown safety consequences. For that reason, experiments involving human materials not screened for HIV pose an enhanced risk for laboratory workers.

Structure of a SIN HIV-1 based vector

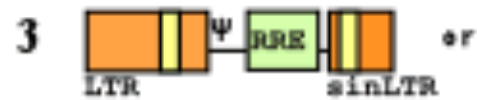
HIV-1 genome



Development of self-inactivating vectors or SIN vectors

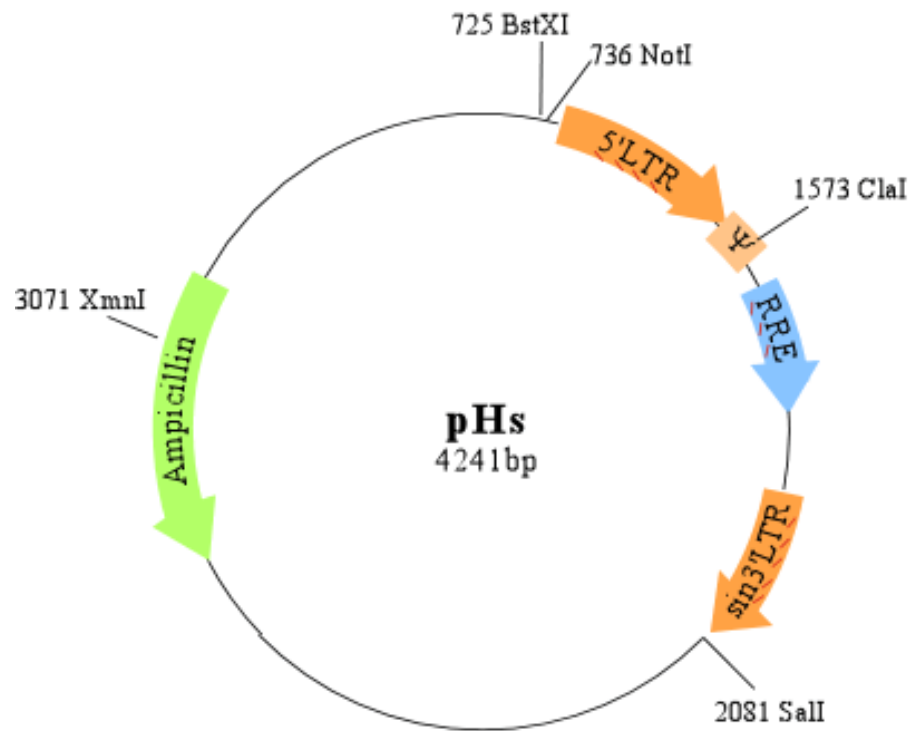


Packaging plasmids

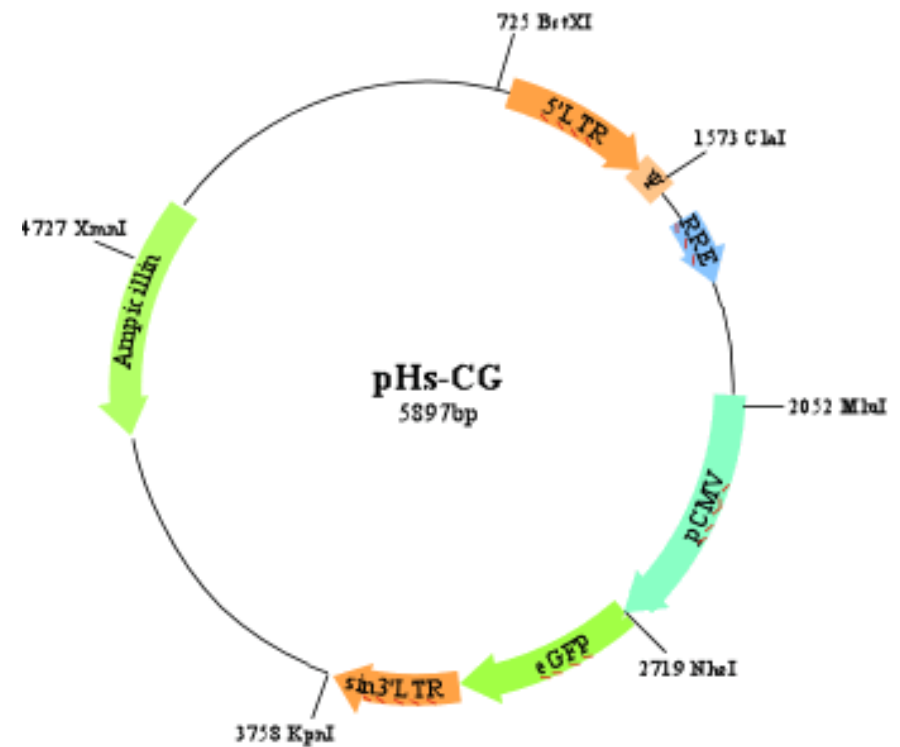


Transfer vectors

Lentiviral SIN vectors



Basic HIV-1 based sin vector.



Basic HIV-1 based sin vector with the GFP marker driven by pCMV.

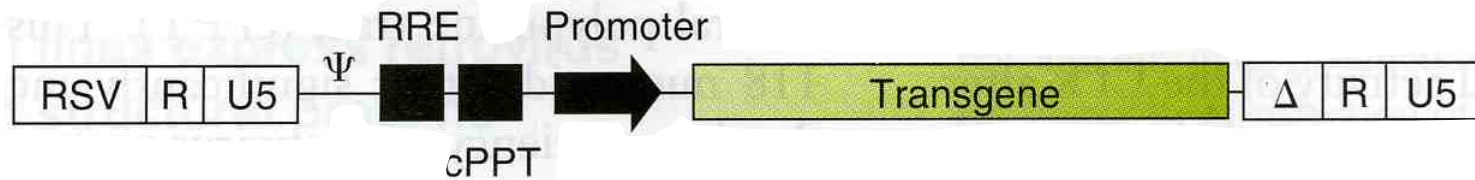
A 3rd generation Lentiviral vector system



Packaging construct

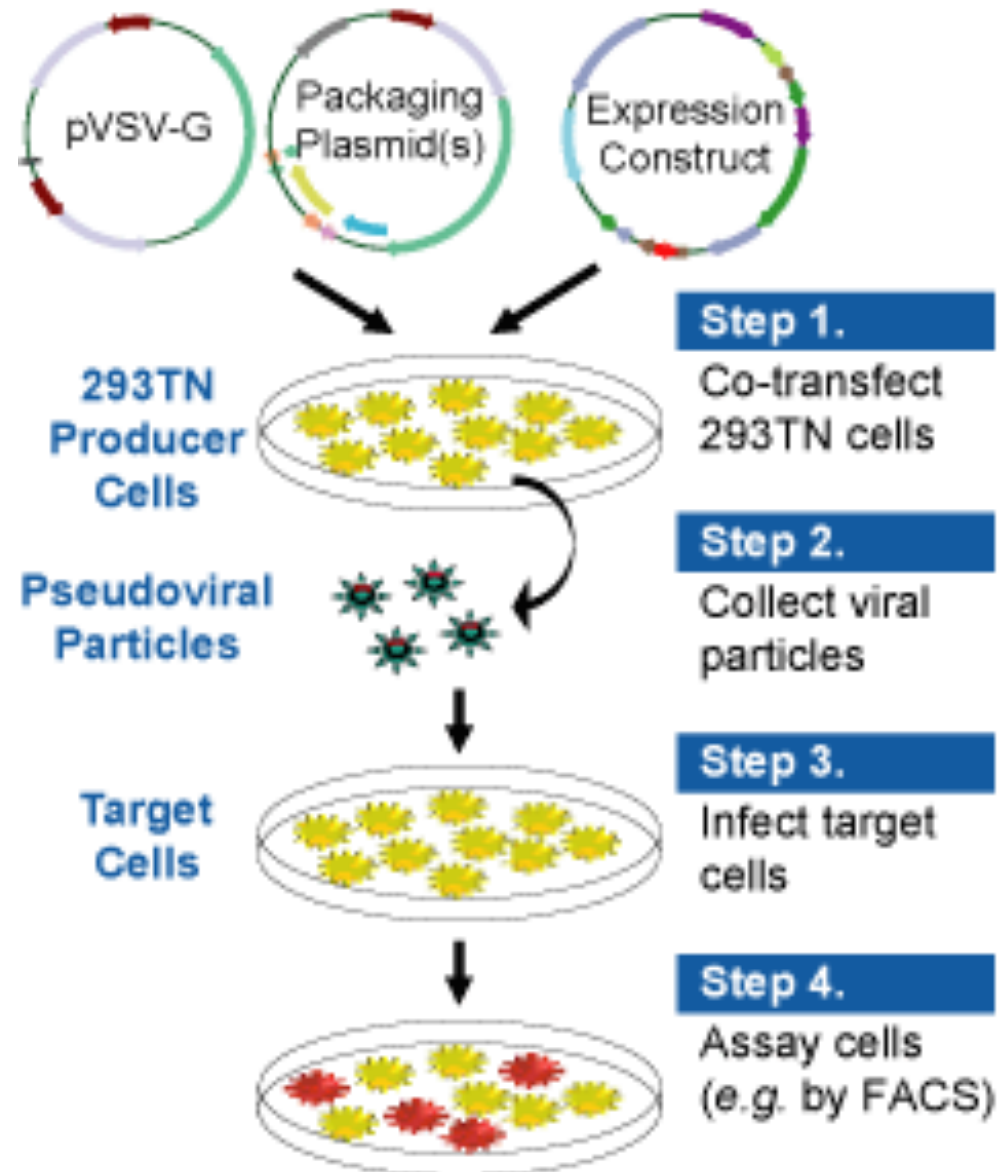


Envelope construct

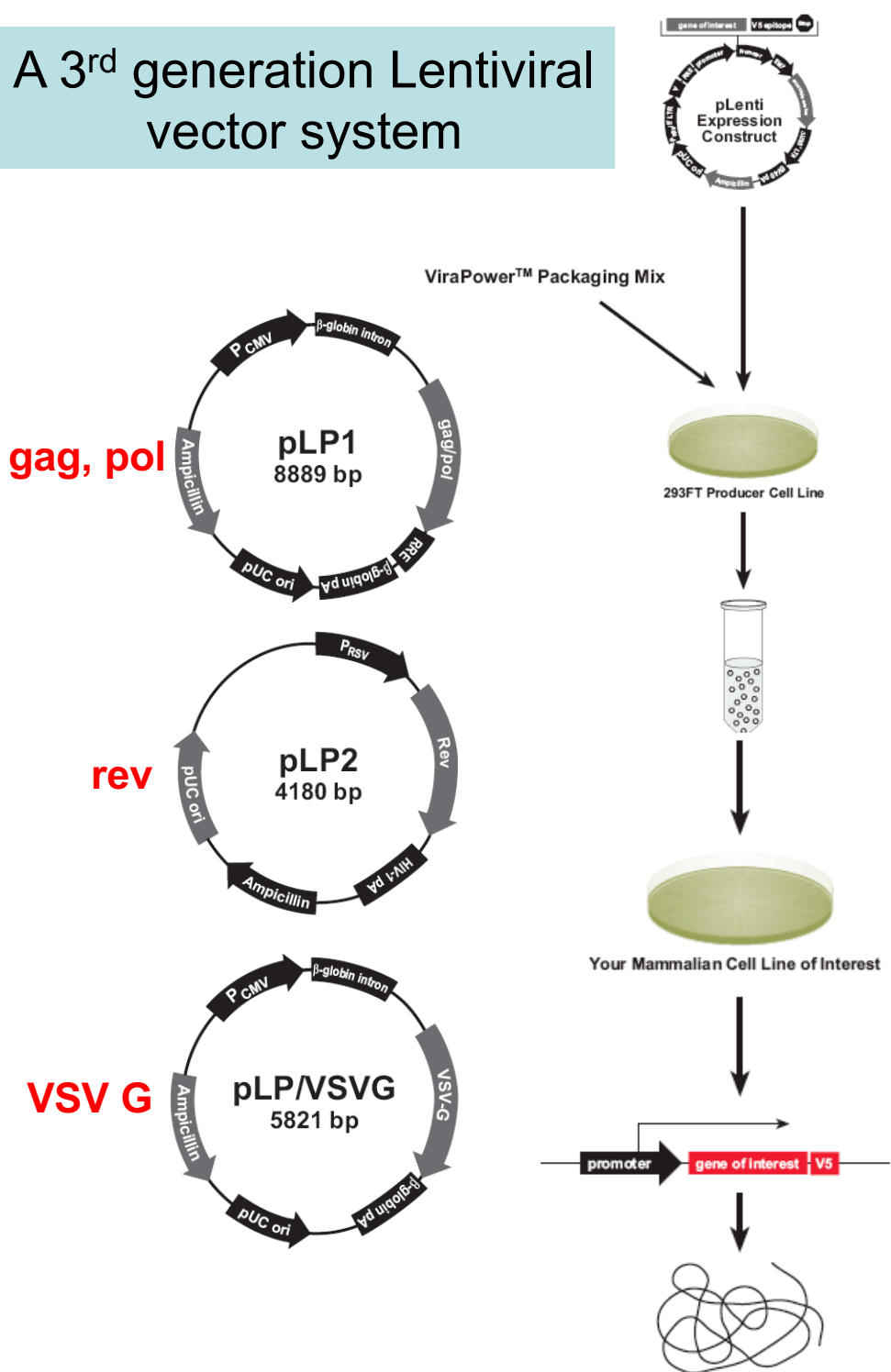


Transfer construct

Flow-chart production of a recombinant lentiviral vector



A 3rd generation Lentiviral vector system



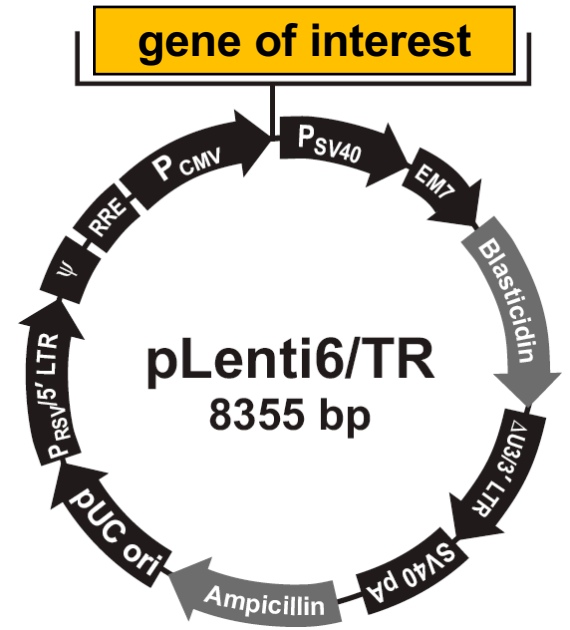
1. Generate the pLenti expression construct containing your gene of interest.

2. Cotransfect the 293FT producer cell line with your pLenti expression construct and the optimized packaging mix.

3. Harvest viral supernatant and determine the titer.

4. Add the viral supernatant to your mammalian cell line of interest. Select for stably transduced cells, if desired.

5. Assay for recombinant protein of interest.



ViraPower™ Lentiviral Expression Systems

Lentiviral systems for high-level expression in dividing and non-dividing mammalian cells

Pros and Cons of Lentiviral Vectors

ADVANTAGES	DISADVANTAGES
Can carry large transgenes (up to 8 Kb)	Potential for generation of RCL
Efficient gene transfer	Potential for insertional mutagenesis: Even replication-incompetent lentiviruses with human tropism are able to infect human cells and integrate their genome into the host cells → risk in case of accidental exposure
Infects dividing and non-dividing cells	
No immunogenic proteins generated	
Stable integration into the host genome and stable expression of the transgene	

Biosafety Considerations for Research Work with Lentiviral Vectors

Biosafety Considerations and Risk Levels

Biosafety Considerations

Higher Risk

Lower Risk



Vector Design

- Vector packaging functions on two plasmids
- Expression of viral genes

- Vector and packaging functions separated onto multiple plasmids
- Deletion of viral genes

Transgene

- Oncogene

- Non-oncogene

Vector Generation

- Large scale

- Laboratory scale

Animal Hosts

- Permissive host
- Animals engrafted with human cells

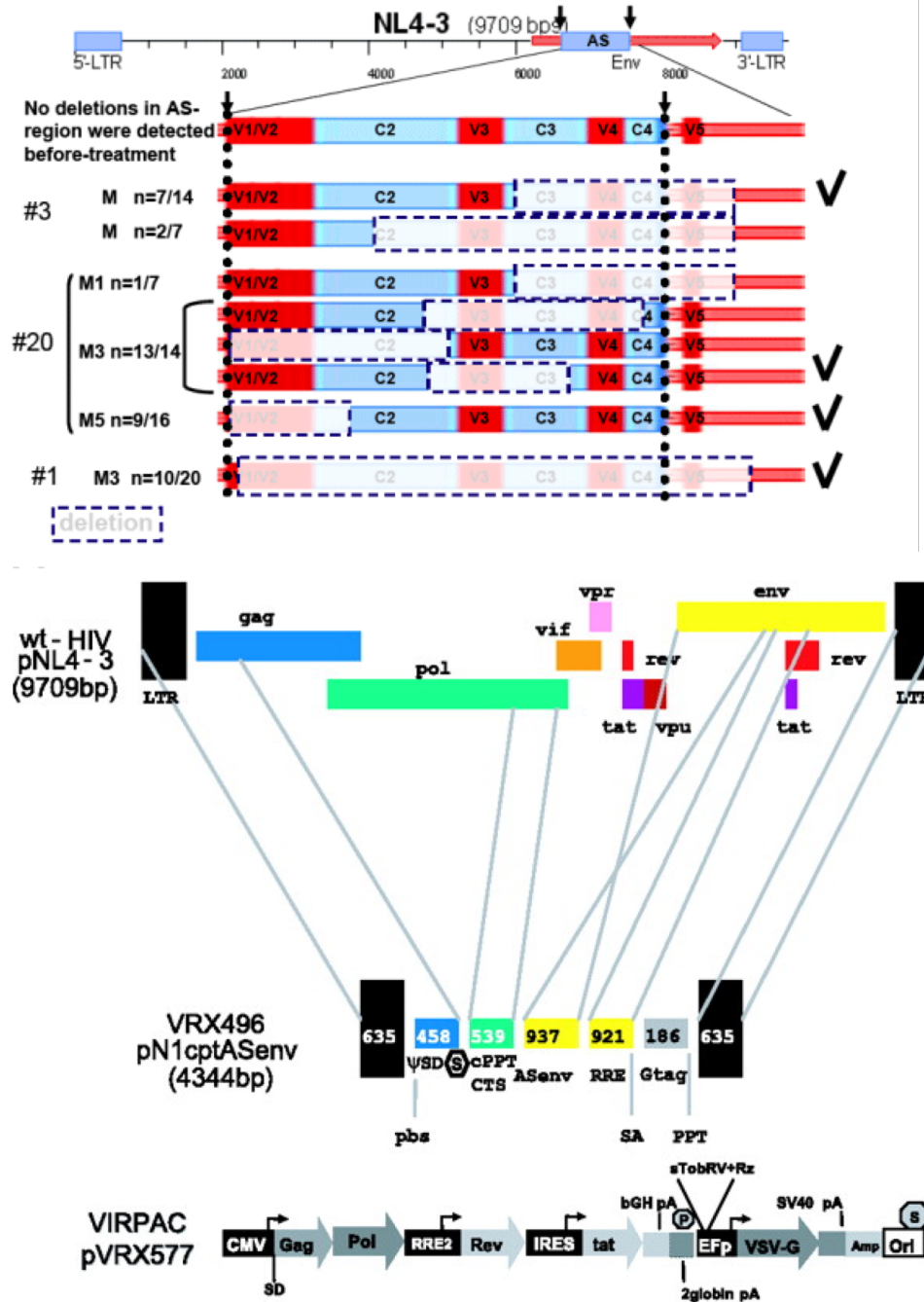
- Non-permissive host

Animal Manipulation

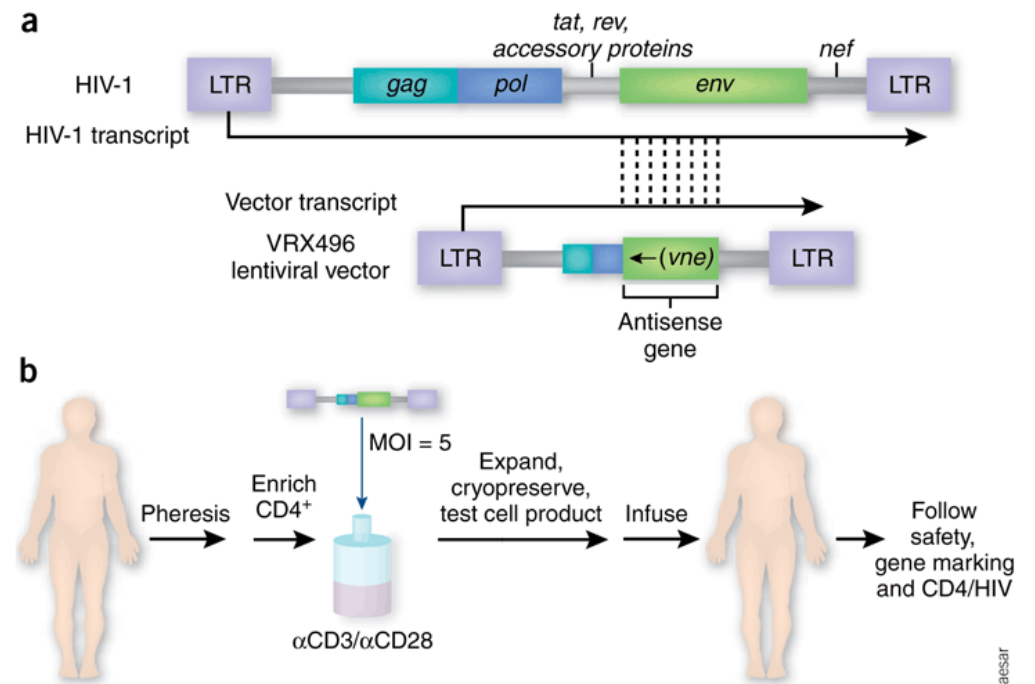
- Vector administration (e.g., use of sharps during injection)

- Housing and husbandry (no use of sharps)

VRX496 Anti-sense Mechanism Deletes Env



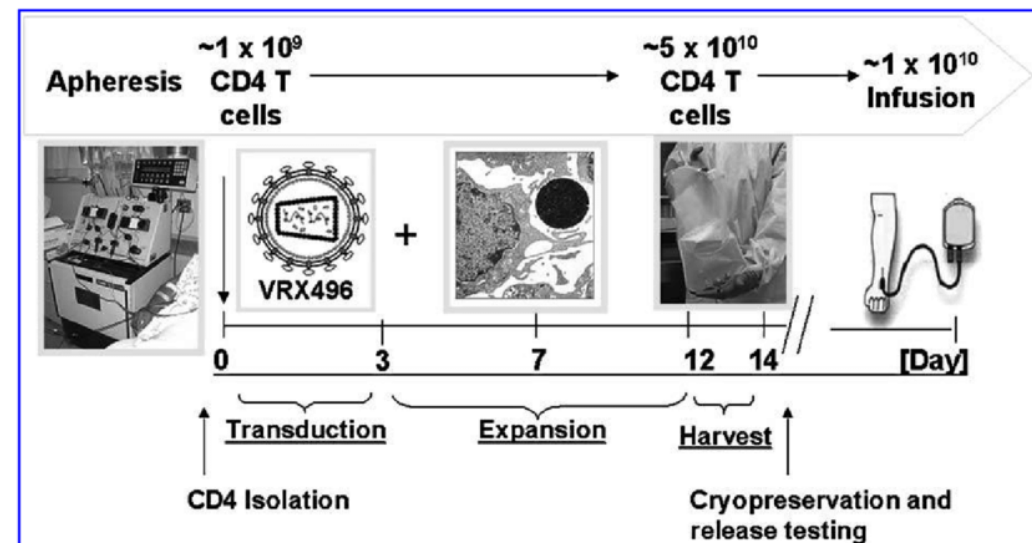
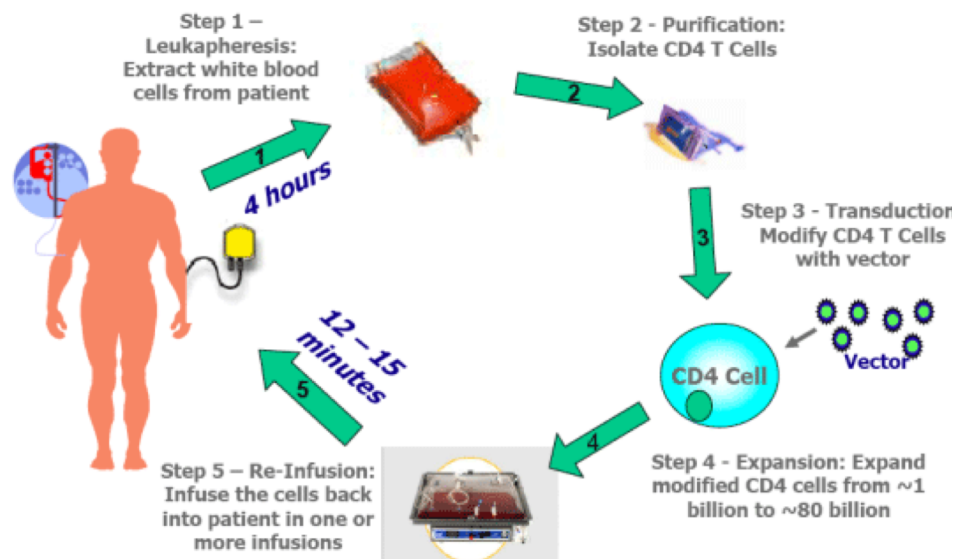
The first clinical trial of a lentiviral vector highlights the promise of this new class of gene-therapy vector



Lexgenleucel-T (VRX496):

Autologous CD4+ Cells Transduced with a Lentiviral Vector Encoding a 937 Base Antisense Sequence Targeting HIV Envelope

VRX496 Anti-HIV T Cell Transplantation



Conclusions from Clinical Trials

Lexgenleucel-T (VRX496):

Autologous CD4+ Cells Transduced with a Lentiviral Vector Encoding a 937 Base Antisense Sequence Targeting HIV Envelope

VRX496 Anti-HIV T Cell Transplantation



- No safety issues
- Reduced viral in treatment failures
- Reduced viral infectivity
- Sustained increases in CD4 counts in 10B bolus
- Additional clinical trials being developed

VRX496 Clinical Studies – Summary

Clinical Trial	Infusion Schedule	Cell Dose	Status
<u>Phase I</u> Failed ≥ 2 HAART CD4 ≥ 150 ; VL ≥ 5000	Single dose	~10 billion	Completed**
<u>Phase II</u> Failed ≥ 1 HAART CD4 ≥ 150 VL ≥ 5000	Repeat 4 or 8 doses	10 billion per dose	Completed
	Single dose	10 billion 20 billion 30 billion	Ongoing
<u>Phase I/II</u> Virologically Controlled CD4 ≥ 350 ; VL ≤ 50	Repeated 6 doses	10 billion per dose	Ongoing

- U.S. multi-center study: University of Pennsylvania, Stanford University, University of Kentucky, Jacobi Medical Center, Mercy Medical Center, Circle Medical Center

** PNAS 103:17372-17377. 2006