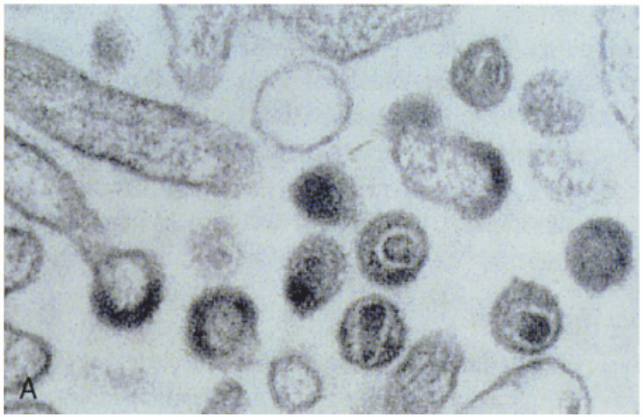
VIROLOGY

Engineering Viral Genomes: Retrovirus Vectors

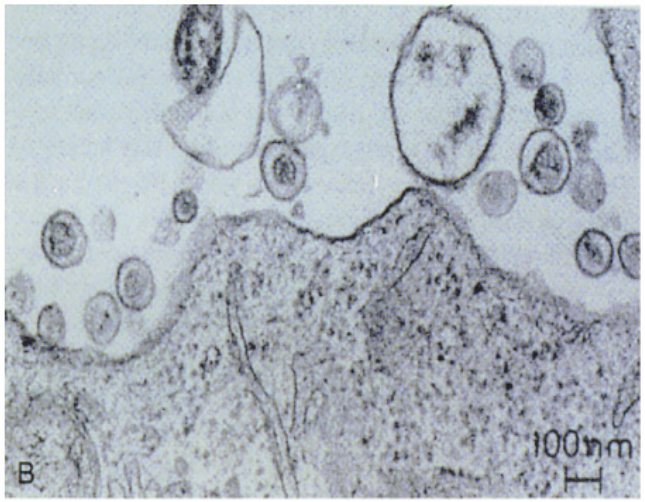
Family *Retroviridae*

Genus	*Examples*	*Features*
Alpharetrovirus	Rous sarcoma virus, Avian leukosis virus	oncogene present in many members
Betaretrovitrus	Mouse mammary tumor virus	mammary carcinomas
Gammaretrovirus	Moloney murine leukemia virus, Feline leukemia virus	oncogene present in many members
Deltaretrovirus	Human T-cell llymphotrophic 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

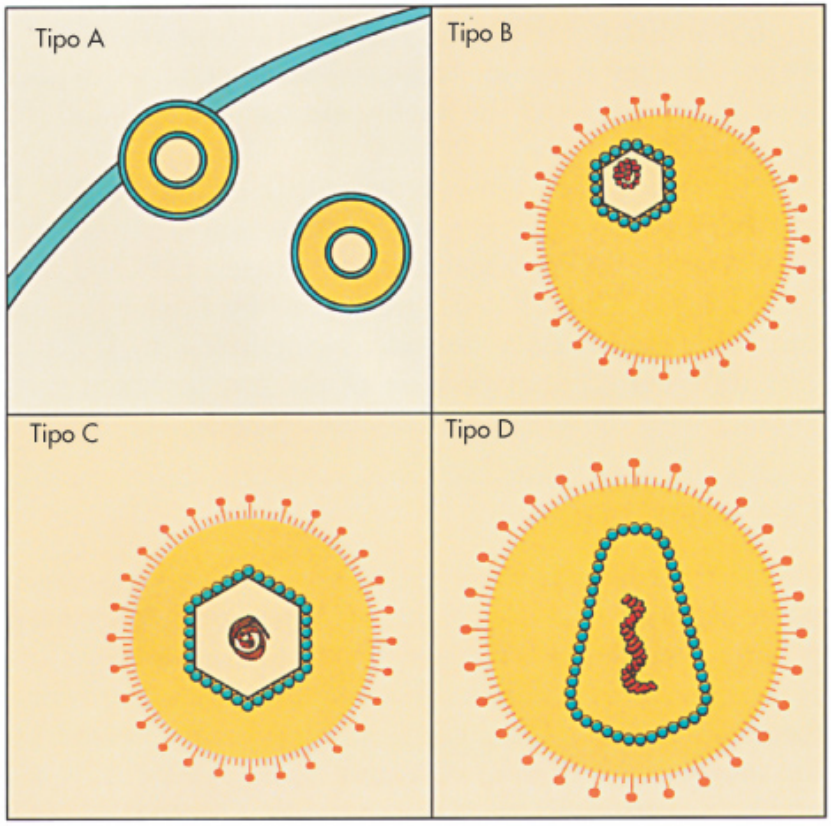
Morphology of Retroviruses



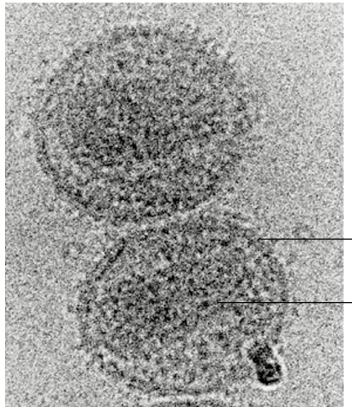
HIV-1



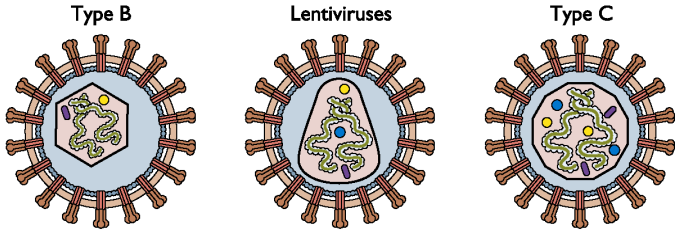
HTLV-1



A



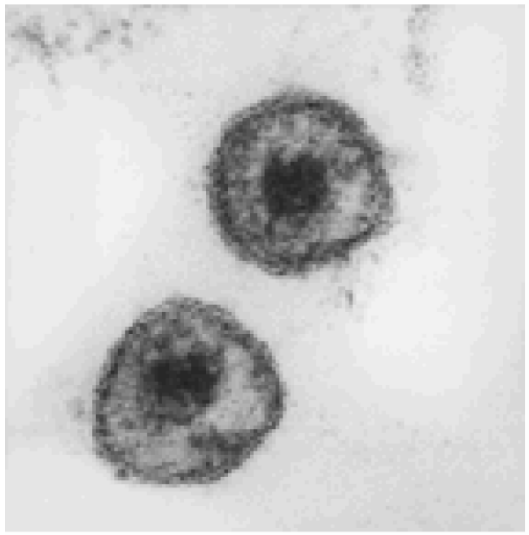
B

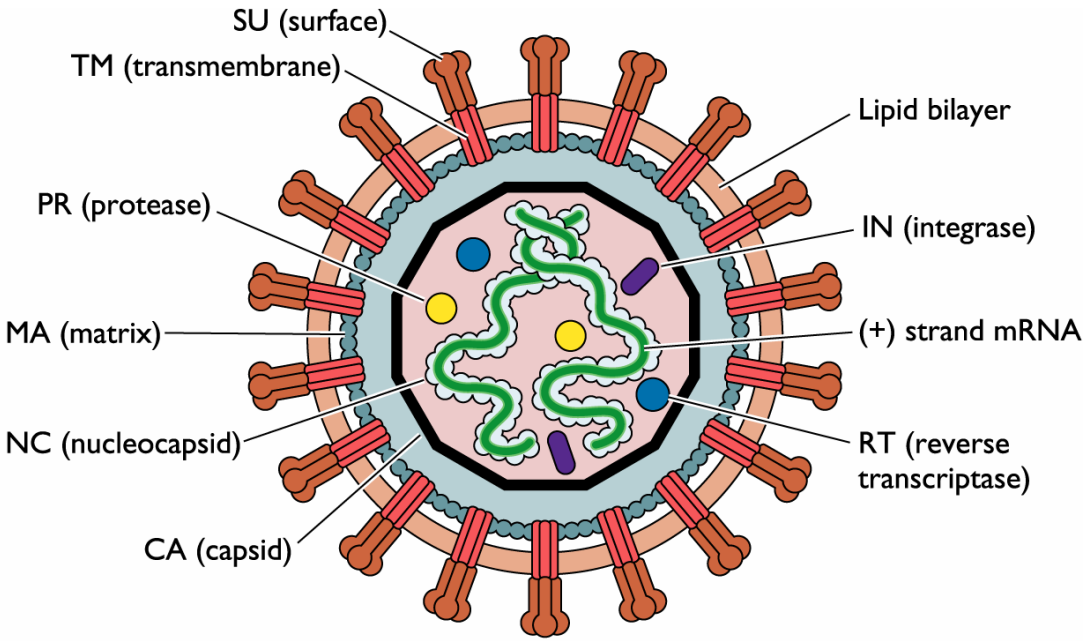


MMTV HIV-1 HTLV-1

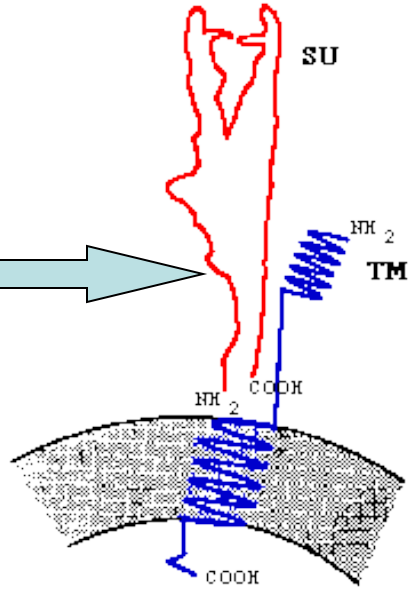
Structure of a Retrovirus

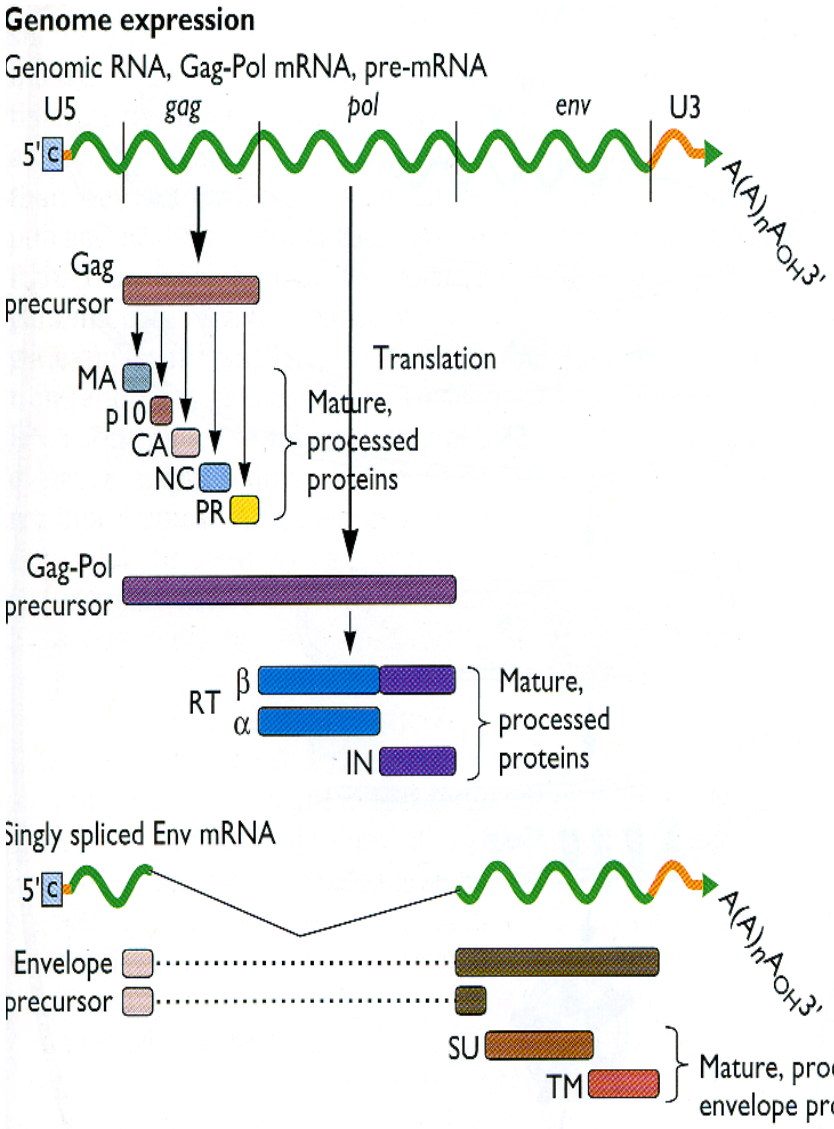
A

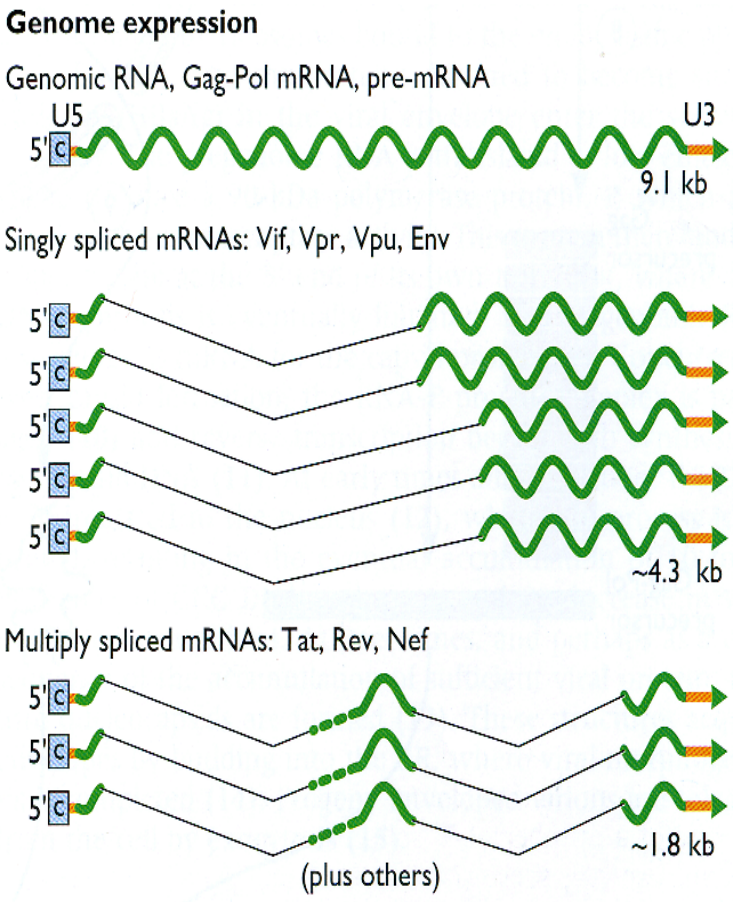


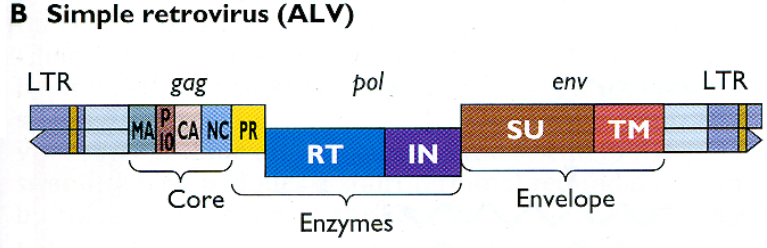


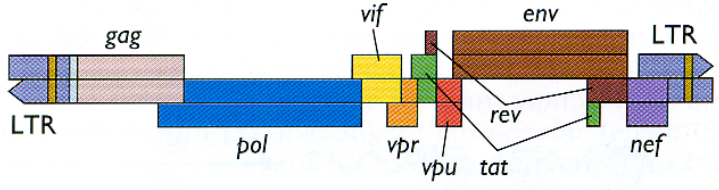
Lollipop-like spikes



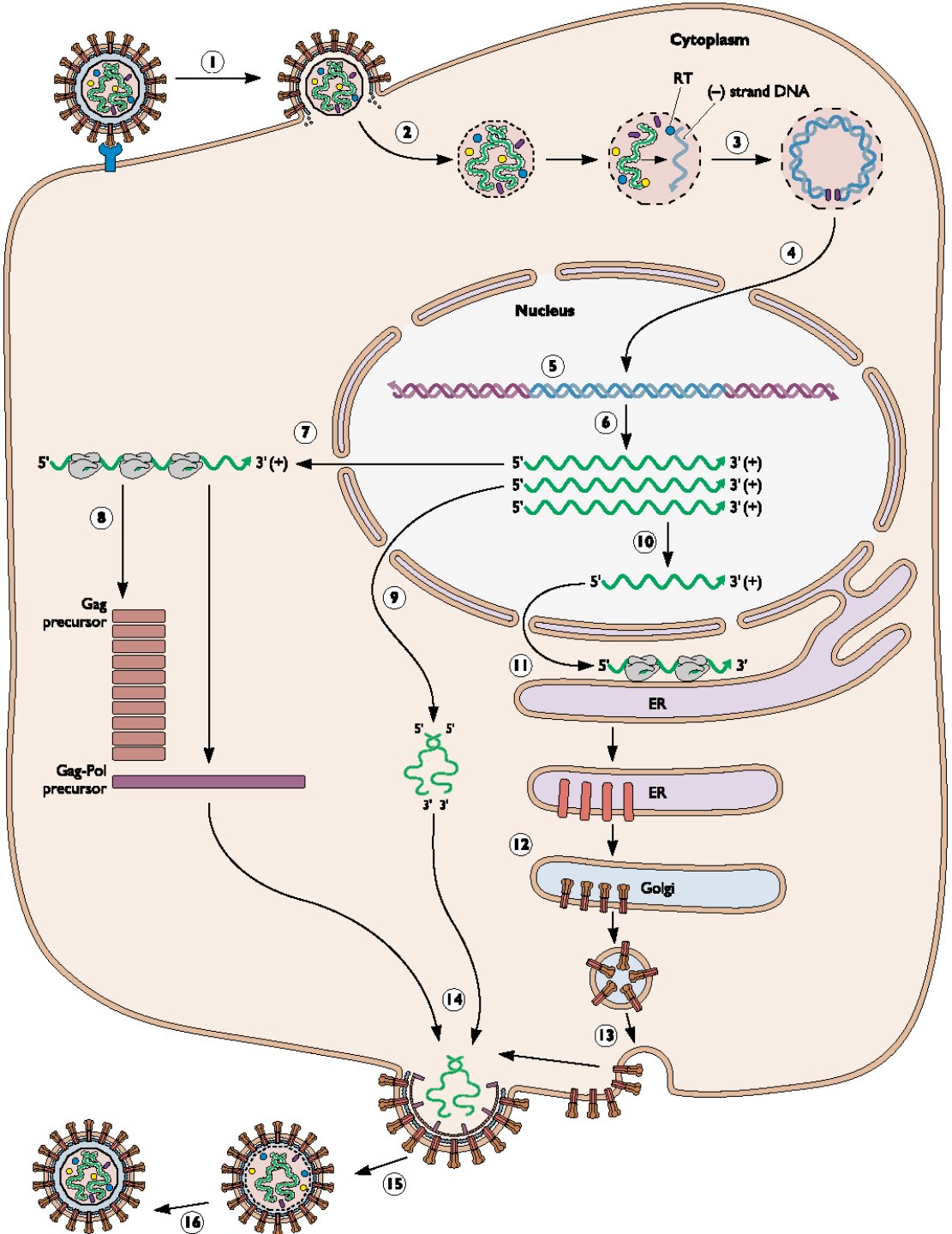

Genome expression
Genomic RNA, Gag-Pol mRNA, pre-mRNA
U5
gag
pol
env
U3
5'
C
A(A)nAOH3'
Gag
precursor
MA
p10
CA
NC
PR
Translation
Mature,
processed
proteins
Gag-Pol
precursor
RT
β
α
IN
Mature,
processed
proteins
Singly spliced Env mRNA
Envelope
precursor
SU
TM
Mature, processed
envelope proteins

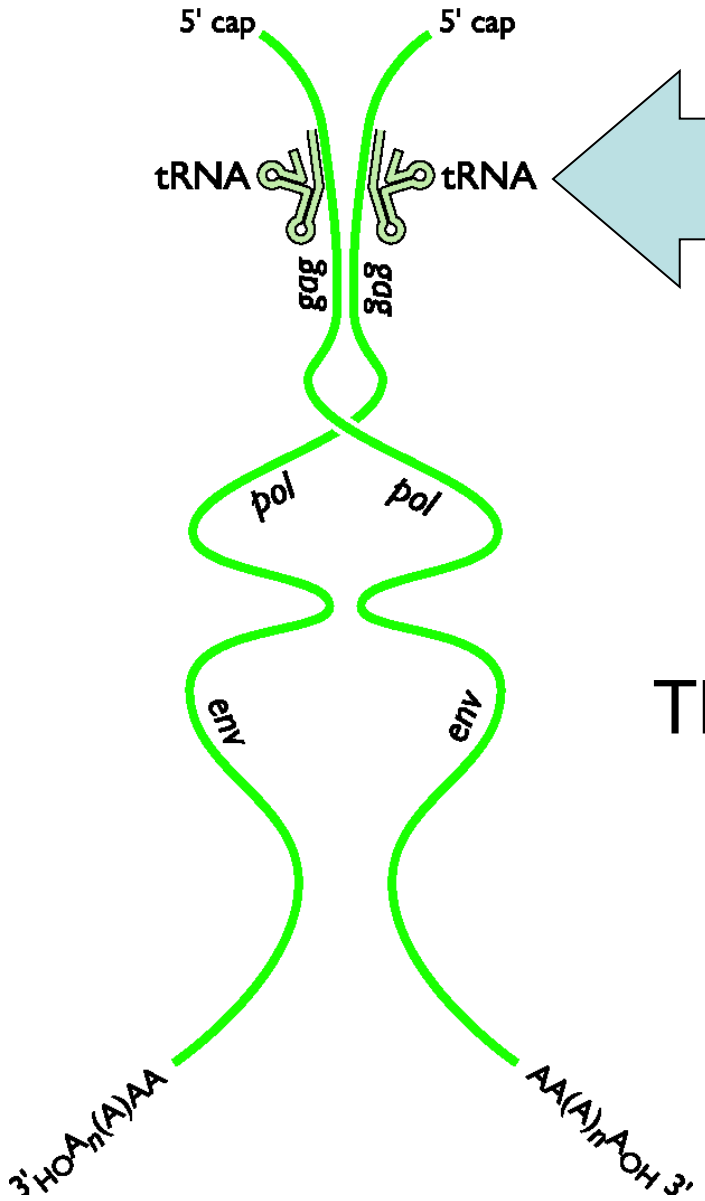

Genome expression
Genomic RNA, Gag-Pol mRNA, pre-mRNA
U5
U3
5'
C
9.1 kb
Singly spliced mRNAs: Vif, Vpr, Vpu, Env
~4.3 kb
Multiply spliced mRNAs: Tat, Rev, Nef
~1.8 kb
(plus others)


B Simple retrovirus (ALV)
LTR
gag
pol
env
LTR
MA
P
10
CA
NC
PR
RT
IN
SU
TM
Core
Enzymes
Envelope


Complex retrovirus (HIV-1)
gag
vif
env
LTR
LTR
rev
pol
vpr
vpu
tat
nef

Retrovirus replicative cycle



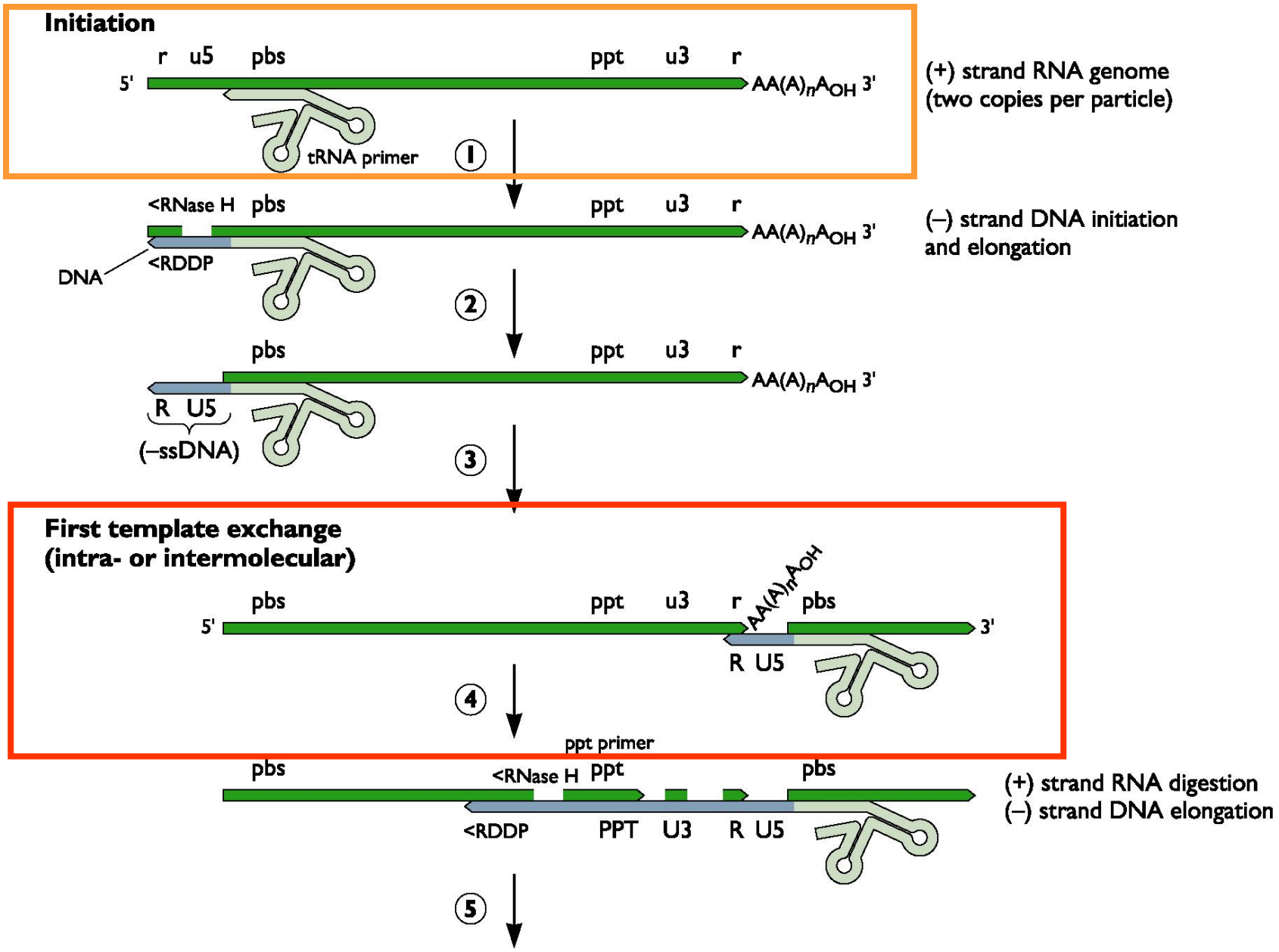


Most mammalian retroviruses use $tRNA^{PRO}$, $tRNA^{Lys3}$, $tRNA^{Lys1,2}$

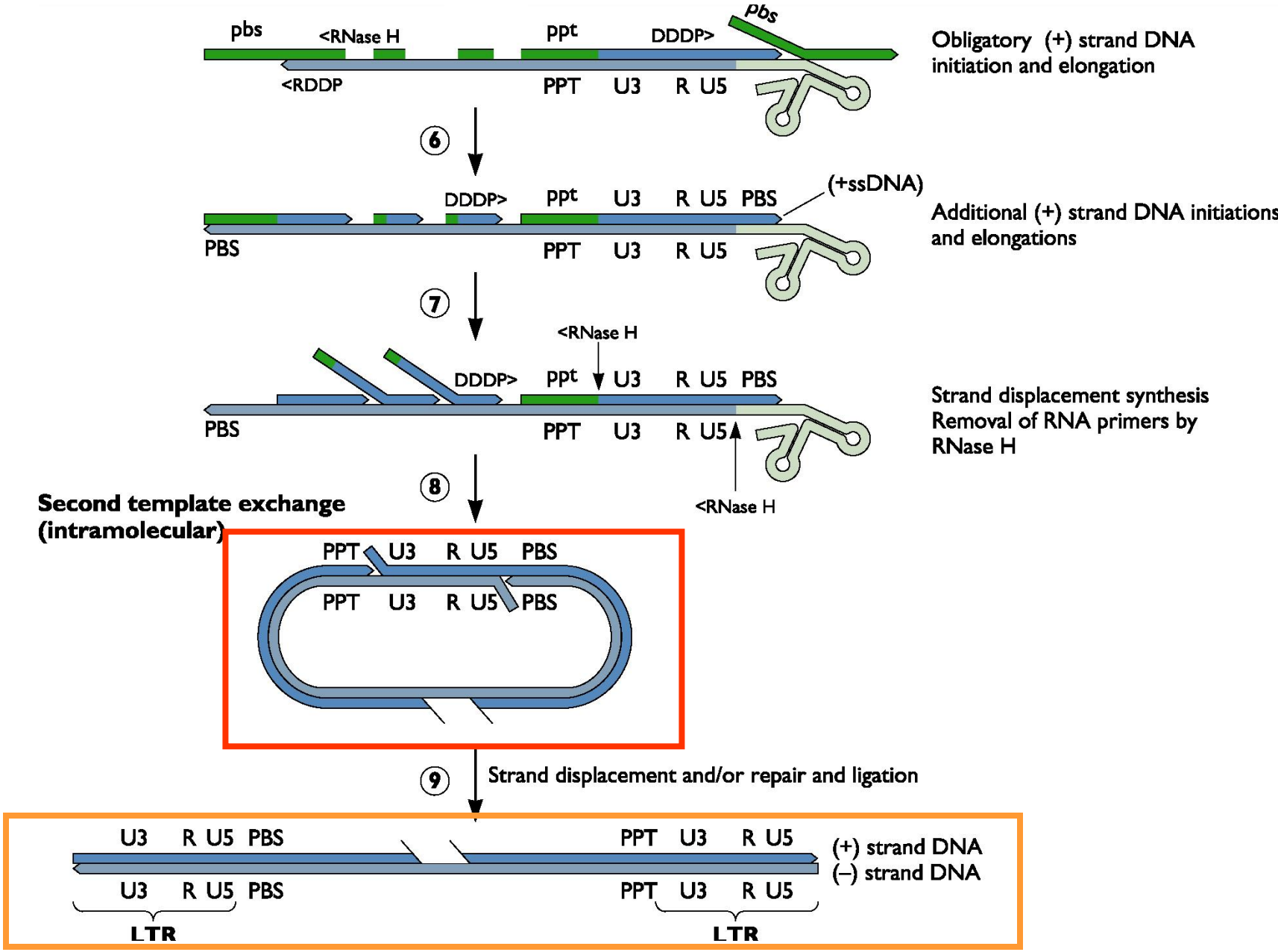
The partially unfolded tRNA is annealed via at least 18 nt at its 3' end to a site on RNA genome called the **primer-binding site (pbs)**

The diploid retroviral genome

The reverse transcription process -1



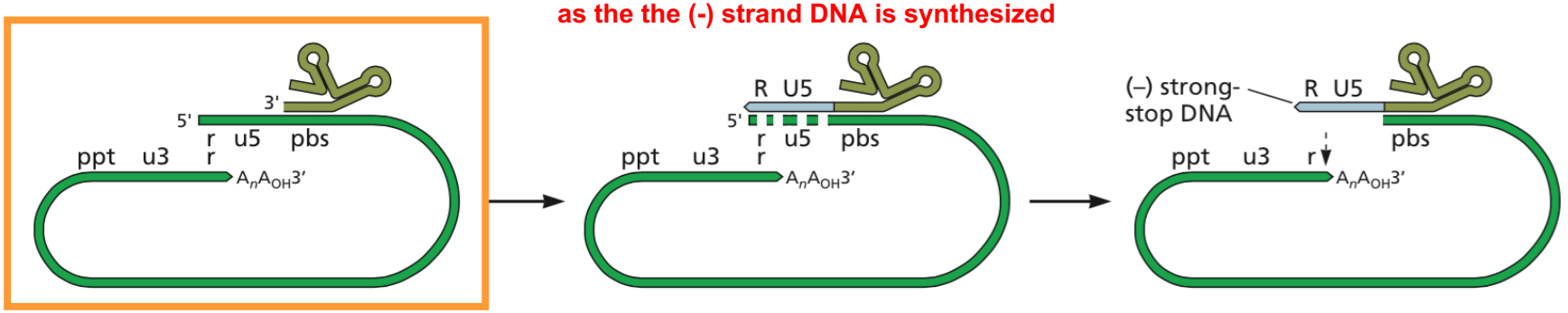
The reverse transcription process -2



Initiation of (–) strand DNA synthesis

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

1

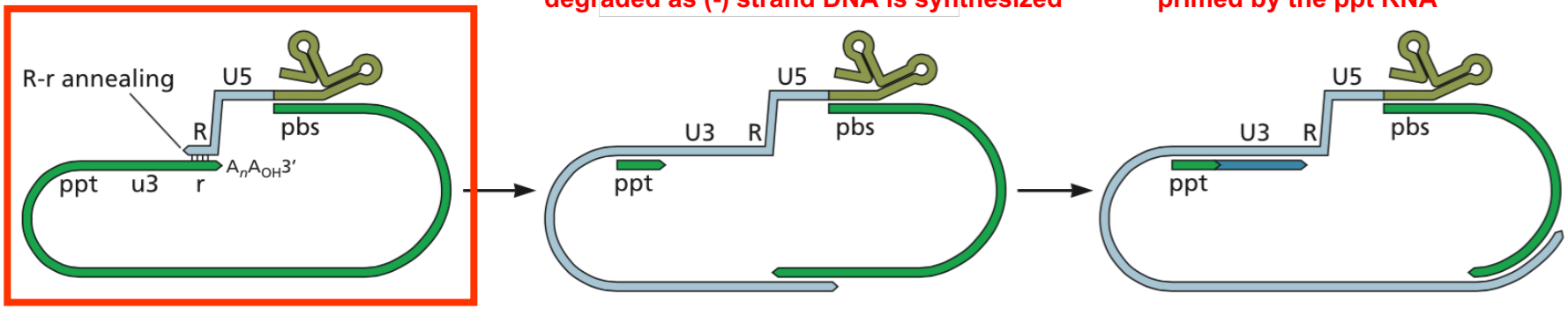


First template exchange

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

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

2



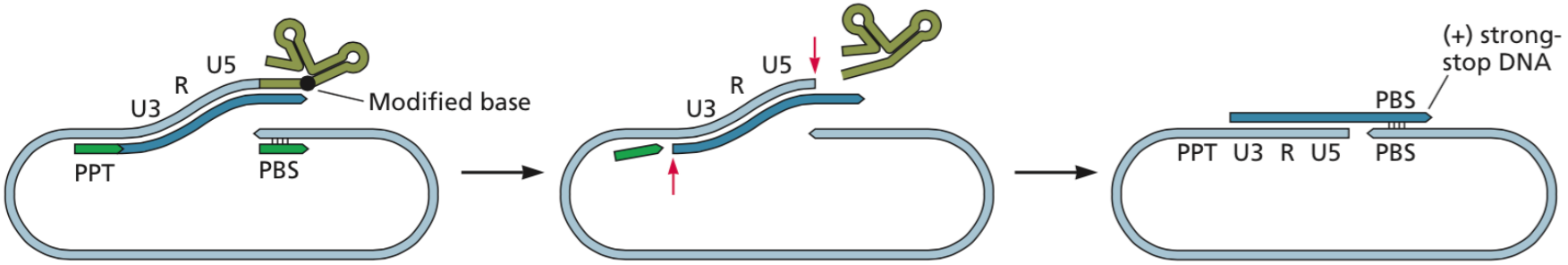
(+) strand DNA synthesis

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

RNAse H activity of RT removes both primer RNAs

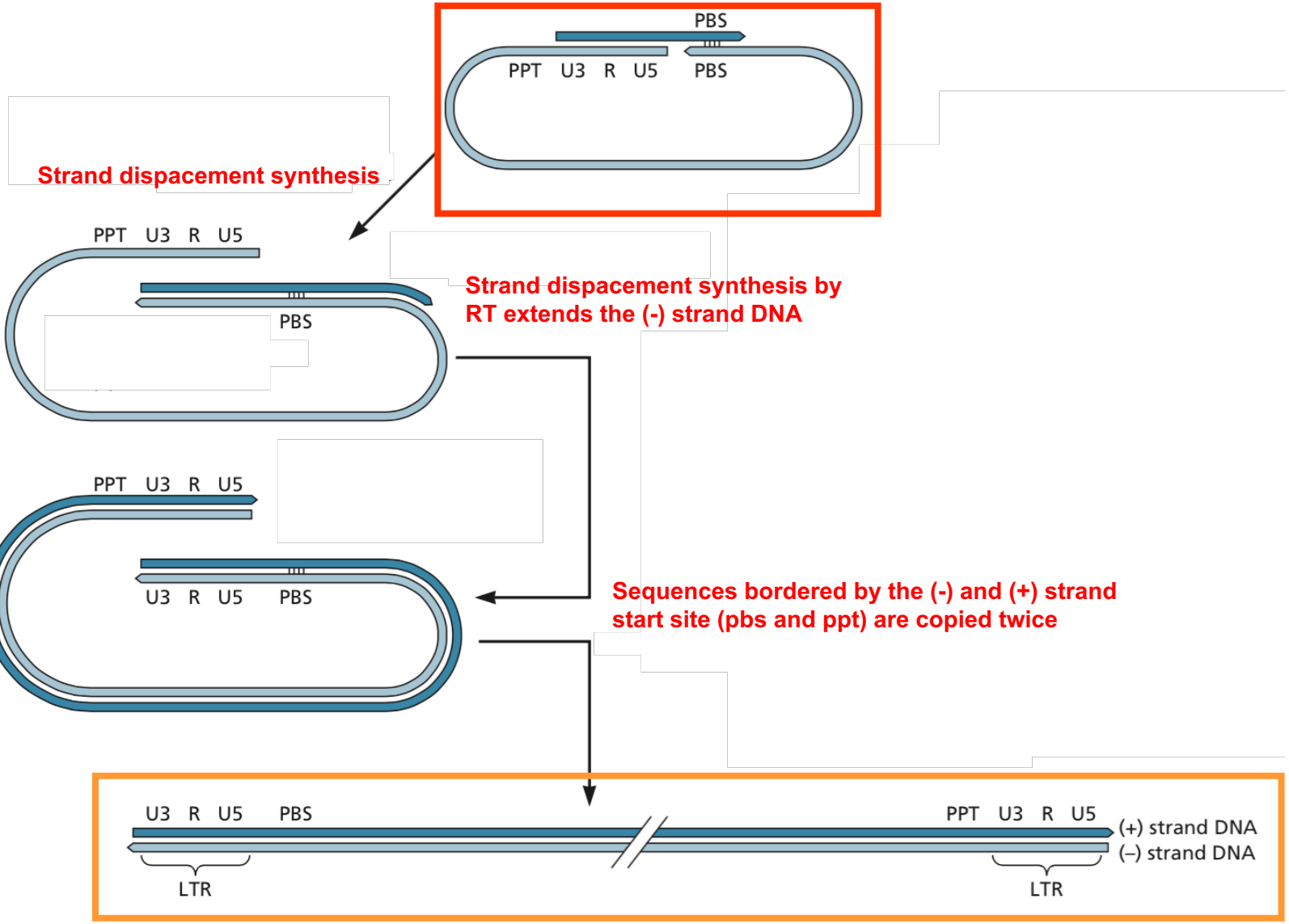
DNA ends are juxtaposed by annealing at complementary PBS sequences

3

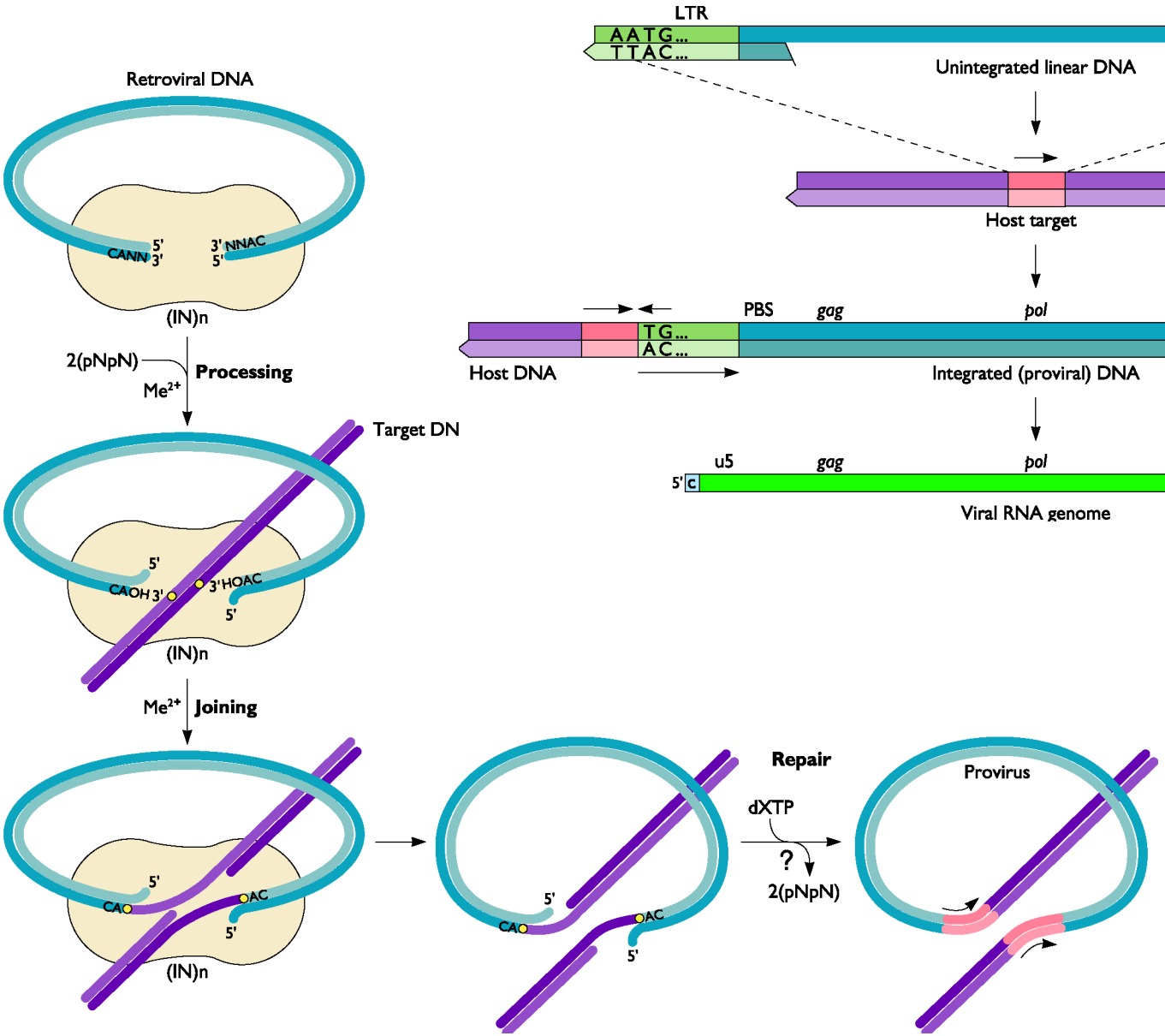


4

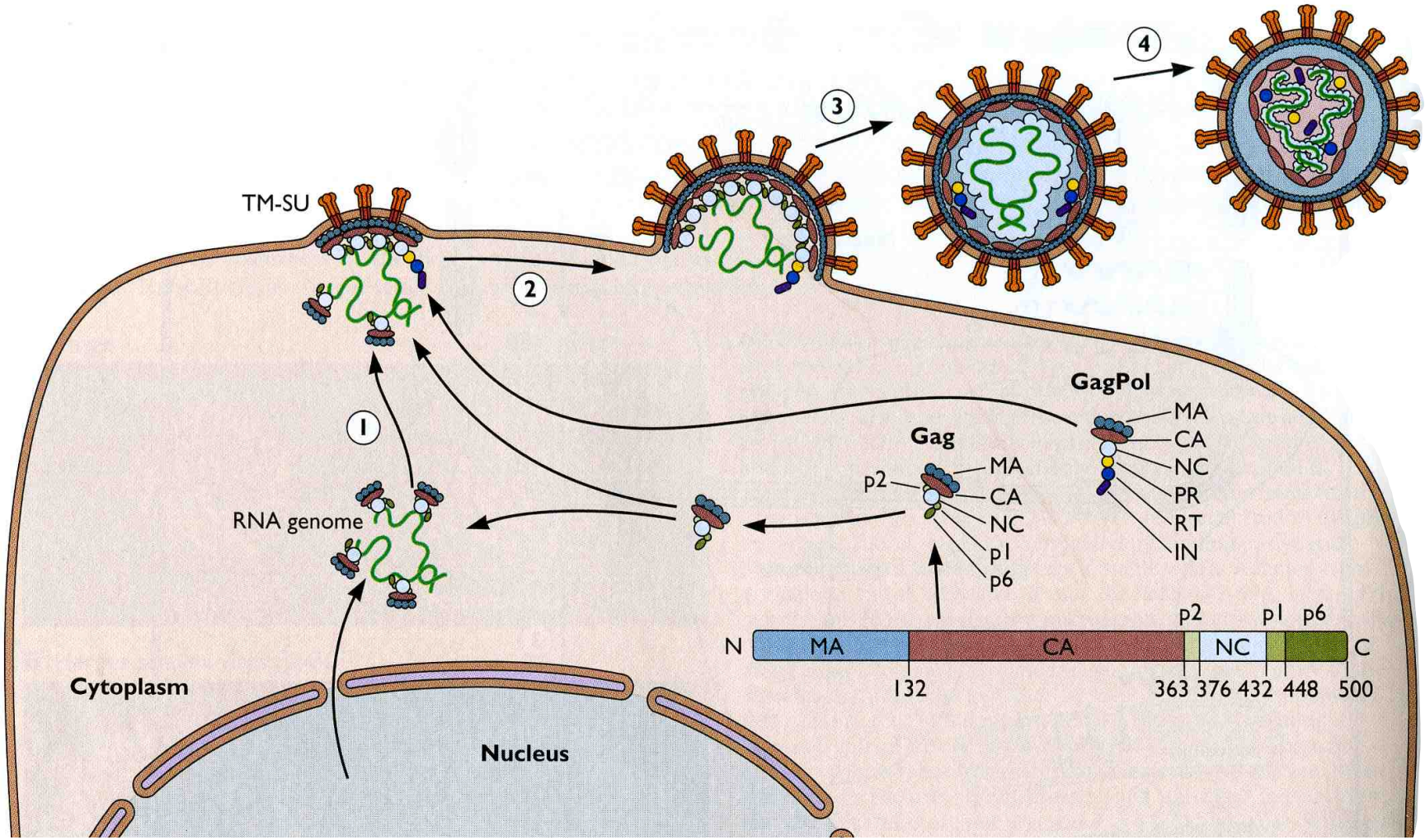
Second template exchange is facilitated by annealing of PBS sequences



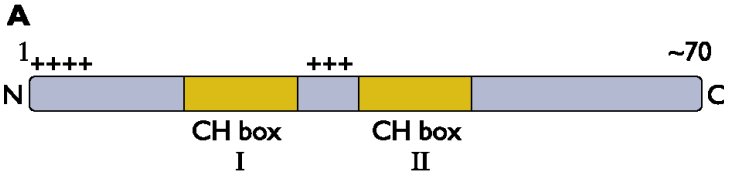
Characteristics of retroviral integration

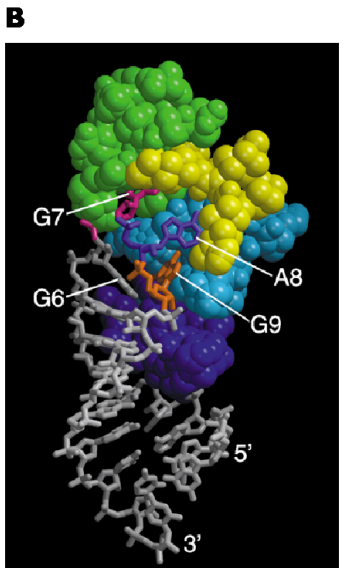


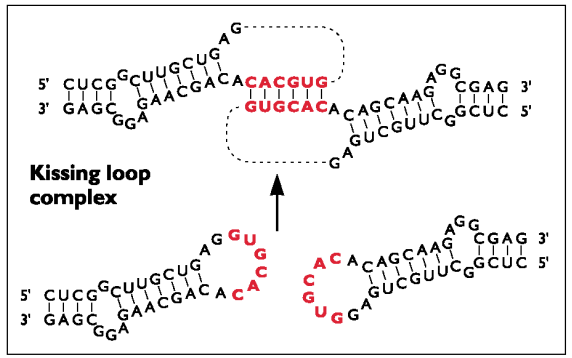
Assembly of a retrovirus from polyprotein precursors

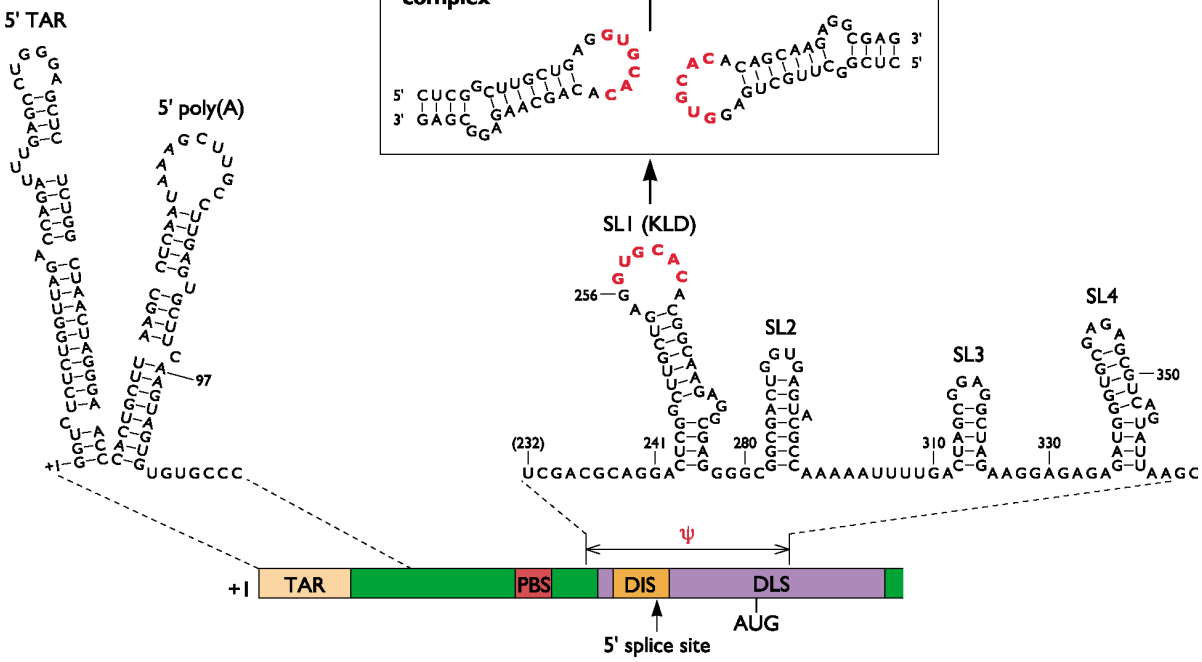


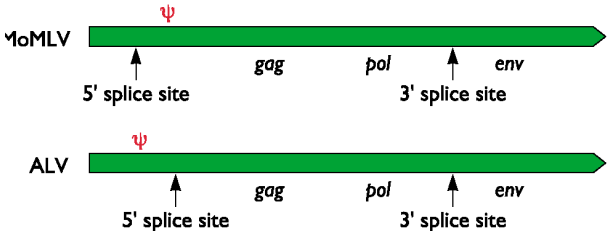
Sequences important in packaging of retroviral genomes











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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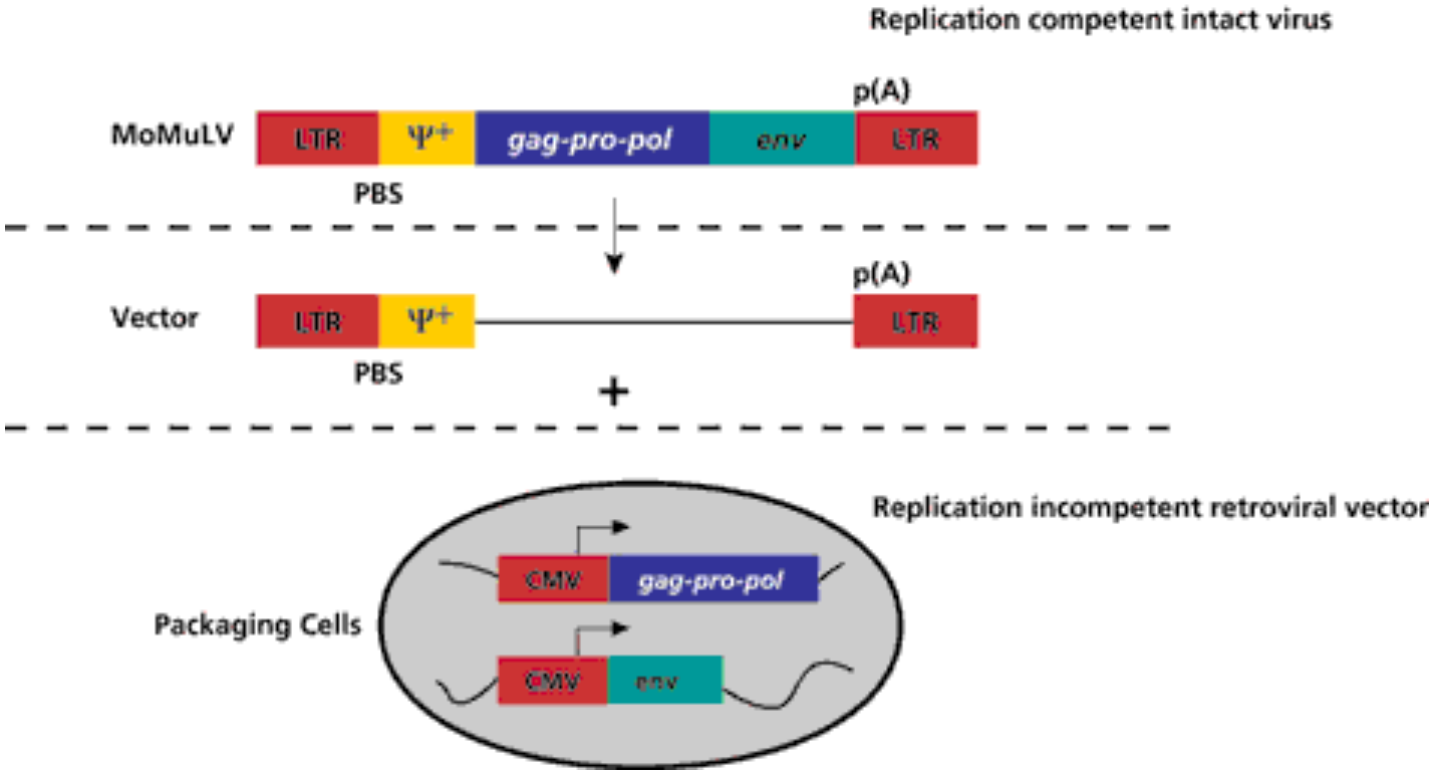
Favorable Features of Retroviruses as Vectors

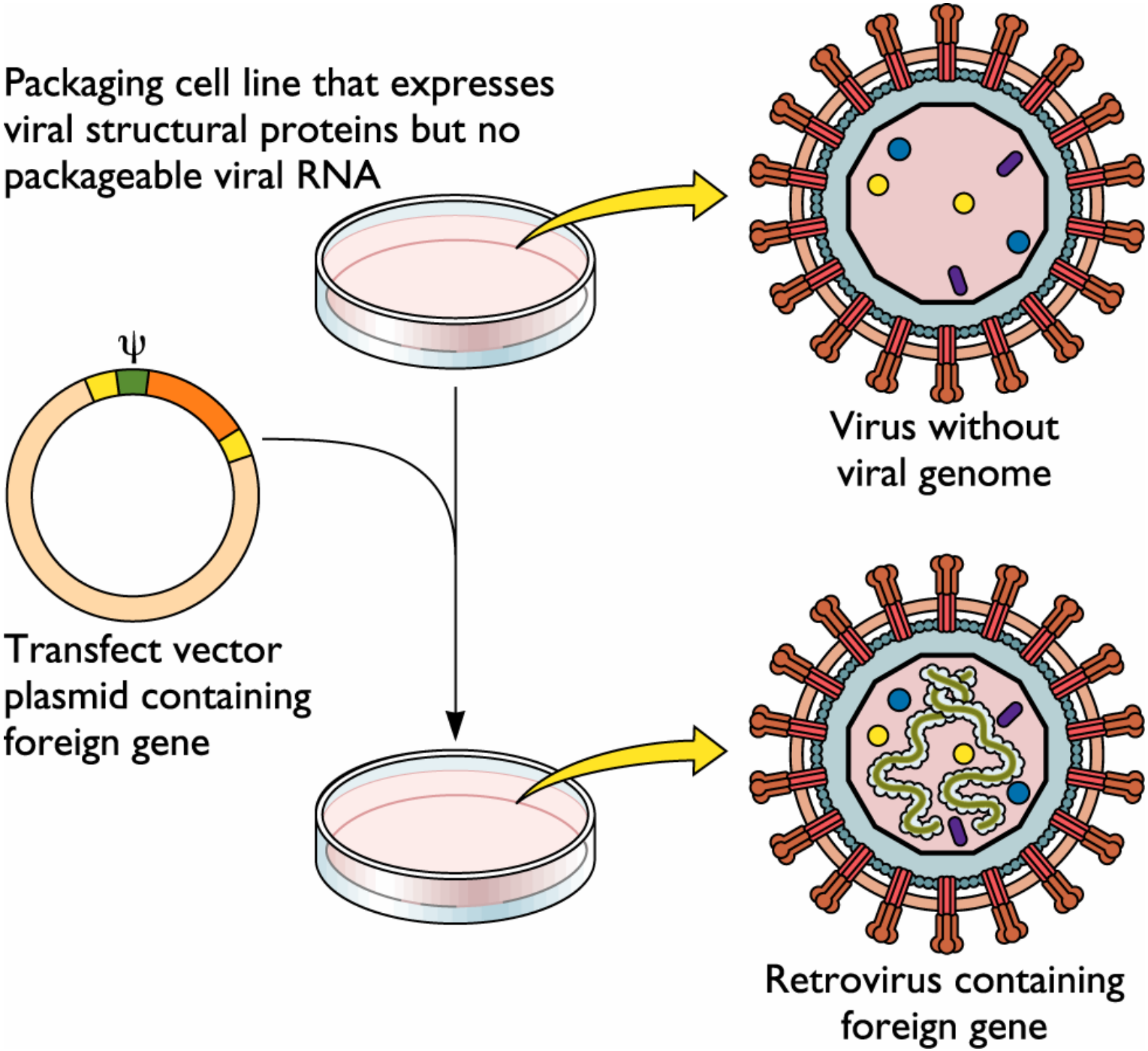
- Well characterized
- Easily to manipulate (genomes 7-9 kb)
- They require 3 trans (gag, pol, env) and 7 major cis-active control elements (U3, R, U5, PBS, SD, Ψ, SA) in order to replicate
- Stability of recombinants vectors (plasmids)
- High efficiency of gene transfer
- Most are replication-defective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

Retrovirus as vectors

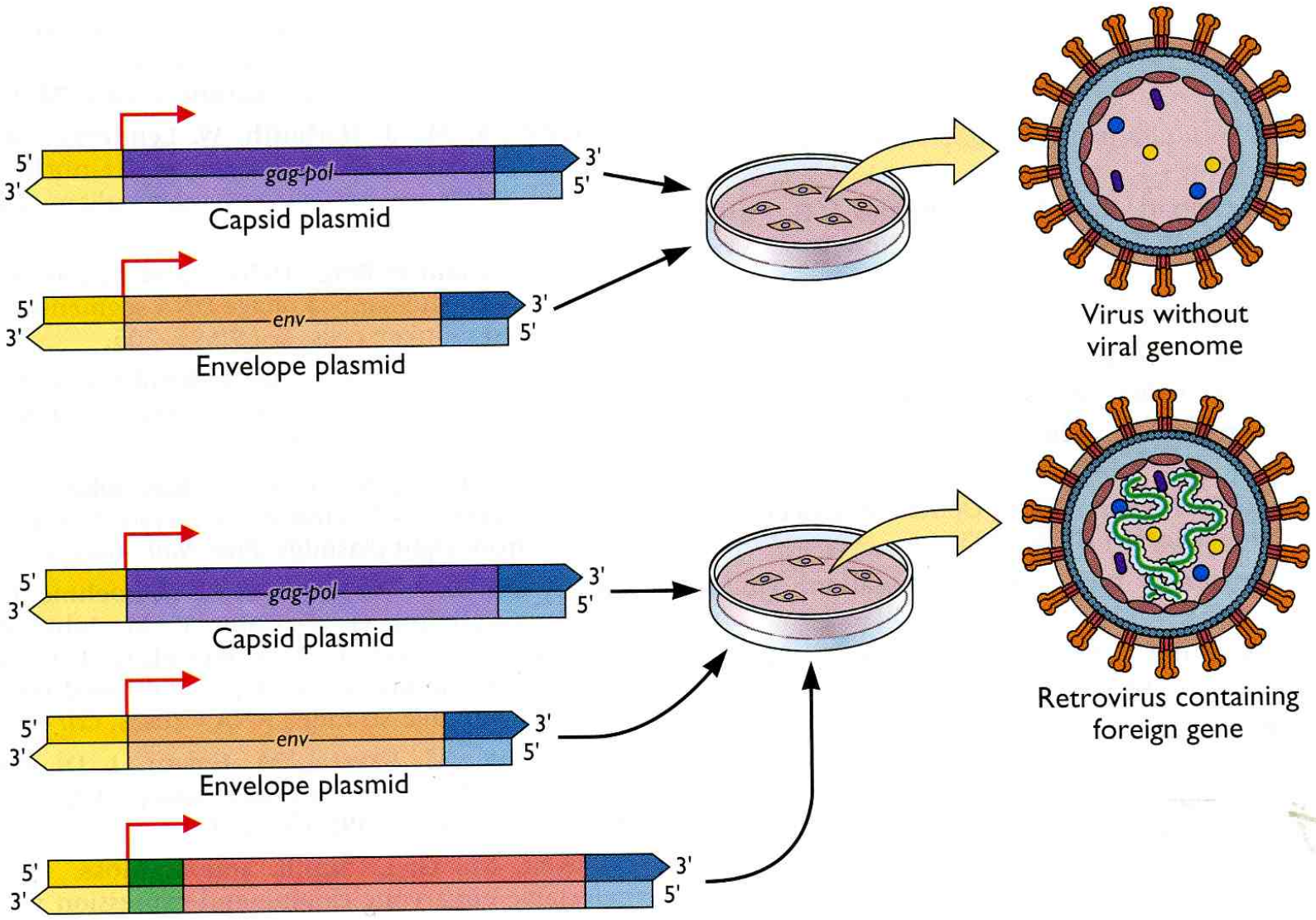
- Replication-incompetent vectors
  - -They bear deletions of some or all of the viral genes
  - -They retain *cis*-acting viral sequences necessary for transmission (U3, R, U5, PBS, SD, Ψ, SA)
  - -They need to be propagated in "packaging" cell lines that provide in *trans* gag, pol, env
- Replication-competent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

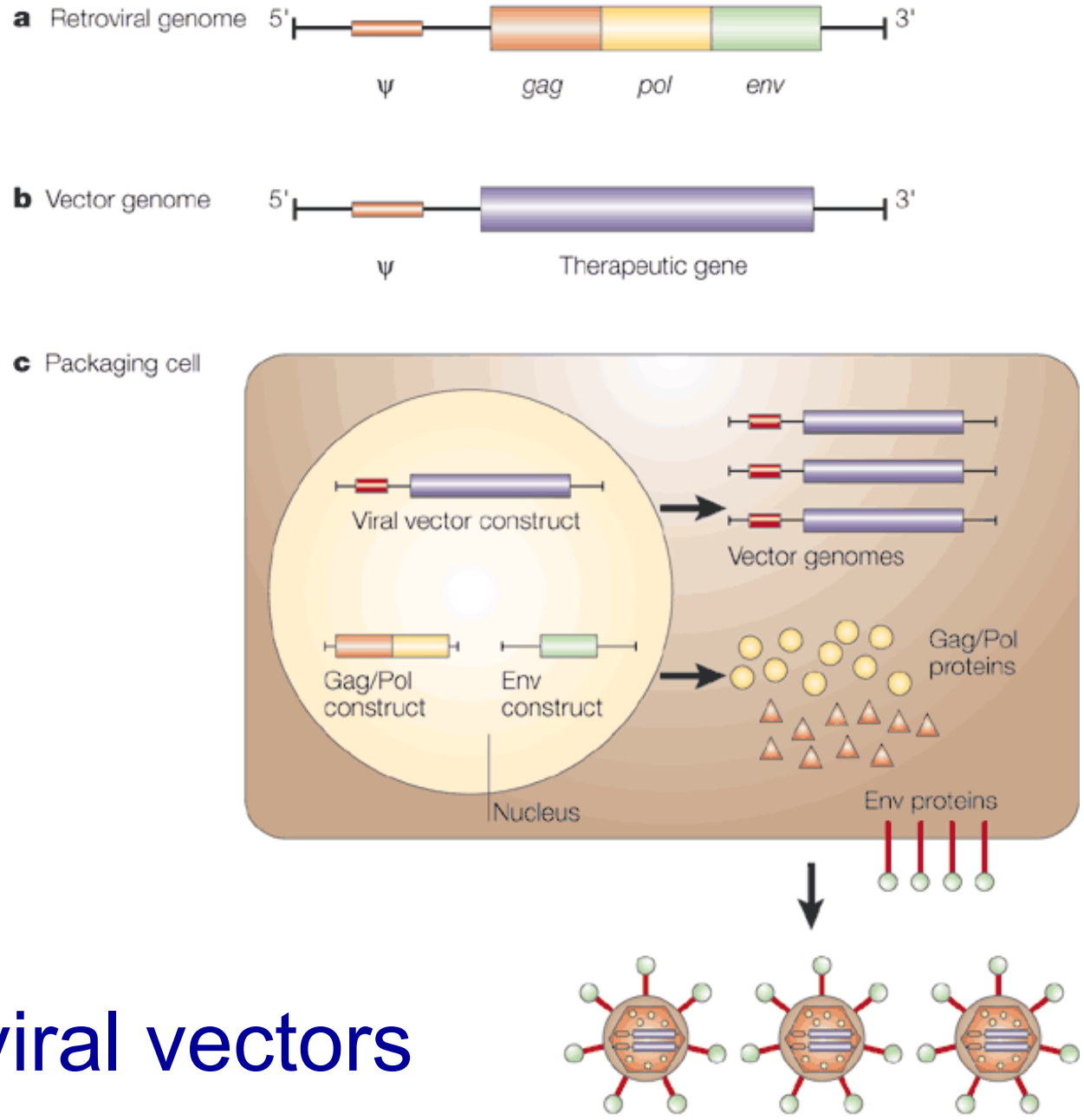
Replication-incompetent vectors




Packaging cell line that expresses viral structural proteins but no packageable viral RNA
ψ
Transfect vector plasmid containing foreign gene
Virus without viral genome
Retrovirus containing foreign gene

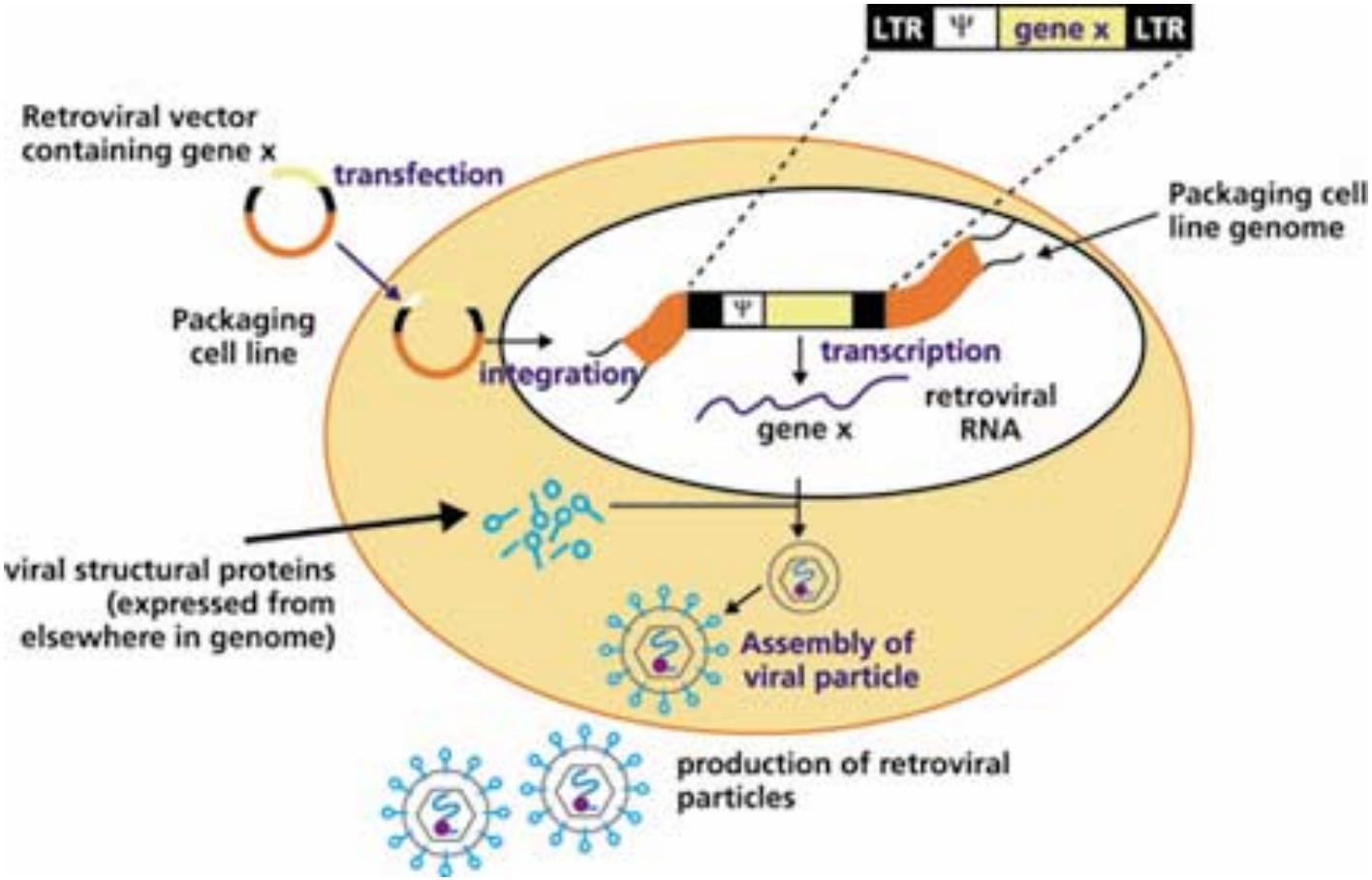
Retroviral vectors



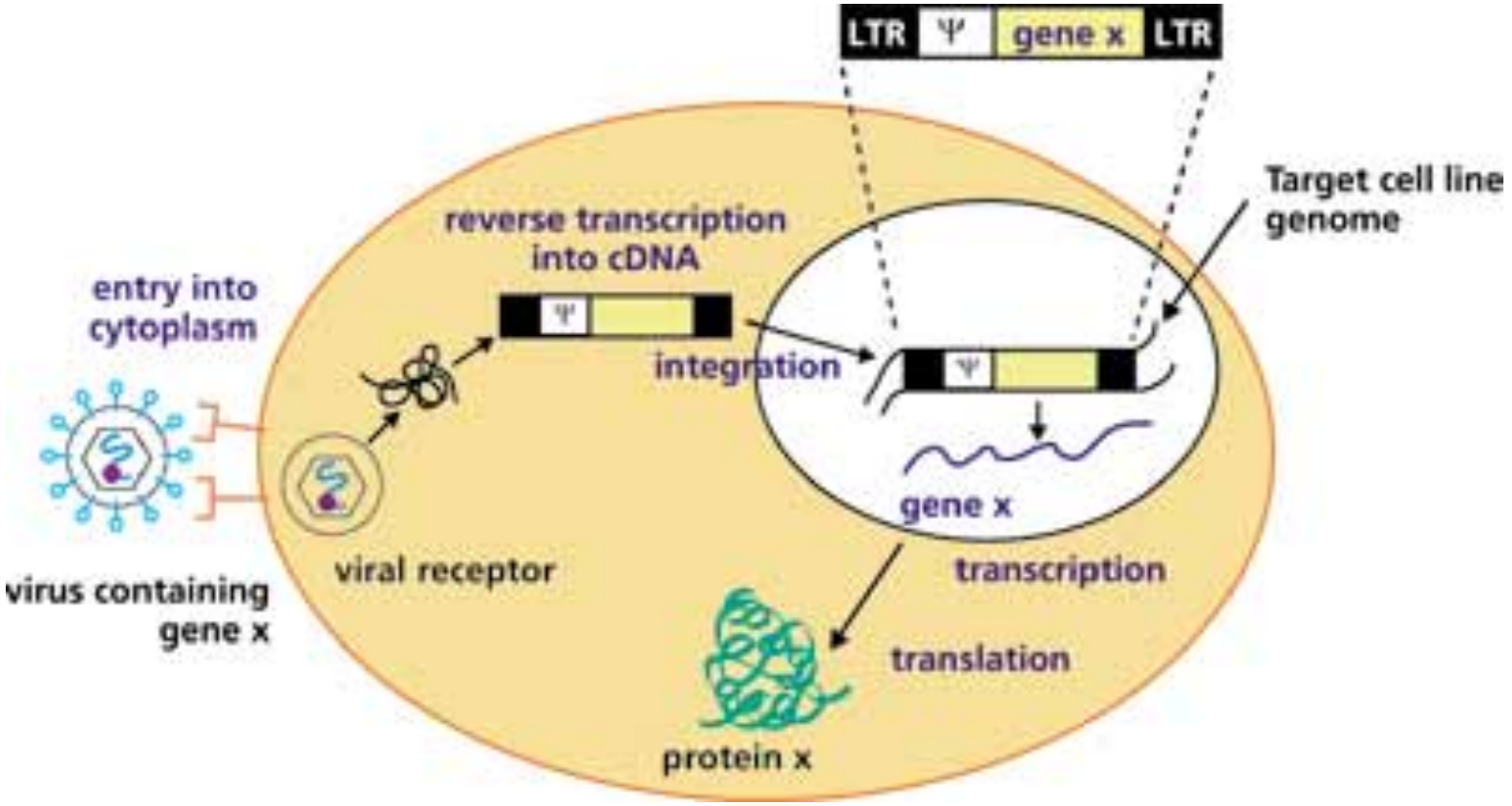


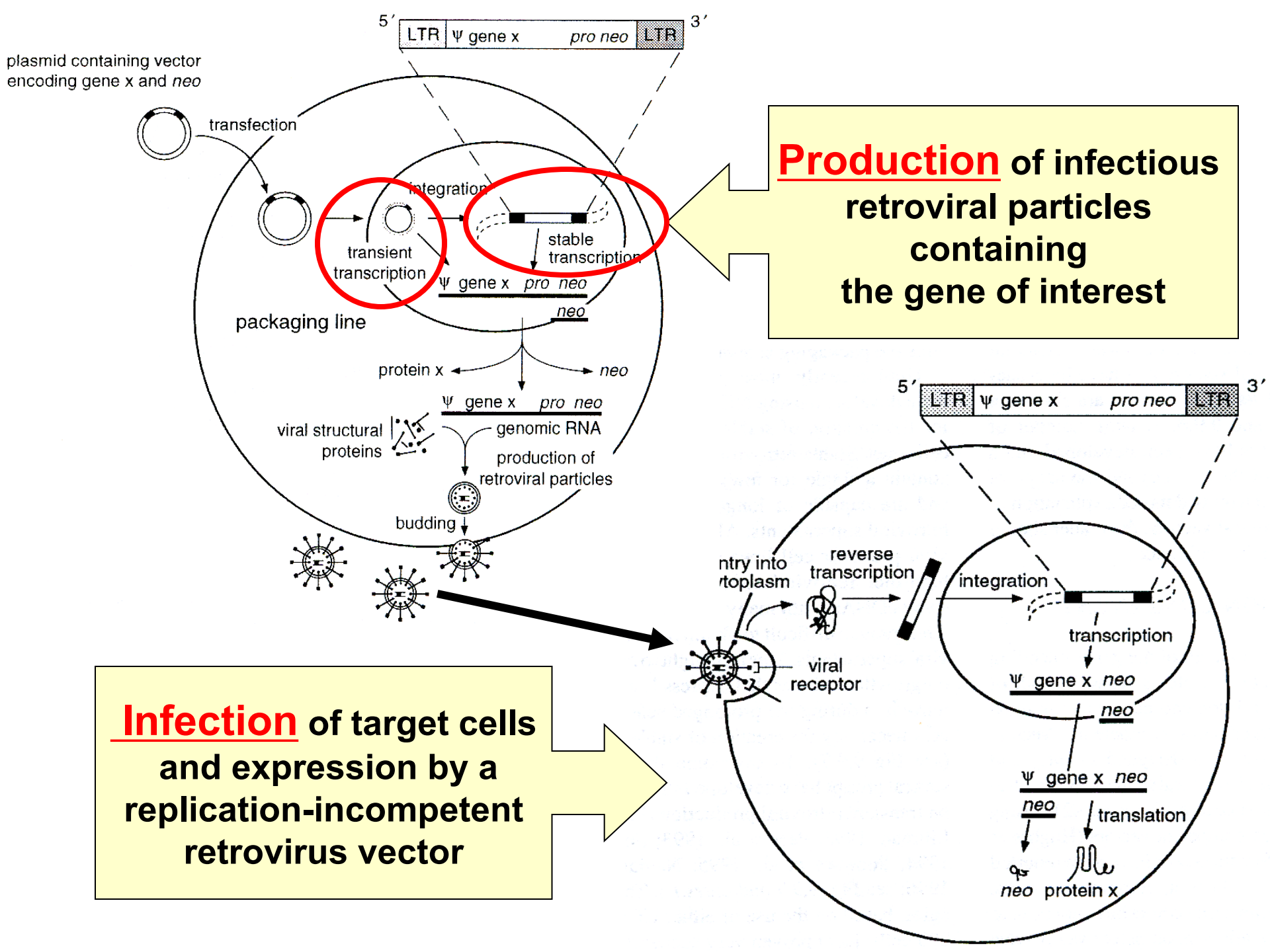
Retroviral vectors

Production of Recombinant Retrovirus in the Packaging Cell



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




plasmid containing vector encoding gene x and neo
5′
LTR
ψ gene x
pro neo
LTR
3′
transfection
integration
transient transcription
stable transcription
ψ gene x pro neo
neo
packaging line
protein x
neo
ψ gene x pro neo
viral structural proteins
genomic RNA
production of retroviral particles
budding
Production of infectious retroviral particles containing the gene of interest
5′
LTR
ψ gene x
pro neo
LTR
3′
ntry into toplasm
reverse transcription
integration
transcription
viral receptor
ψ gene x neo
neo
ψ gene x neo
neo
translation
neo
protein x
Infection of target cells and expression by a replication-incompetent retrovirus vector

Tropism of Retrovirus Vectors

Retrovirus tropism is determined at three levels:

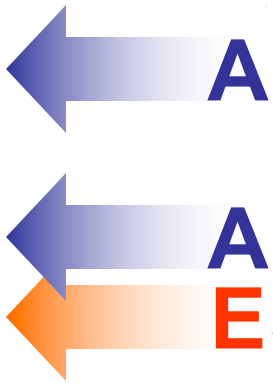
1) Viral envelope proteins (gpSU);

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)
	Galactosylceramide	Glycolipid	
Human immunodeficiency virus type 2	CD4	Ig-like	Chemokine receptors
	Cxcr4	7-transmembrane superfamily	
Simian immunodeficiency virus	CD4	Ig-like	Chemokine receptors
Gibbon ape leukemia virus	Glvr1	Sodium-dependent phosphate transport protein	
Feline leukemia virus B	Glvr1	Sodium-dependent phosphate transport protein	
Amphotropic murine leukemia virus	Ram-1	Sodium-dependent phosphate transport protein	
Ecotropic murine leukemia virus	Cat	Cationic amino acid transport protein	
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

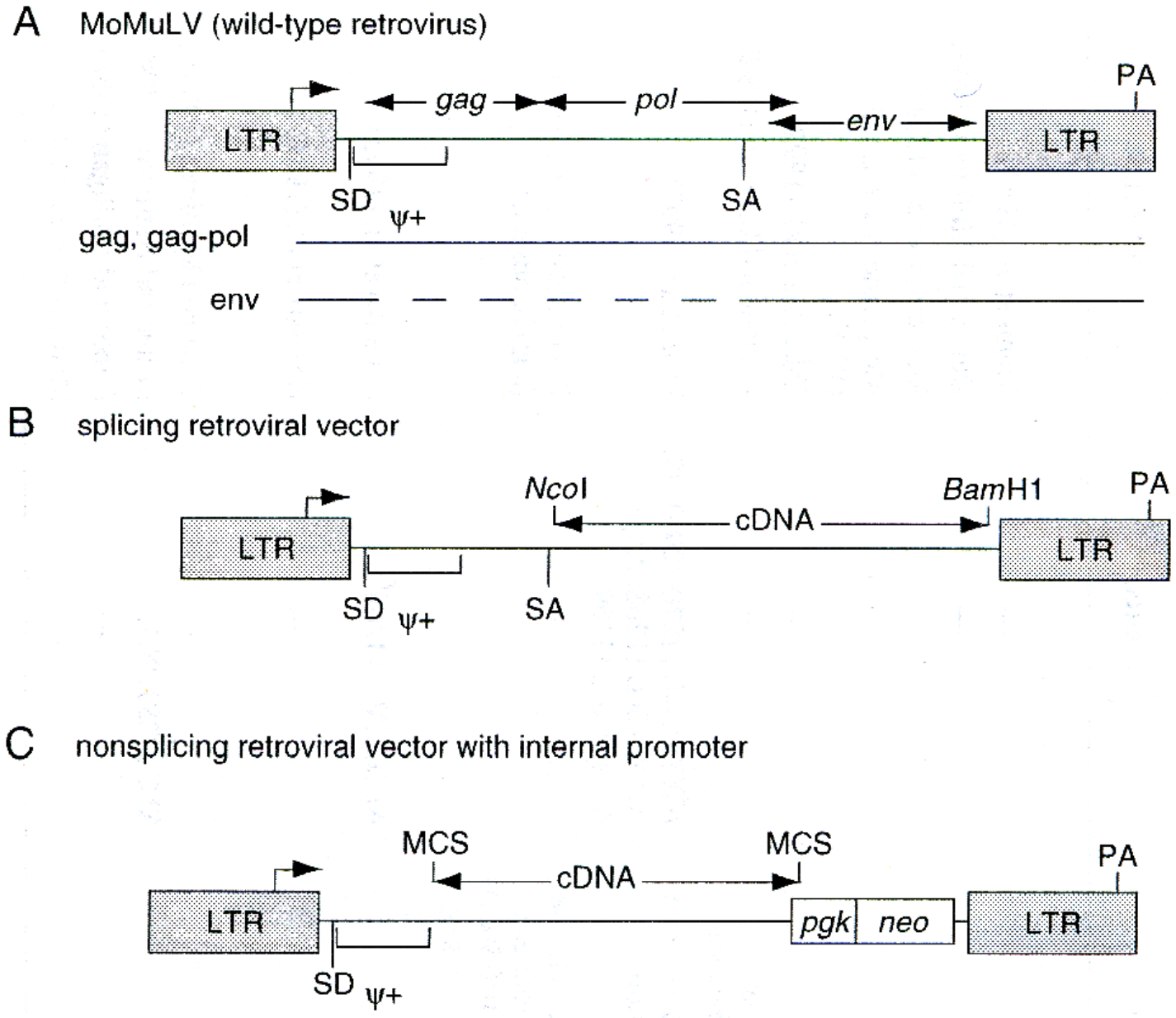
Host-range of MoMuLV-derived Vectors

- **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

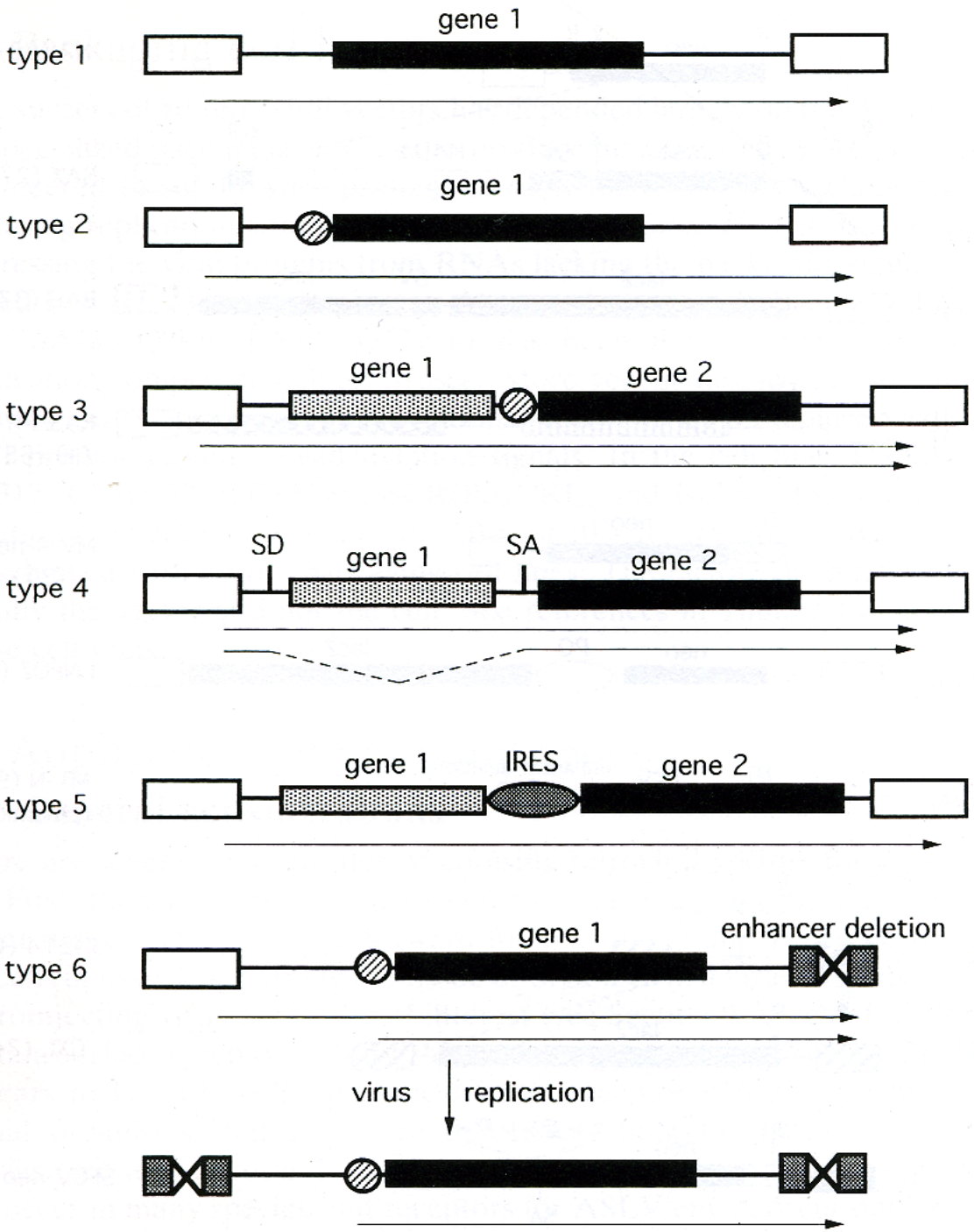
Host-range of Retroviral Vectors

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

Development of retroviral vector design



Replication-defective retroviral vectors

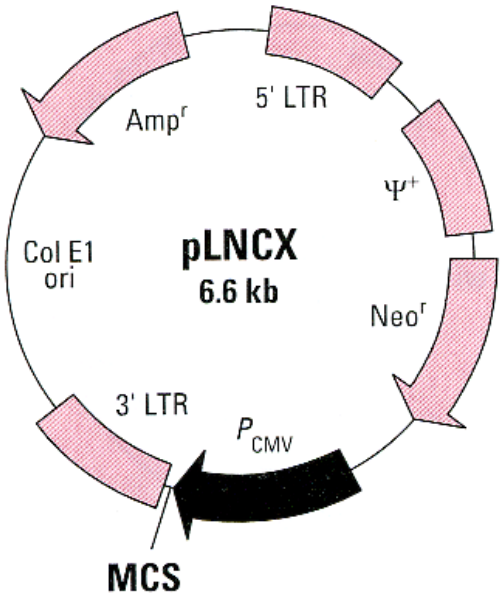


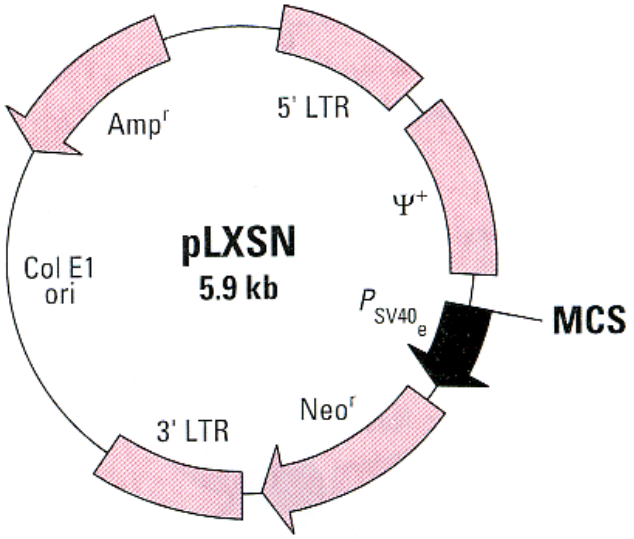
LTR

Internal promoter

IRES

Retroviral expression vectors





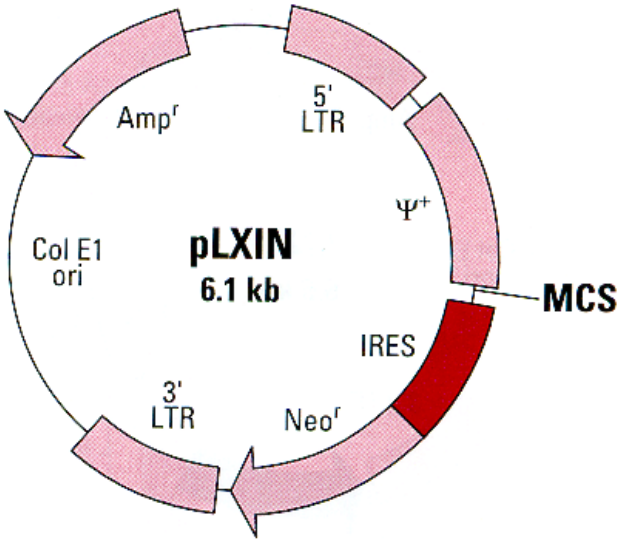
GCGGCCCCAAGCTTGTTAACATCGATAAAATA

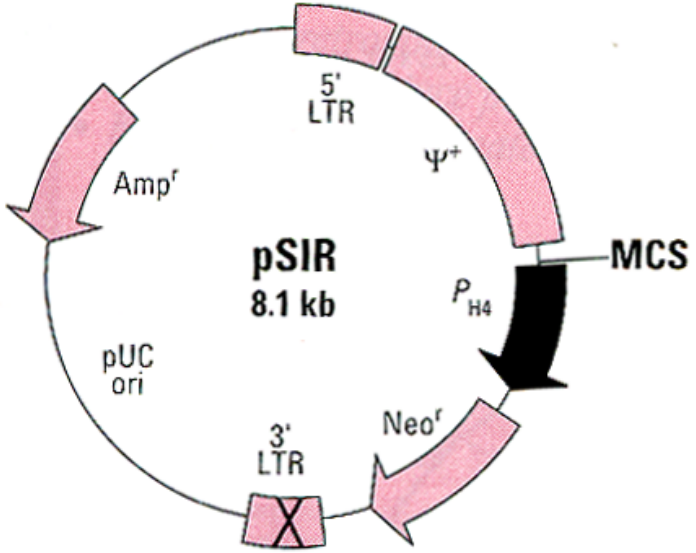
Hind III *Hpa* I *Cla* I

GCGCCGGAATTCGTTAACTCGAGGATCCGGCTGTG

*Eco*R I *Hpa* I *Xho* I *Bam*H I

Retroviral expression vectors



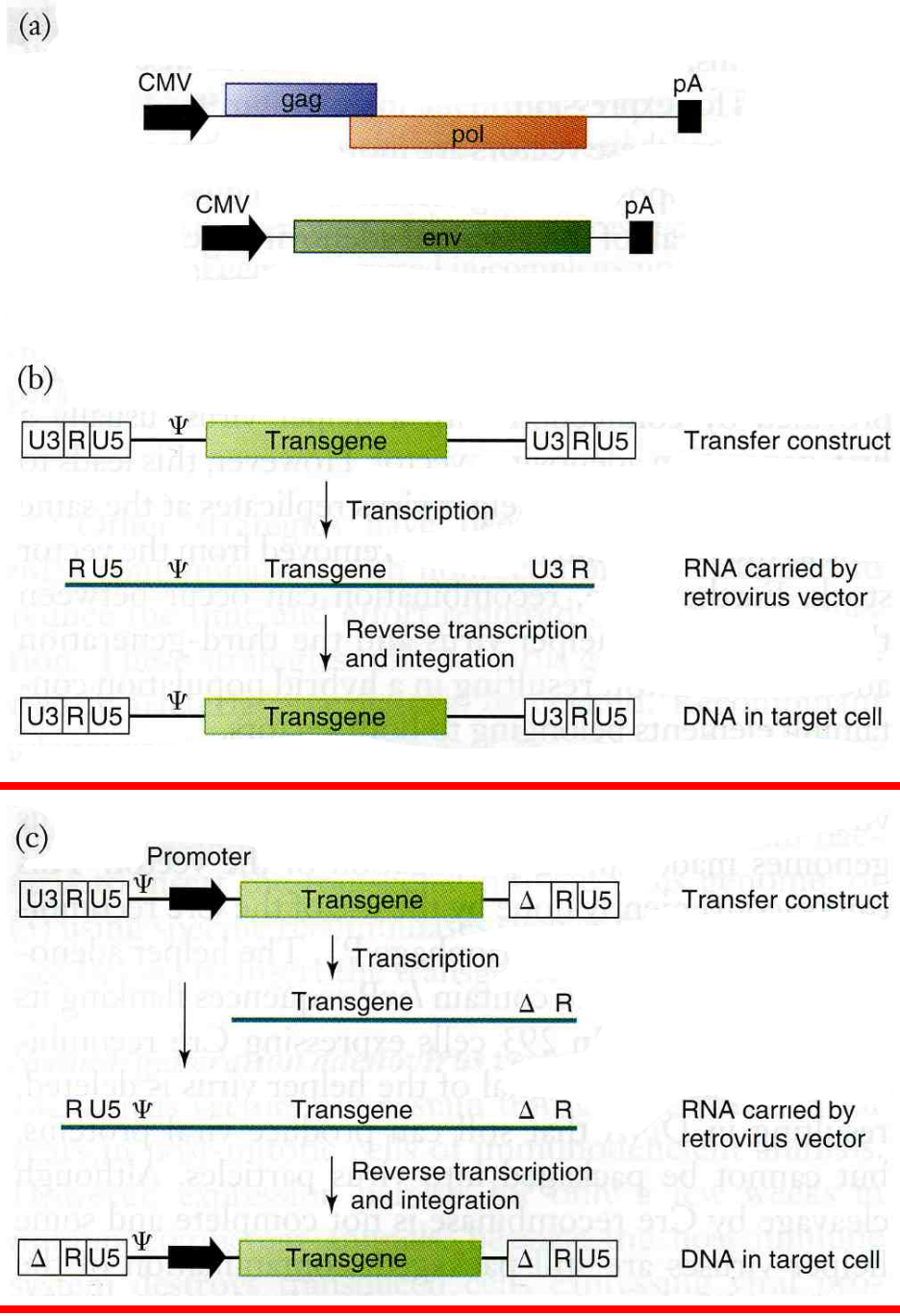


GGAATTCGTTAACTCGAGGATCCACTAGTAACGGCCGCCAGAATTCG

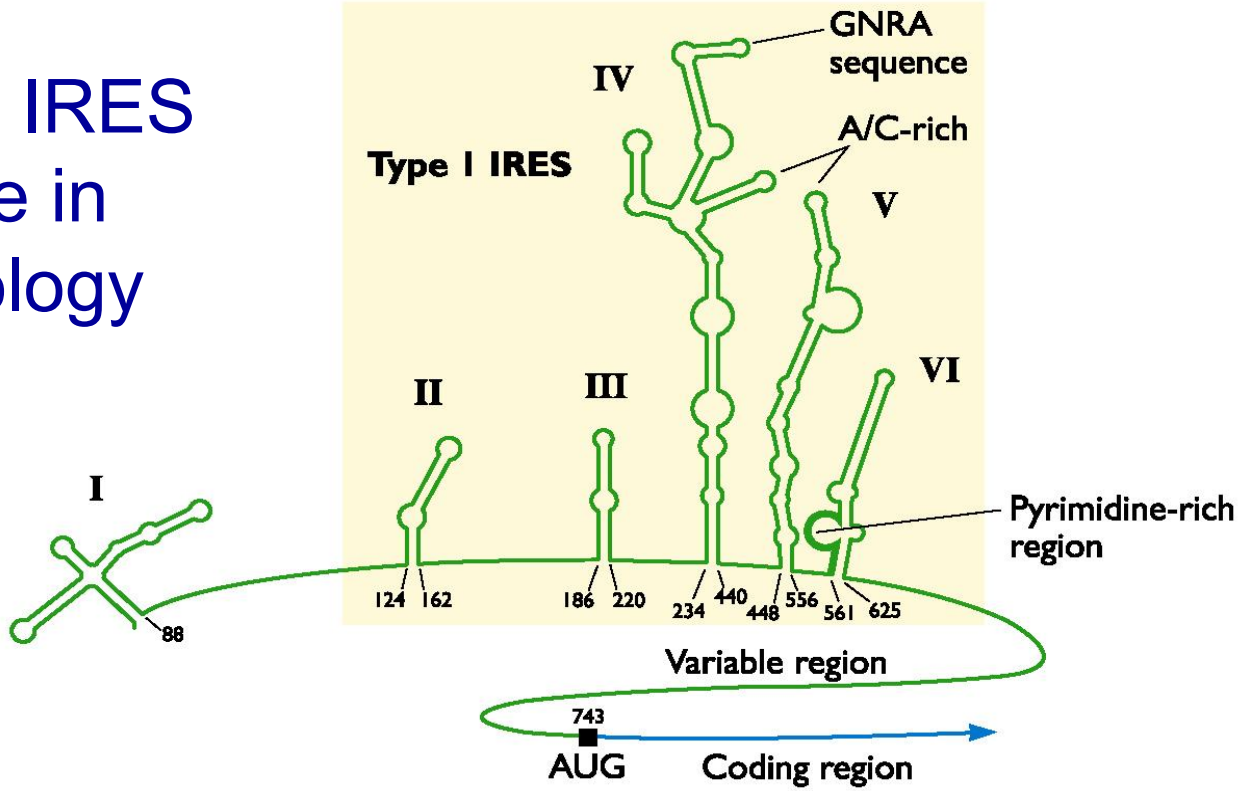
*Eco*R I *Hpa* I *Xho* I *Bam*H I *Eco*R I

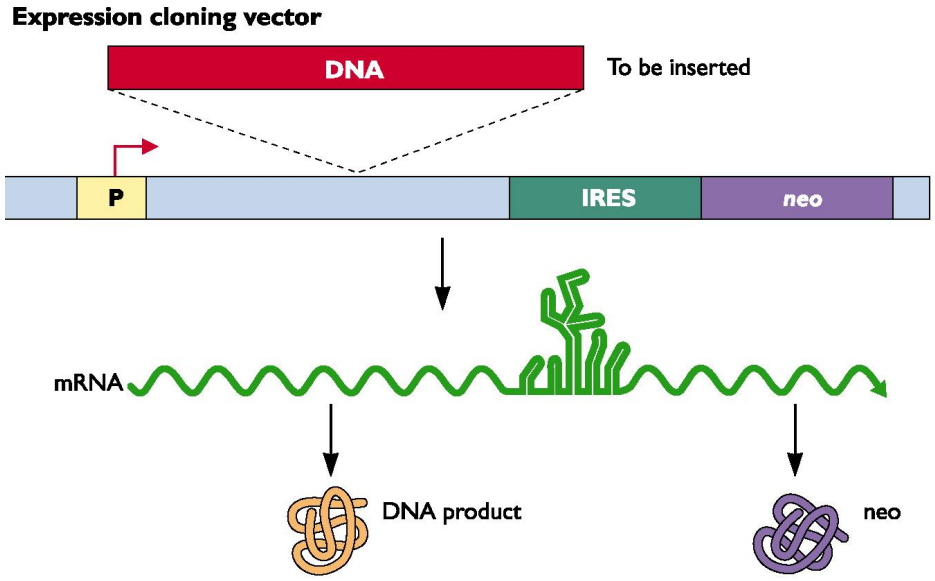
CCCCTCGAGAAGCTTGTCGACGGATCCGAATTC

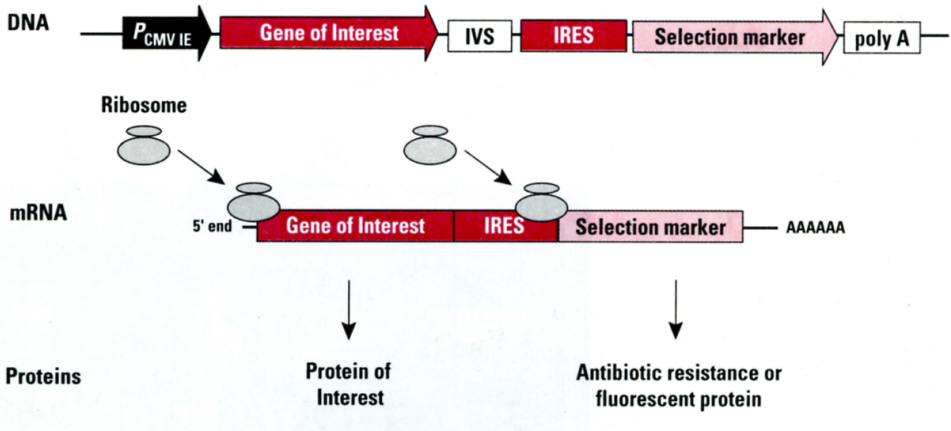
Xho I *Hind* III *Bam*H I *Eco*R I

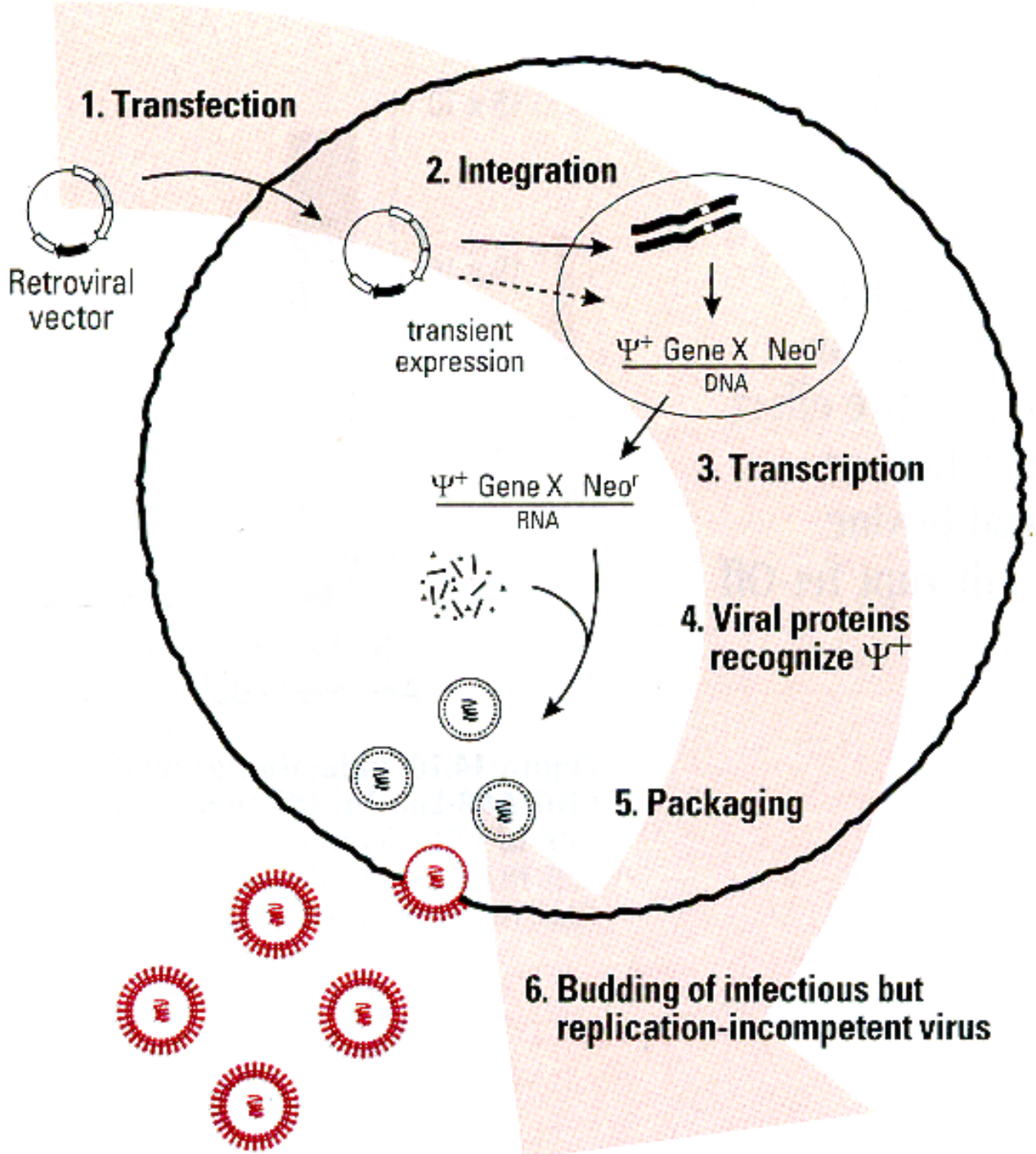

(a)
CMV
gag
pol
pA
CMV
env
pA
(b)
U3 R U5
Ψ
Transgene
U3 R U5
Transfer construct
Transcription
R U5
Ψ
Transgene
U3 R
RNA carried by retrovirus vector
Reverse transcription and integration
U3 R U5
Ψ
Transgene
U3 R U5
DNA in target cell
(c)
Promoter
U3 R U5
Ψ
Transgene
Δ R U5
Transfer construct
Transcription
Transgene
Δ R
R U5
Ψ
Transgene
Δ R
RNA carried by retrovirus vector
Reverse transcription and integration
Δ R U5
Ψ
Transgene
Δ R U5
DNA in target cell

Picornaviruses IRES and their use in molecular biology



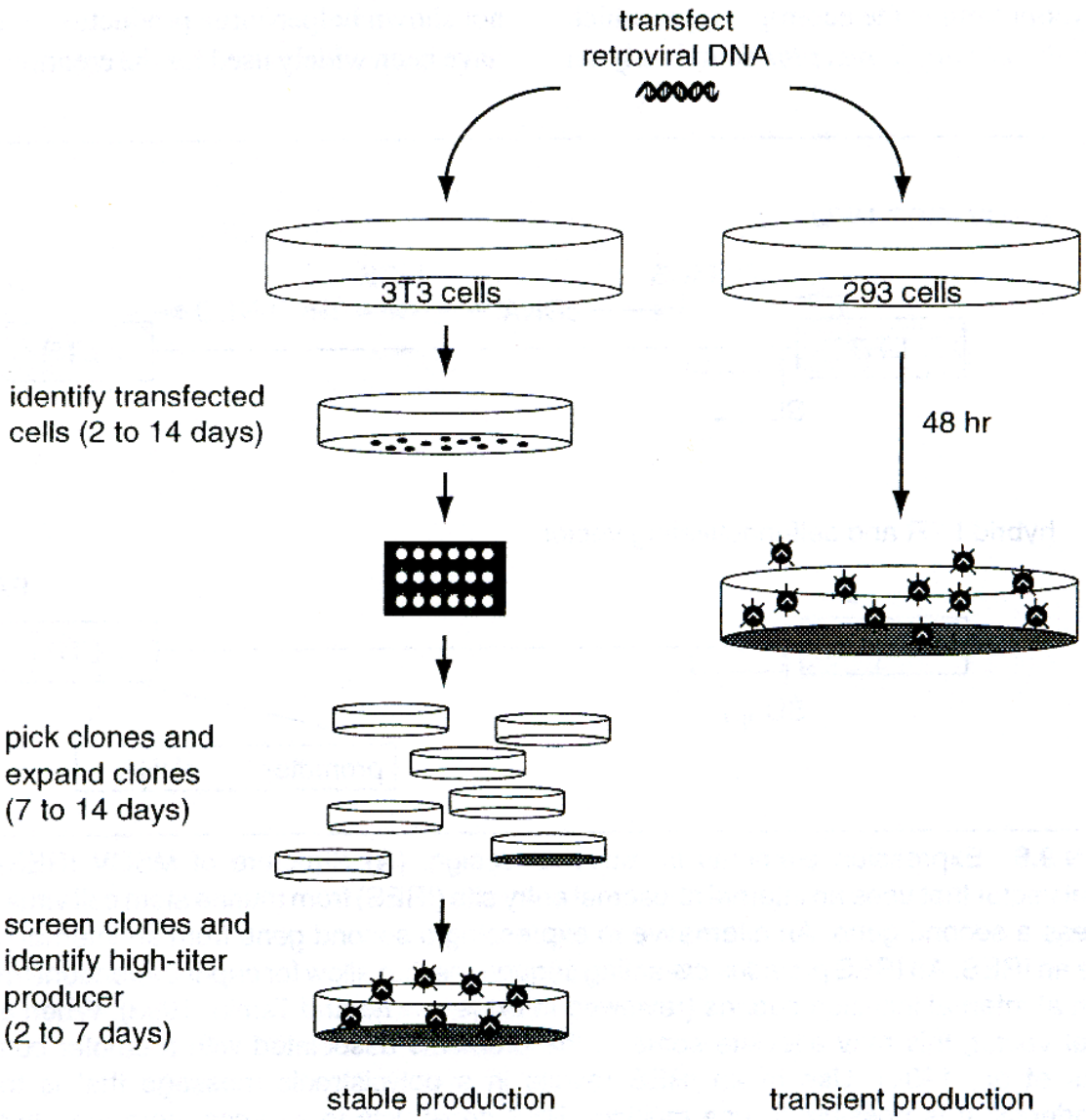


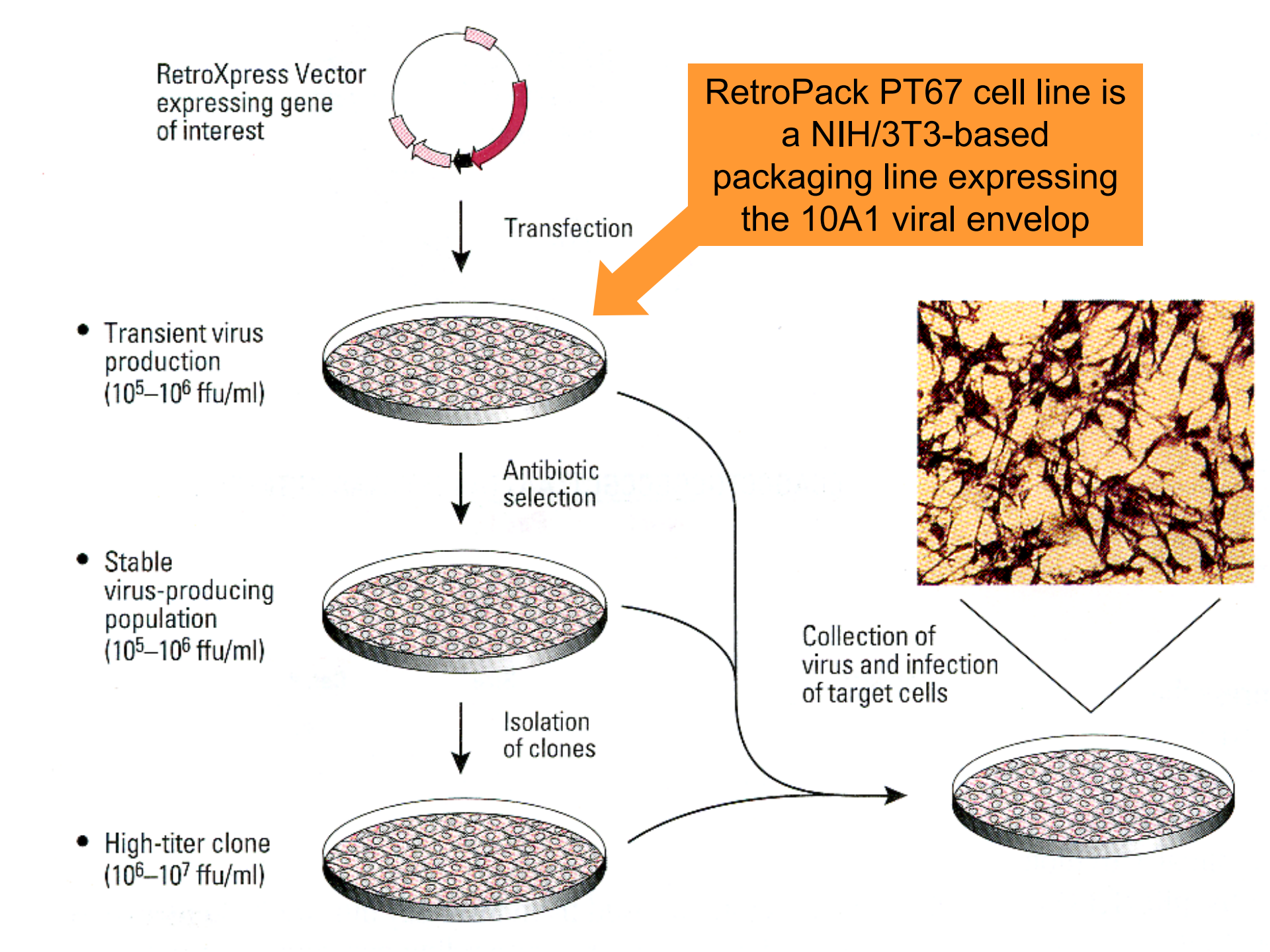




Packaging cell: produces viral proteins from stably integrated genes

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



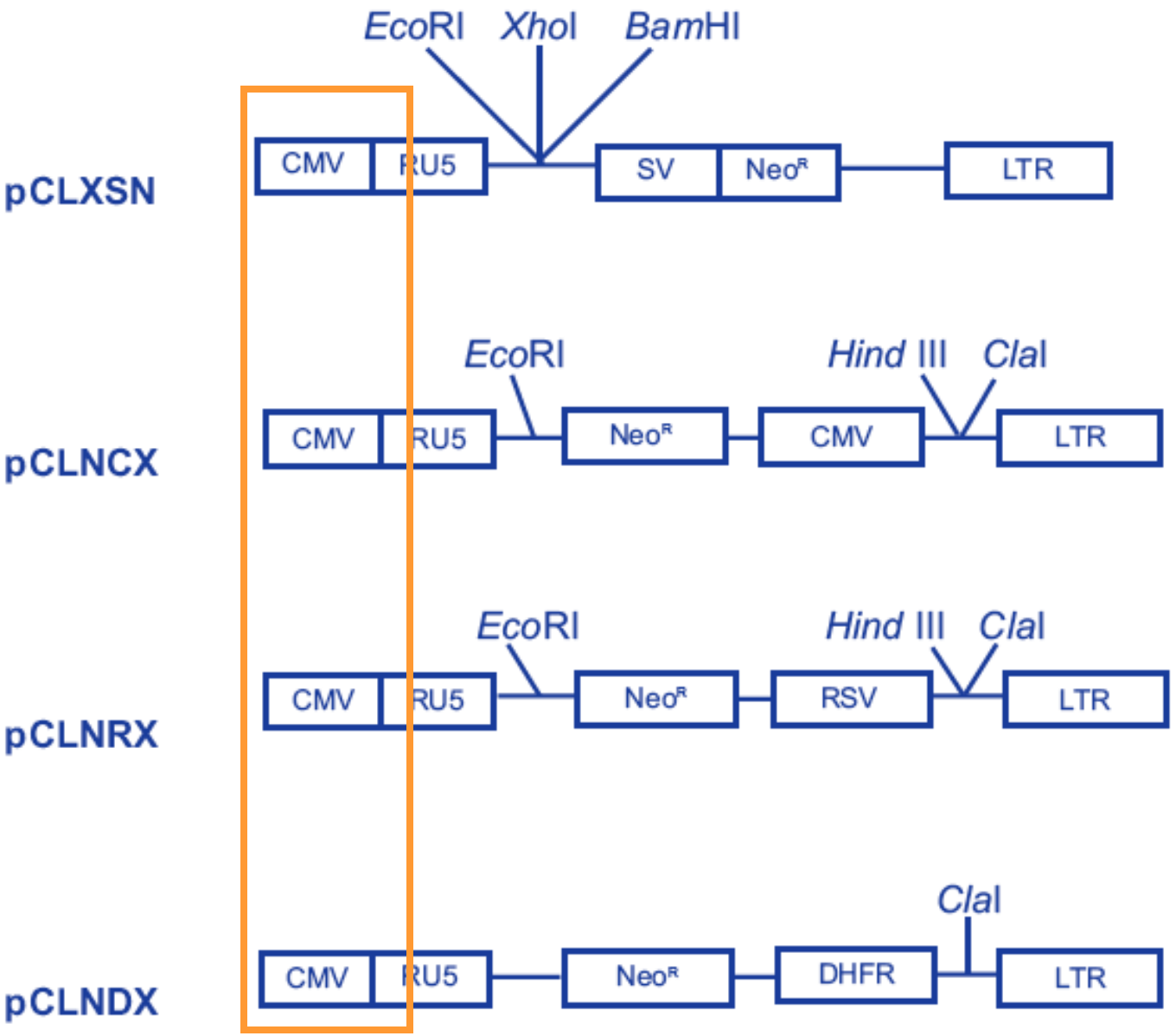

RetroXpress Vector expressing gene of interest
Transfection
RetroPack PT67 cell line is a NIH/3T3-based packaging line expressing the 10A1 viral envelop
• Transient virus production (10^5–10^6 ffu/ml)
Antibiotic selection
• Stable virus-producing population (10^5–10^6 ffu/ml)
Isolation of clones
• High-titer clone (10^6–10^7 ffu/ml)
Collection of virus and infection of target cells

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

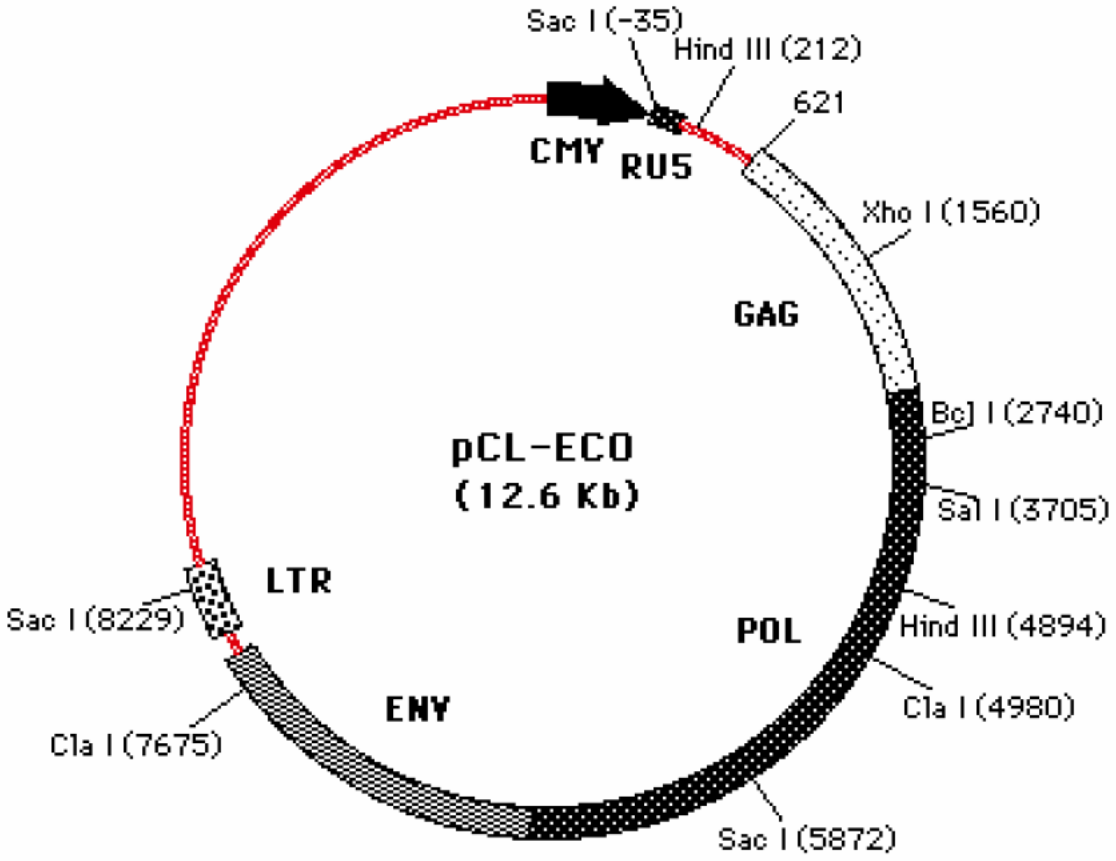
Retromax system: choice of vectors



Retromax system: choice of packaging vectors

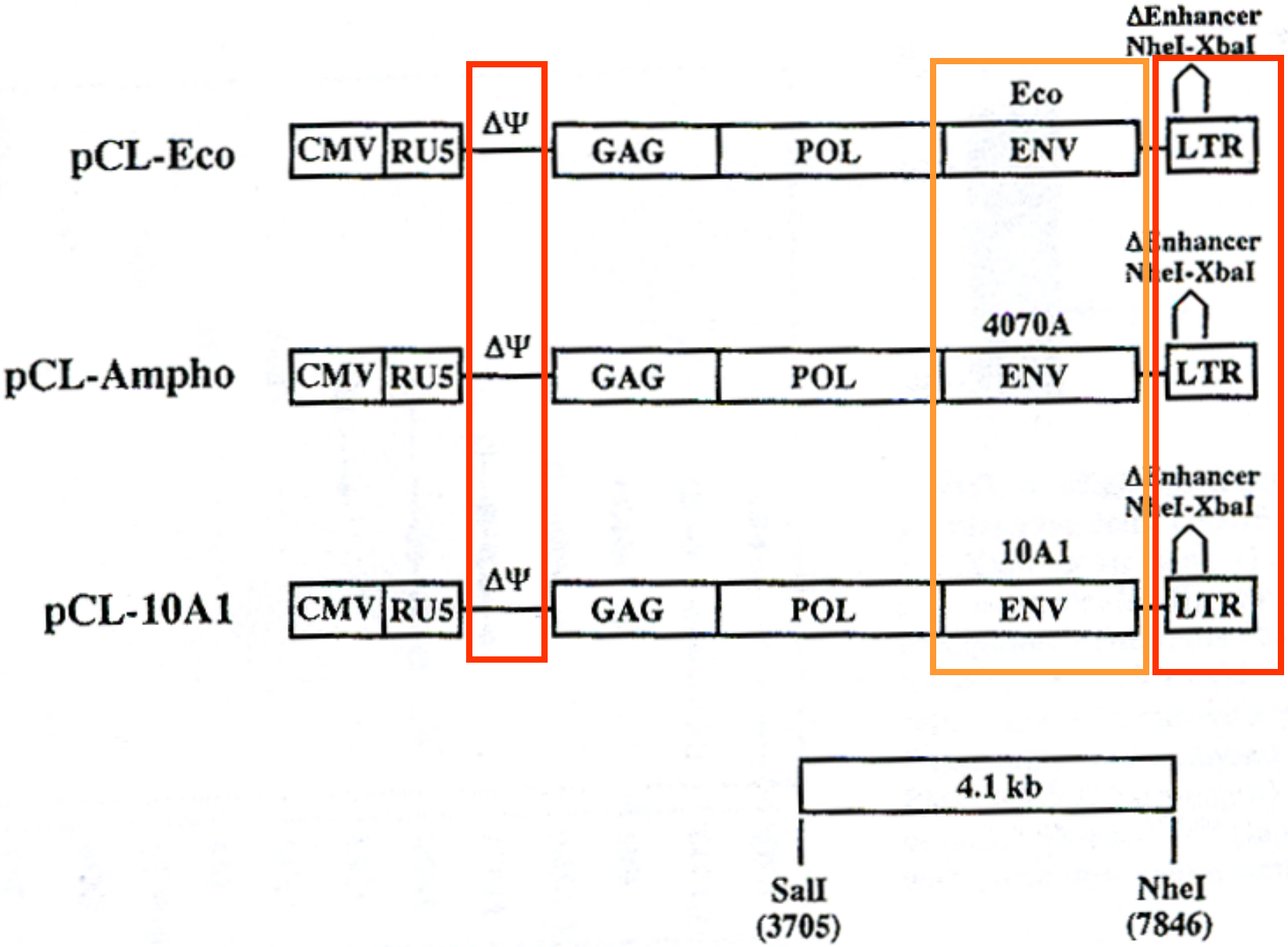
Ecotropic (usually (MoMuLV)	mouse and rat cells only (not human)
Amphotropic (from 4070 MuIV)	most mammalian cells (no hamster)
Gibbon Ape leukemia virus (GALV)	many mammalian cells (including hamster)
10A1 (MuLV)	most mammalian cells (including hamster)

Retromax system: choice of packaging vectors

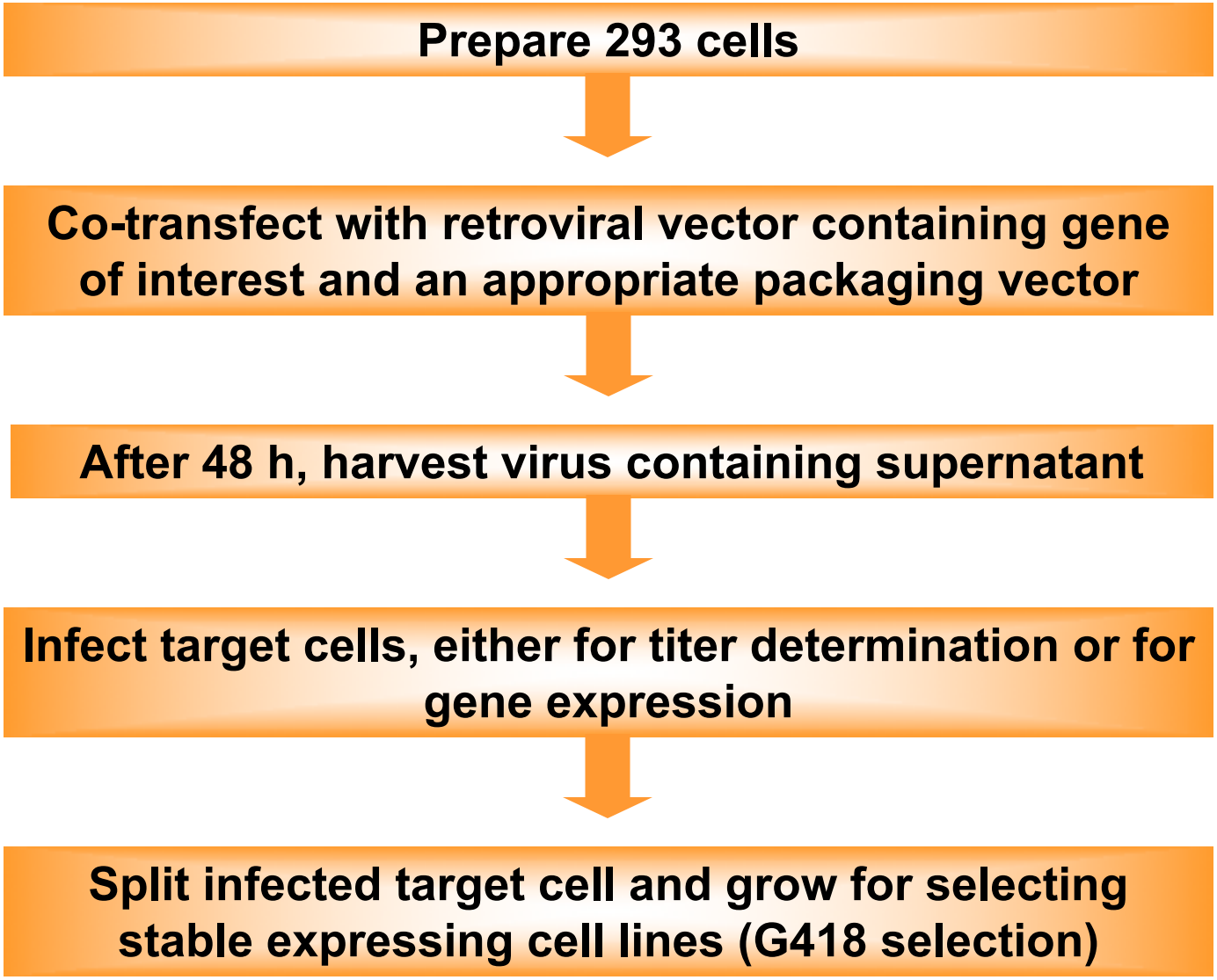


The **pCL-Eco** packaging vector. The gene coding for **env** was replaced with env gene from different MULV strains (4070A and 10A1) to create **pCL-Ampho** and **pCL-10A1** packaging vectors

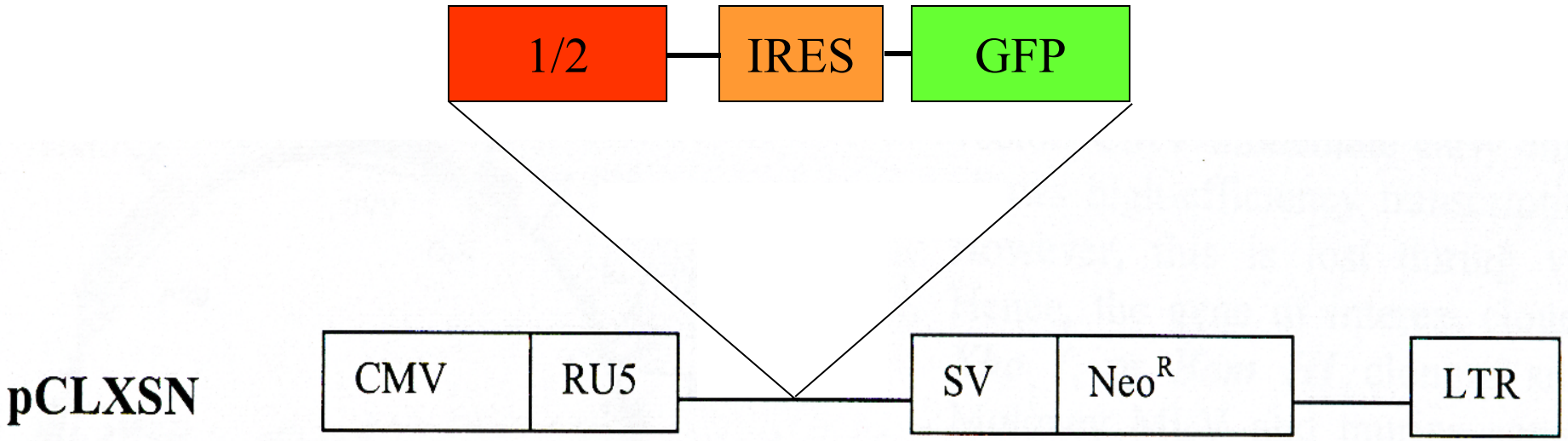
Retromax system: pCL packaging vectors



Retromax system: outline of the procedure

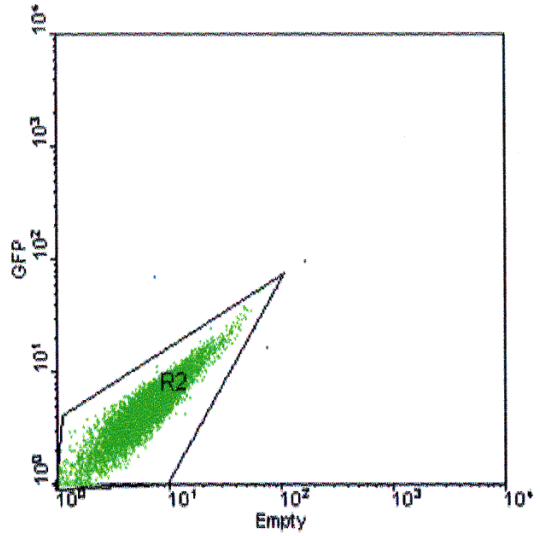


Retromax system: construction of pCLXSN-GFP vectors

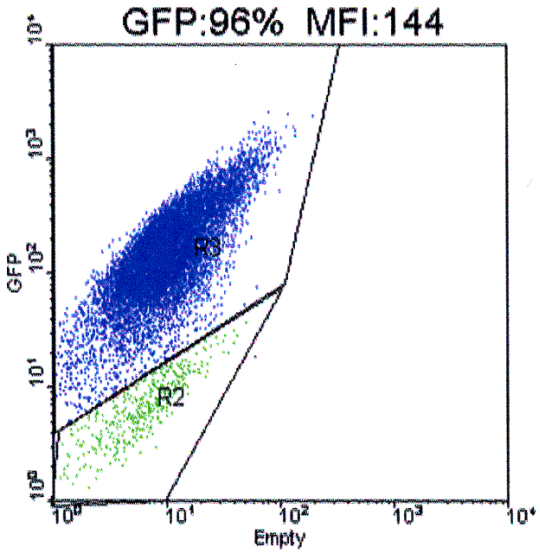


GFP exp in infected HUVEC after 1 wk of G418 selection

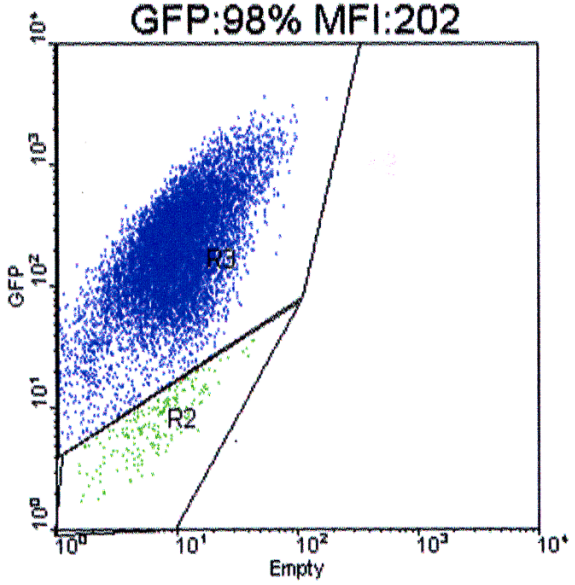
Mock



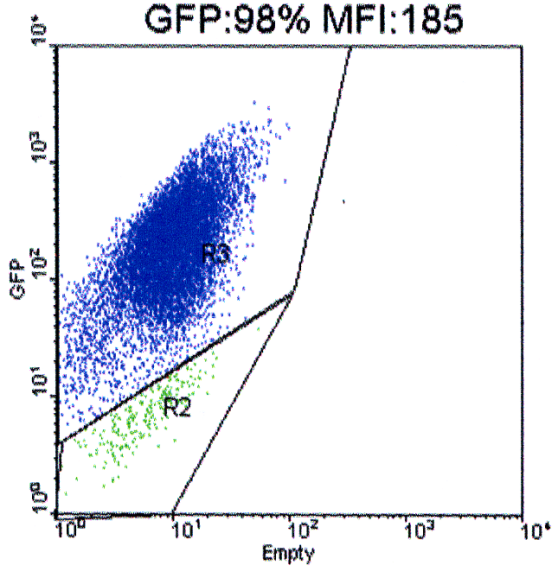
P1



P2



PE



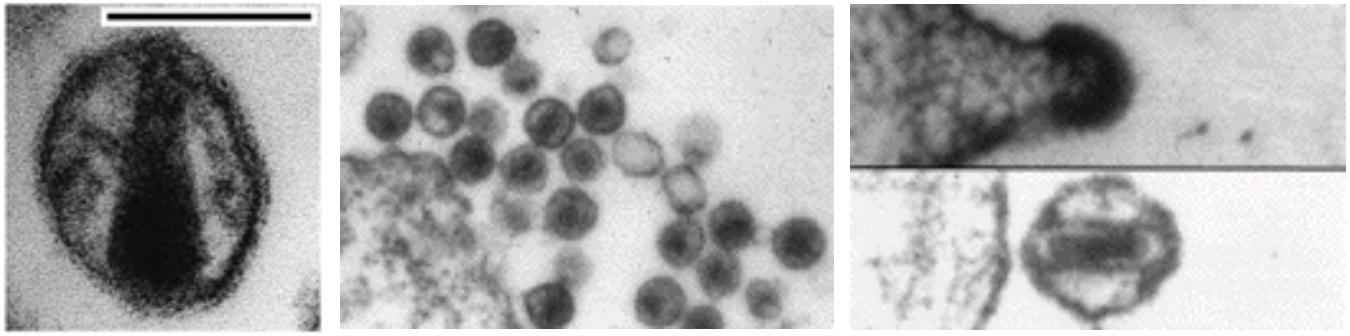
VIROLOGY

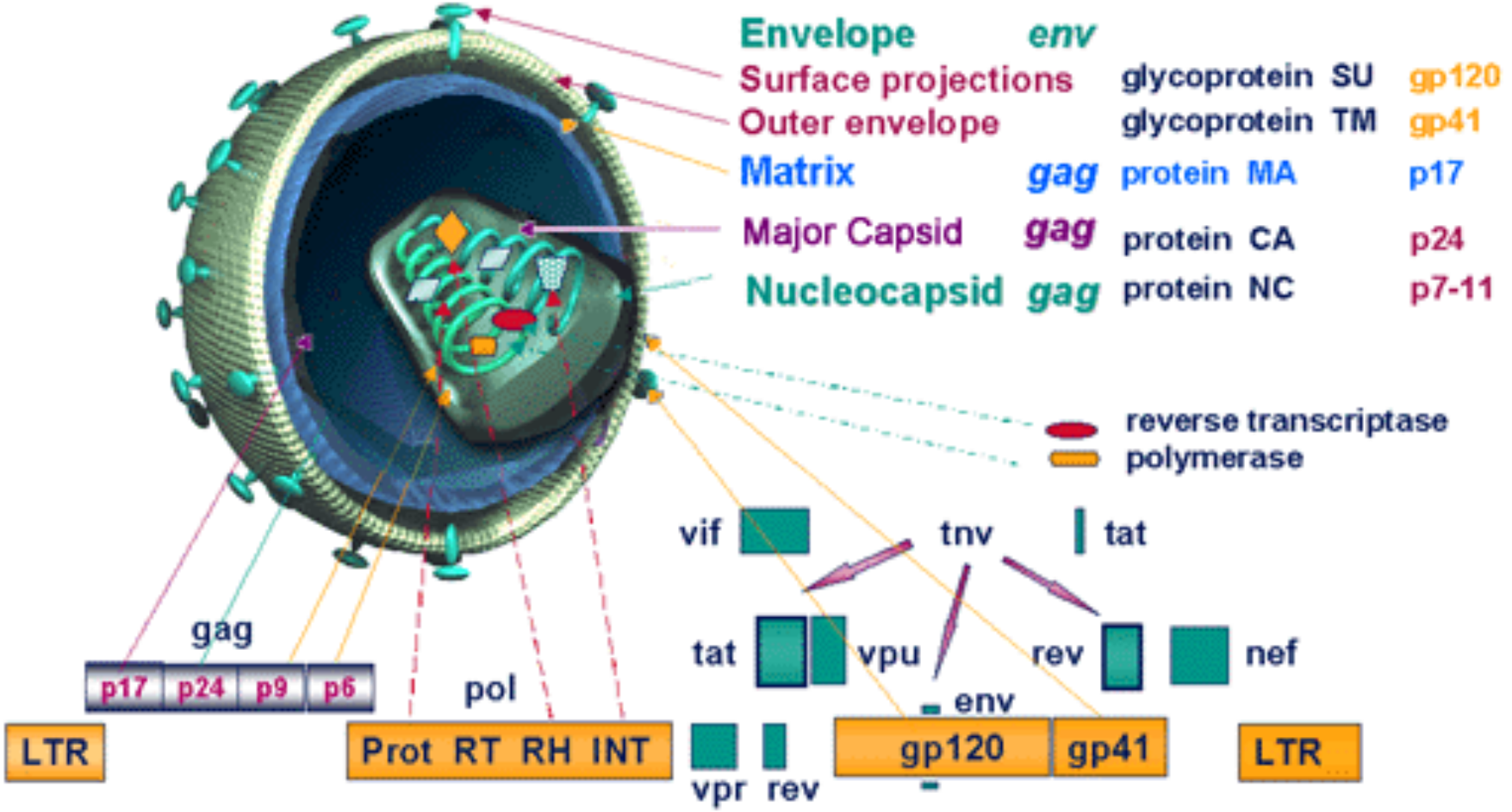
Engineering Viral Genomes: Lentivirus Vectors

Viral vectors

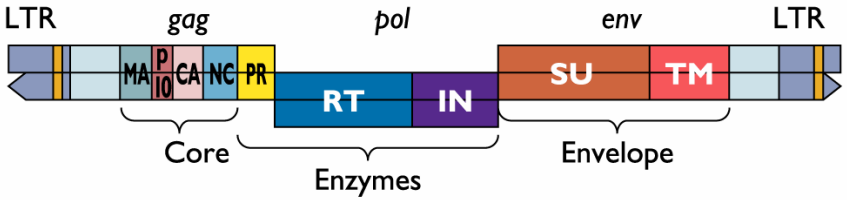
Virus	Insert size	Integration	Duration of expression	Advantages	Disadvantages
Adeno-associated virus	~4.5–9 (?) kb	Low efficiency	Long	Nonpathogenic, episomal, infects nondividing cells	Immunogenic, toxicity
Adenovirus	2–38 kb	No	Short	Efficient gene delivery	Transient, immunogenic
Alphavirus	~5 kb	No	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

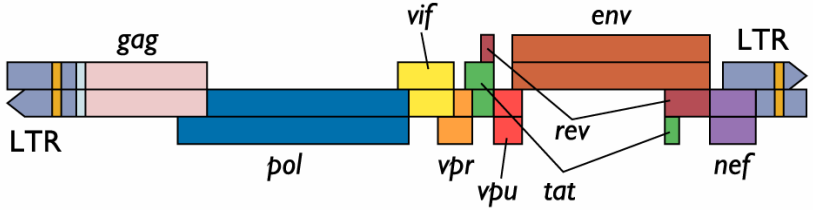



Envelope env
Surface projections glycoprotein SU gp120
Outer envelope glycoprotein TM gp41
Matrix gag protein MA p17
Major Capsid gag protein CA p24
Nucleocapsid gag protein NC p7-11
reverse transcriptase
polymerase
vif
tnv
tat
gag
p17 p24 p9 p6
tat
vpu
rev
nef
pol
env
LTR
Prot RT RH INT
gp120
gp41
LTR
vpr rev

B Simple retrovirus (ALV)

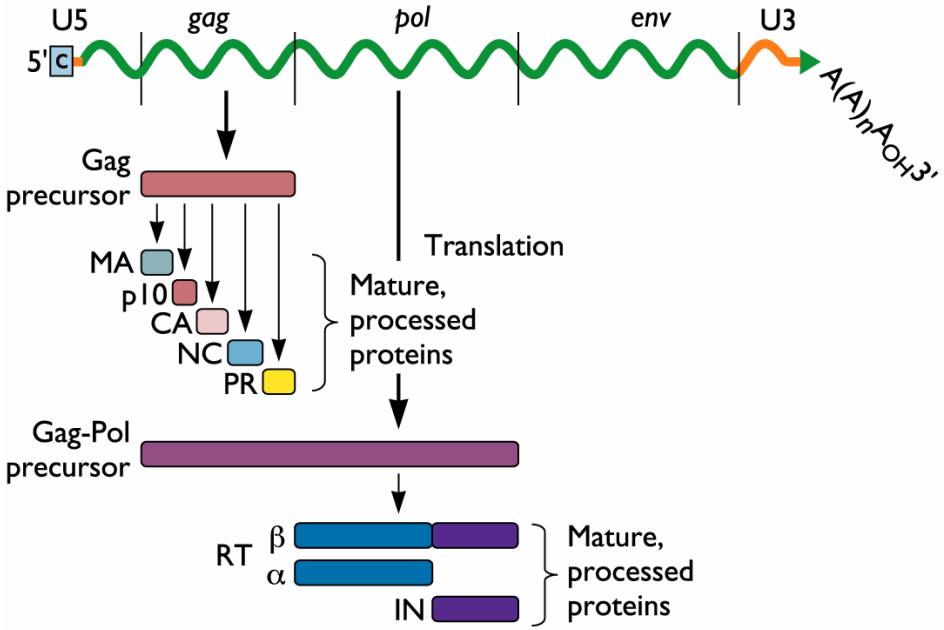


Complex retrovirus (HIV-1)

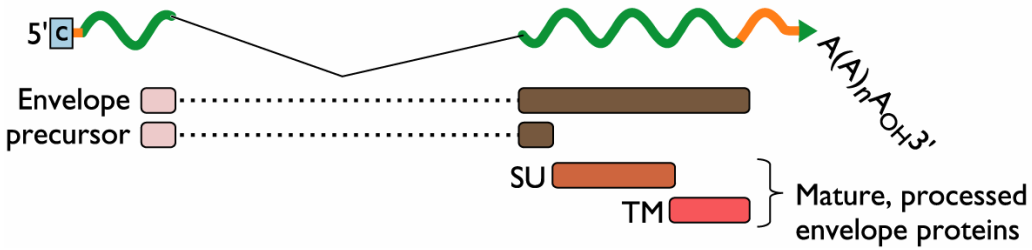


Genome expression

Genomic RNA, Gag-Pol mRNA, pre-mRNA



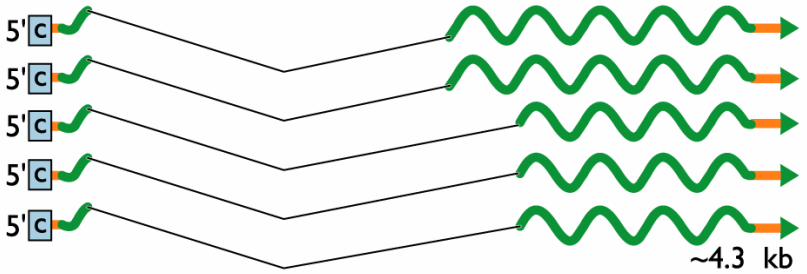
Singly spliced Env mRNA



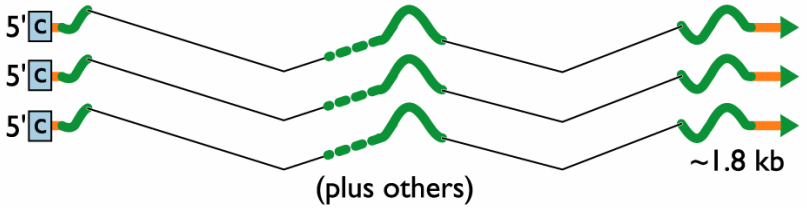
Genome expression

Genomic RNA, Gag-Pol mRNA, pre-mRNA

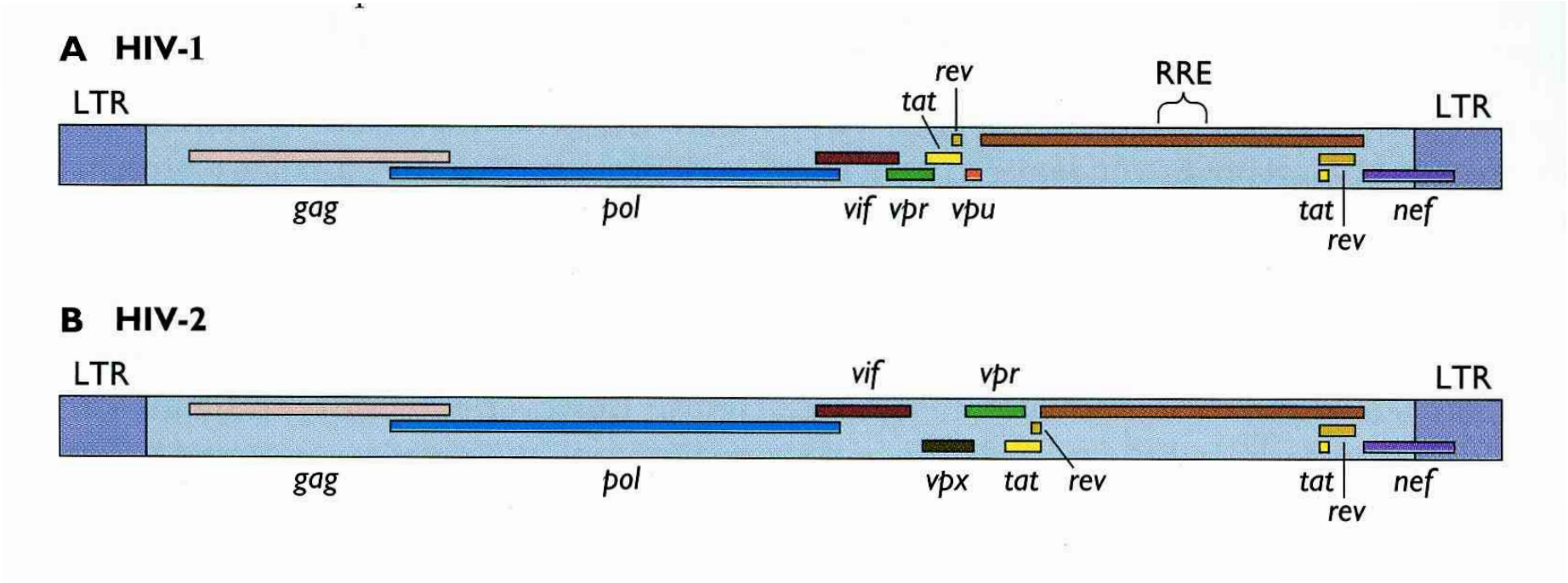
Singly spliced mRNAs: Vif, Vpr, Vpu, Env



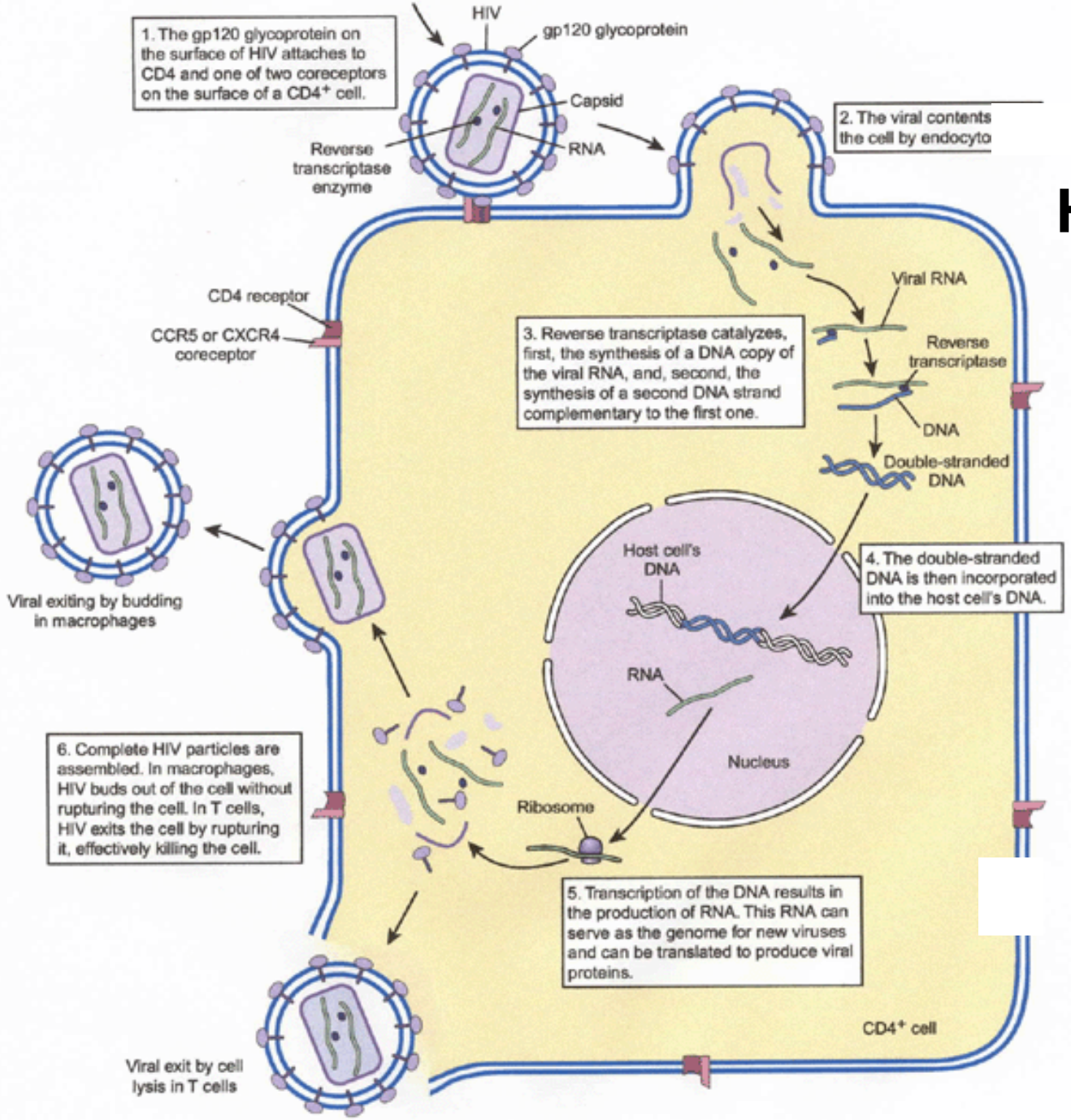
Multiply spliced mRNAs: Tat, Rev, Nef



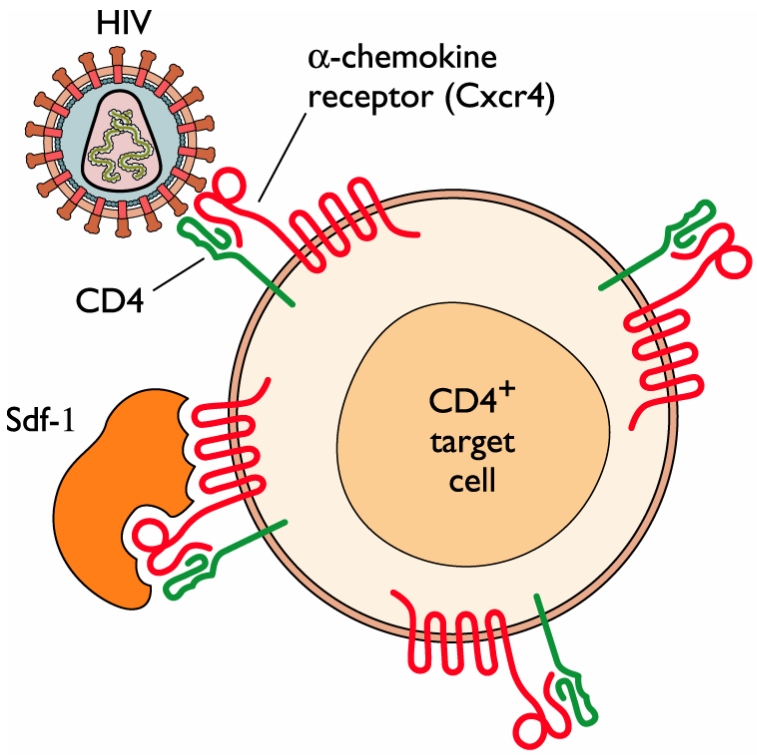
Genome organization of HIV-1 and HIV-2



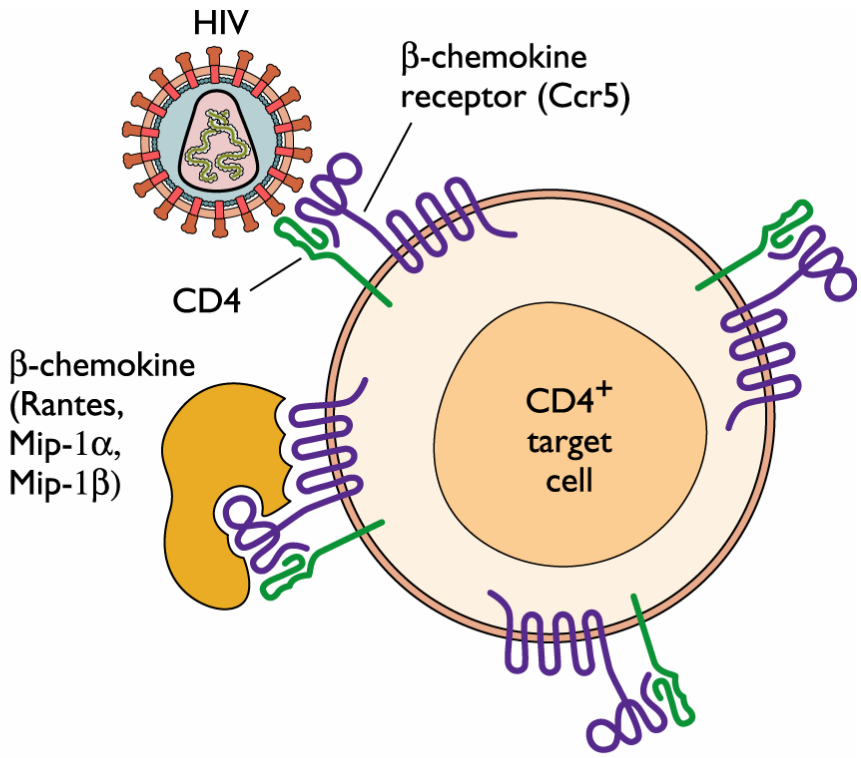
HIV lyfe cycle



HIV receptor and coreceptors

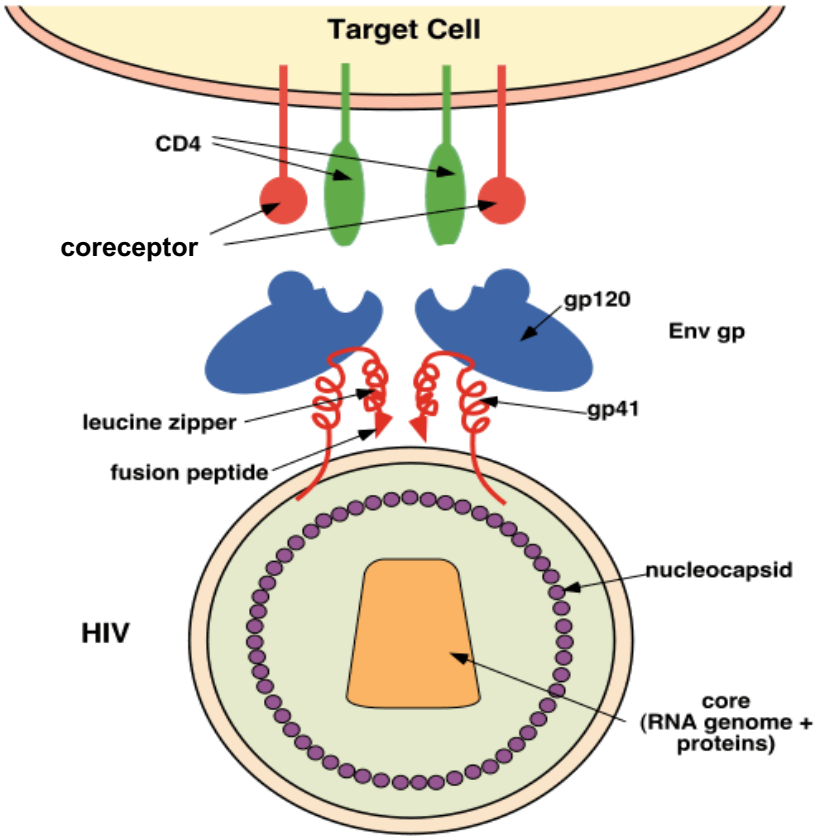


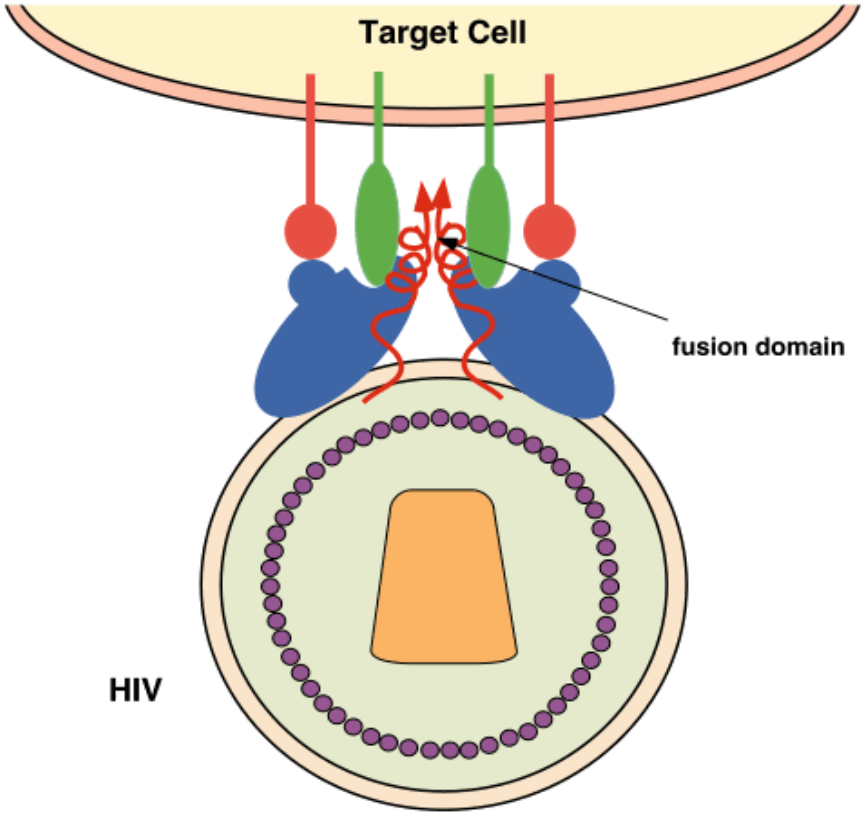
T-cell-line-tropic strain of HIV-1



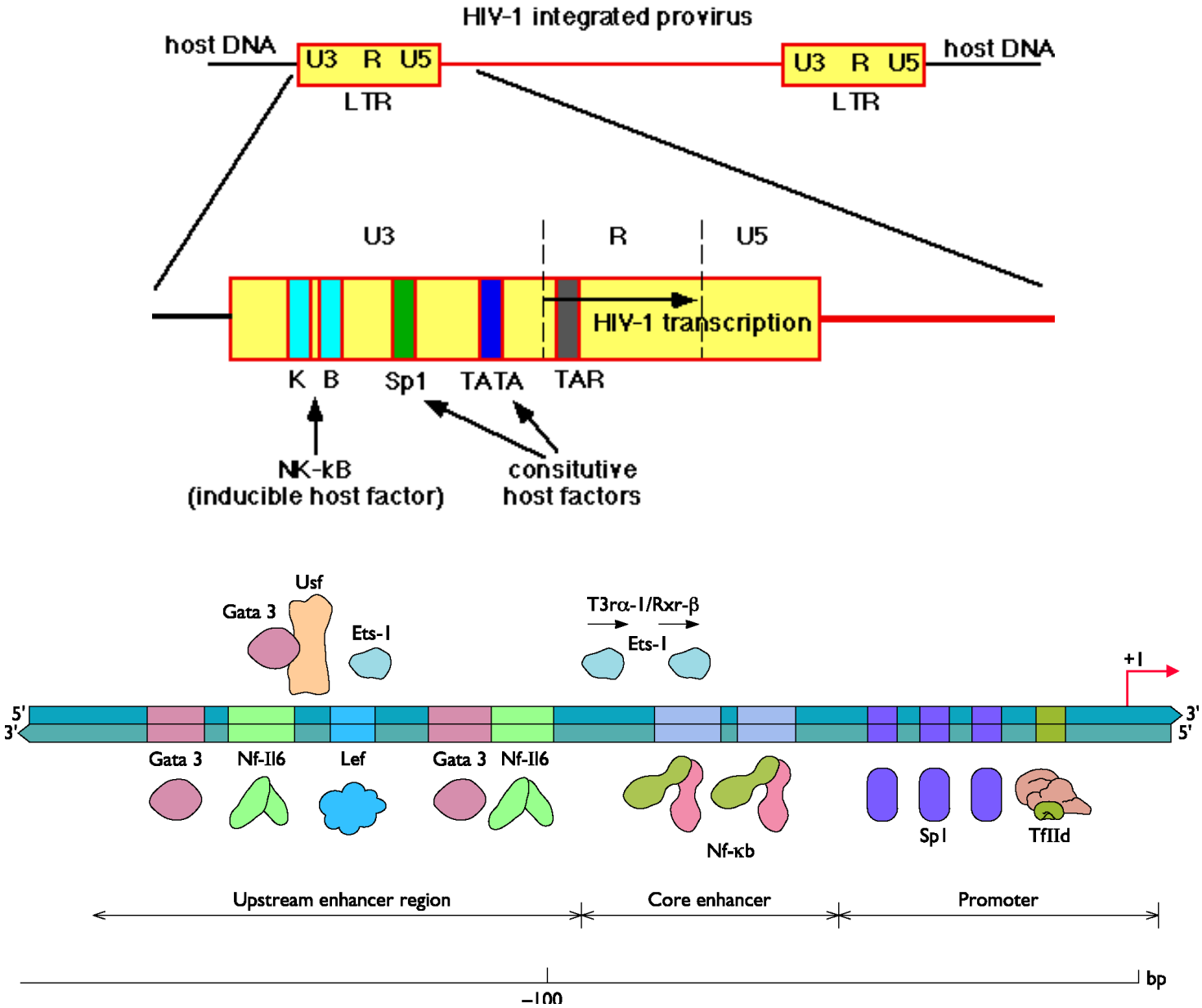
Macrophage-tropic strain of HIV-1

HIV attachment and entry into cells

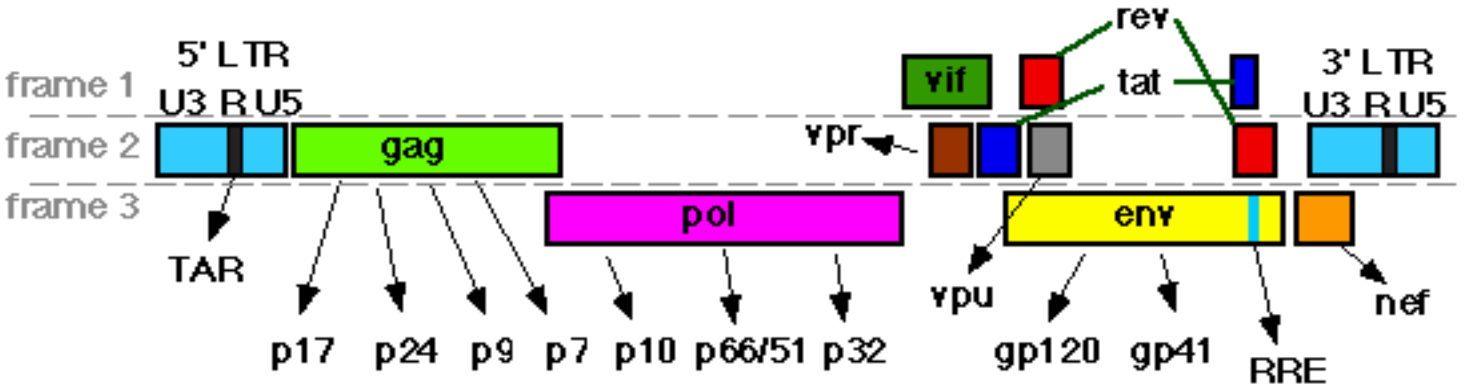




HIV-1 LTR


HIV-1 integrated provirus
host DNA
U3 R U5
LTR
U3 R U5
LTR
host DNA
U3
R
U5
HIV-1 transcription
K B
Sp1
TATA
TAR
NK-kB
(inducible host factor)
consitutive
host factors
Usf
Gata 3
Ets-1
T3rα-1/Rxr-β
Ets-1
+1
5'
3'
3'
5'
Gata 3
Nf-Il6
Lef
Gata 3
Nf-Il6
Nf-κb
Sp1
TfIId
Upstream enhancer region
Core enhancer
Promoter
bp
-100

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 (transmebrane)

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

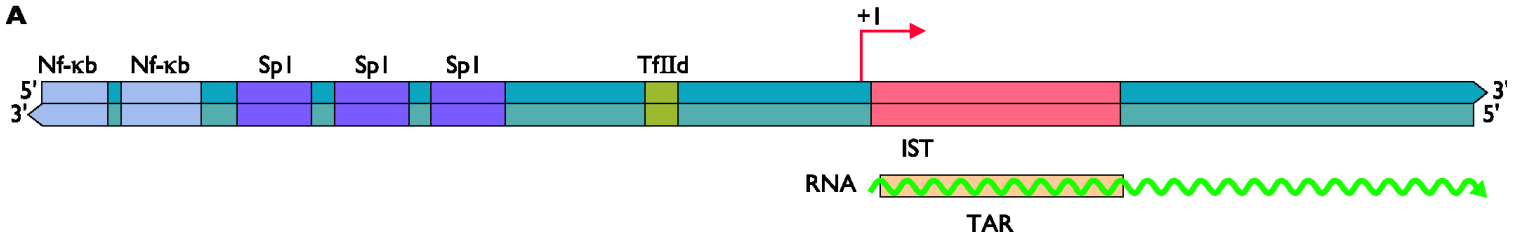
HIV auxiliary proteins

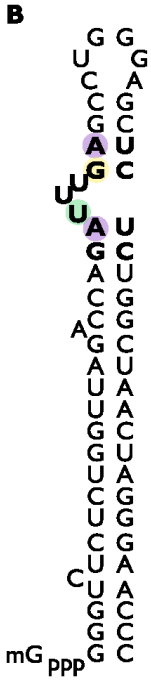
Protein[b]	Size (kDa)	Function	Location
Regulatory			
Tat	14	Transactivation; binds TAR to facilitate initiation and elongation of viral transcription	Primarily in cell nucleus
Rev	19	Regulation of viral mRNA expression; binds RRE and facilitates nuclear export of unspliced or singly spliced RNAs	Primarily 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

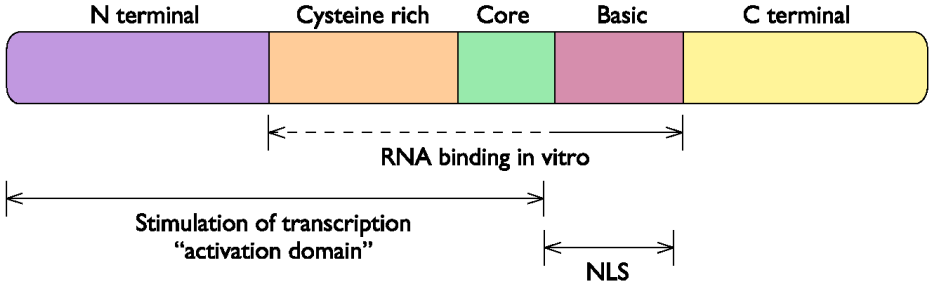
HIV Tat: features and functions

- 14 kDa protein encodes by a multispliced 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 lenght mRNA)
- Stimulates production of viral RNA as much as 100 fold

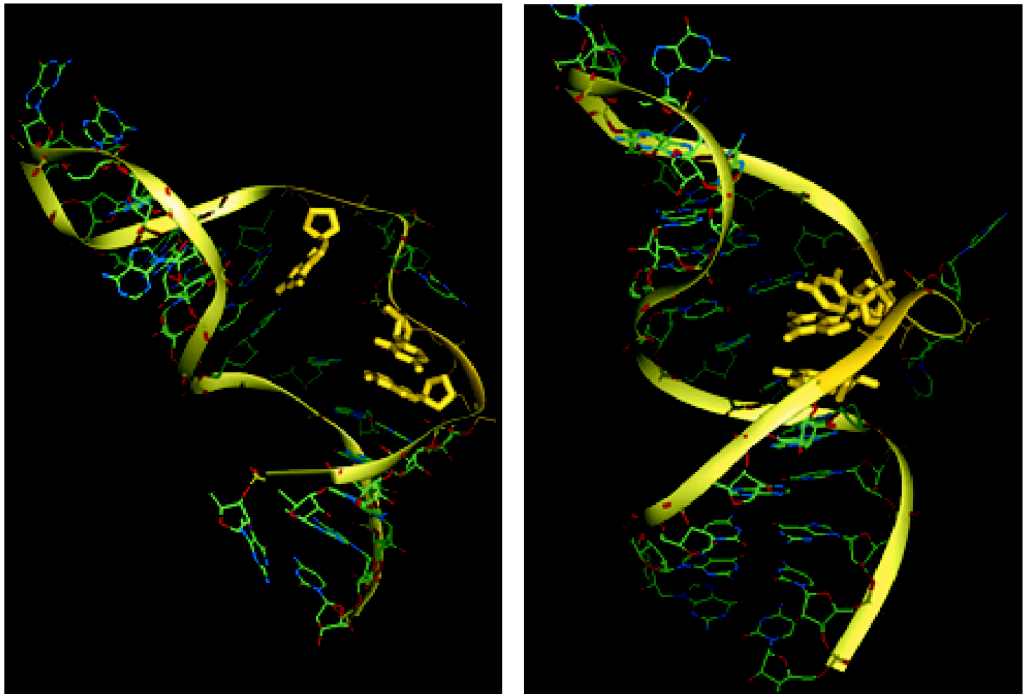
HIV TRASCRIPTION - Tat and TAR



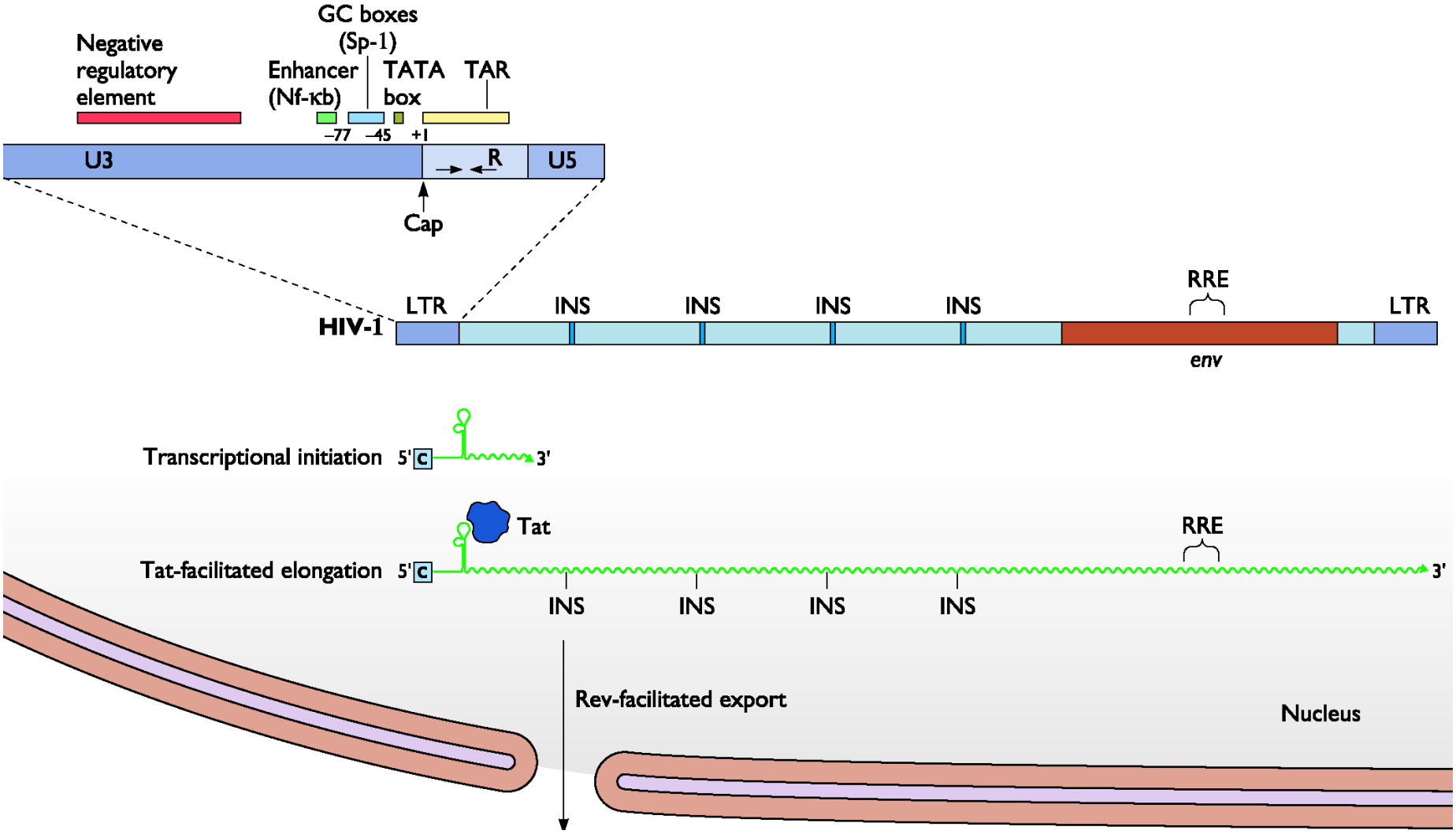




C

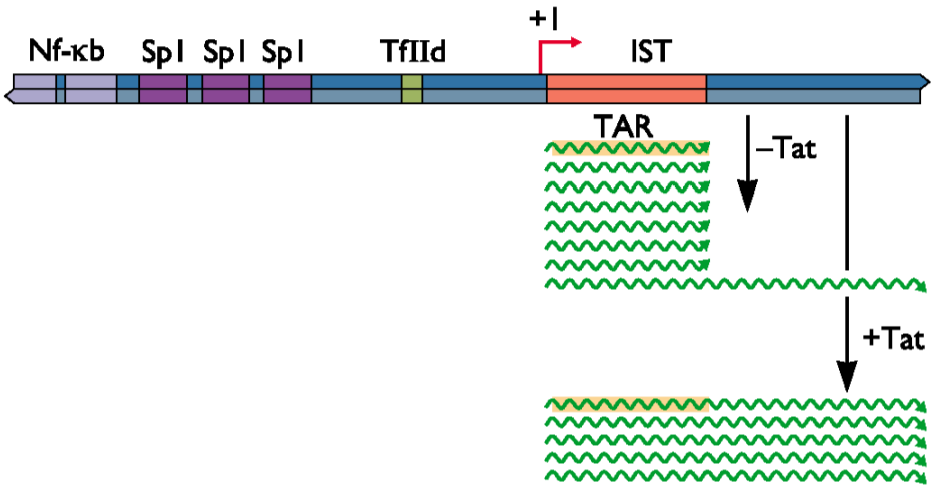


HIV TRASCRIPTION - Tat and TAR

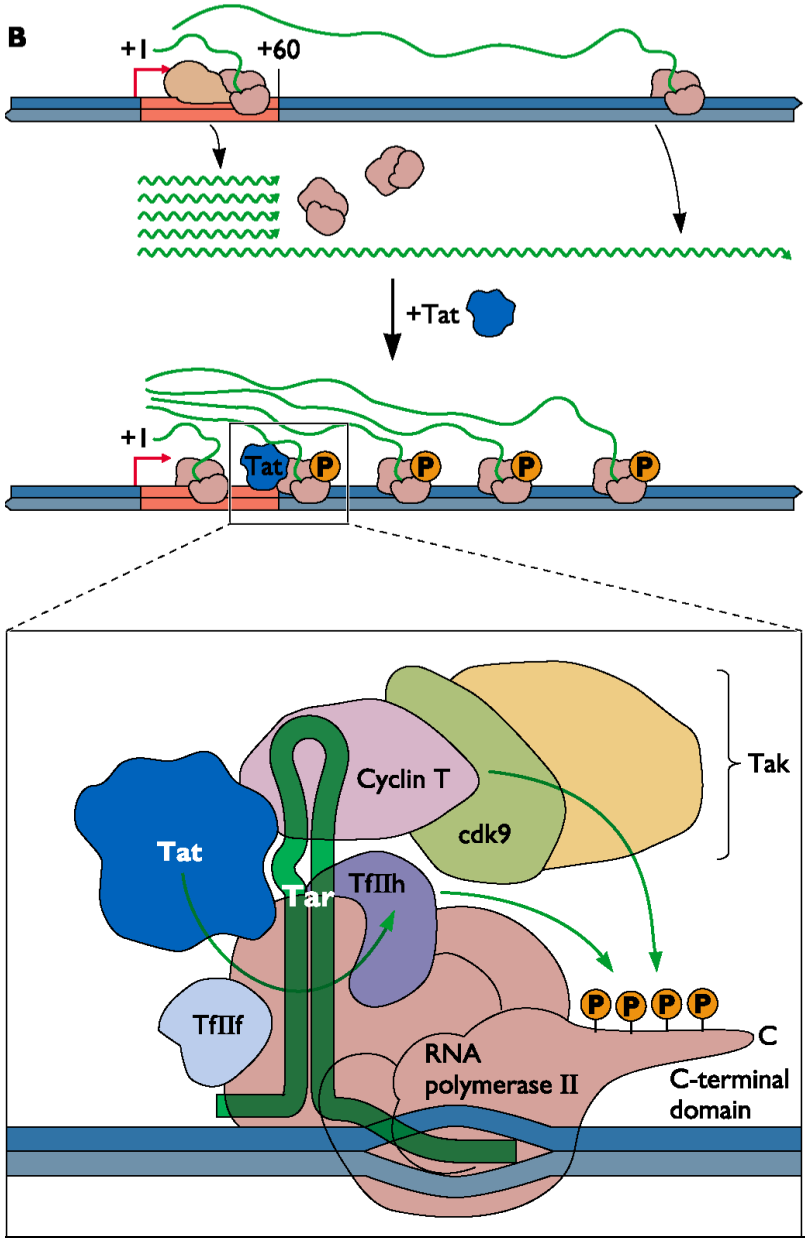


HIV TRANSCRIPTION

Tat and TAR

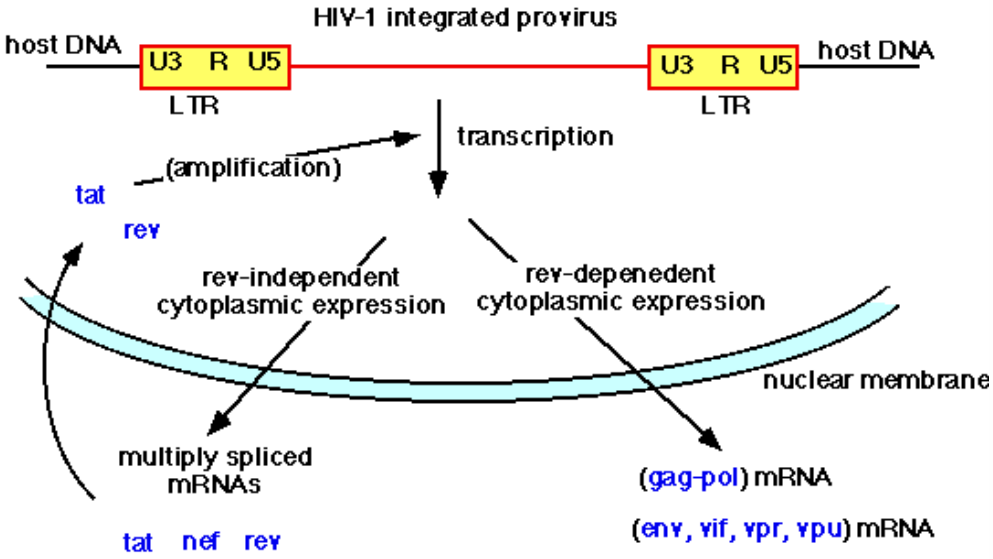


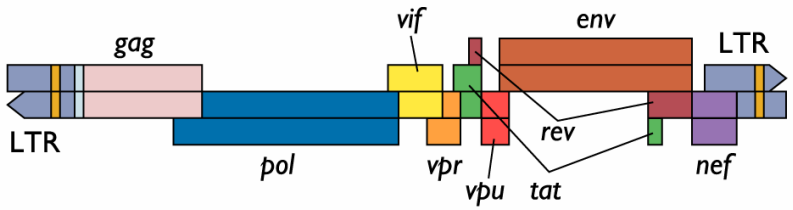
HIV-1 transcription in the presence or absence of Tat



Stimulation of transcription of HIV-1 proviral DNA by Tat

HIV TRANSCRIPTION
mRNA splicing

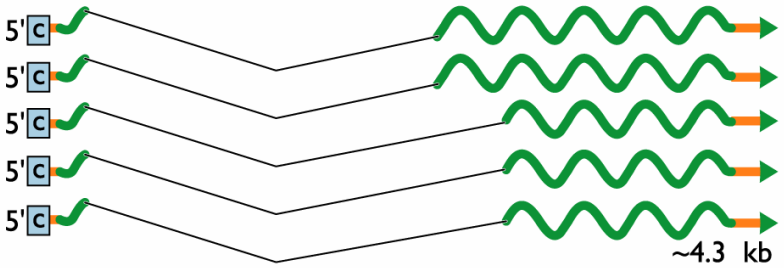




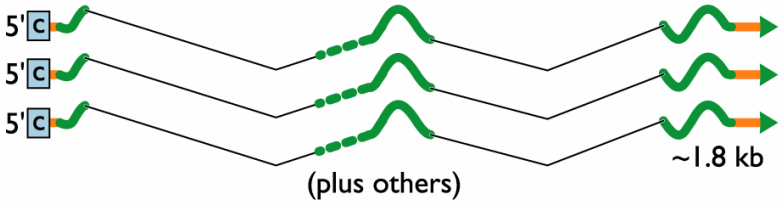
Genome expression

Genomic RNA, Gag-Pol mRNA, pre-mRNA

Singly spliced mRNAs: Vif, Vpr, Vpu, Env



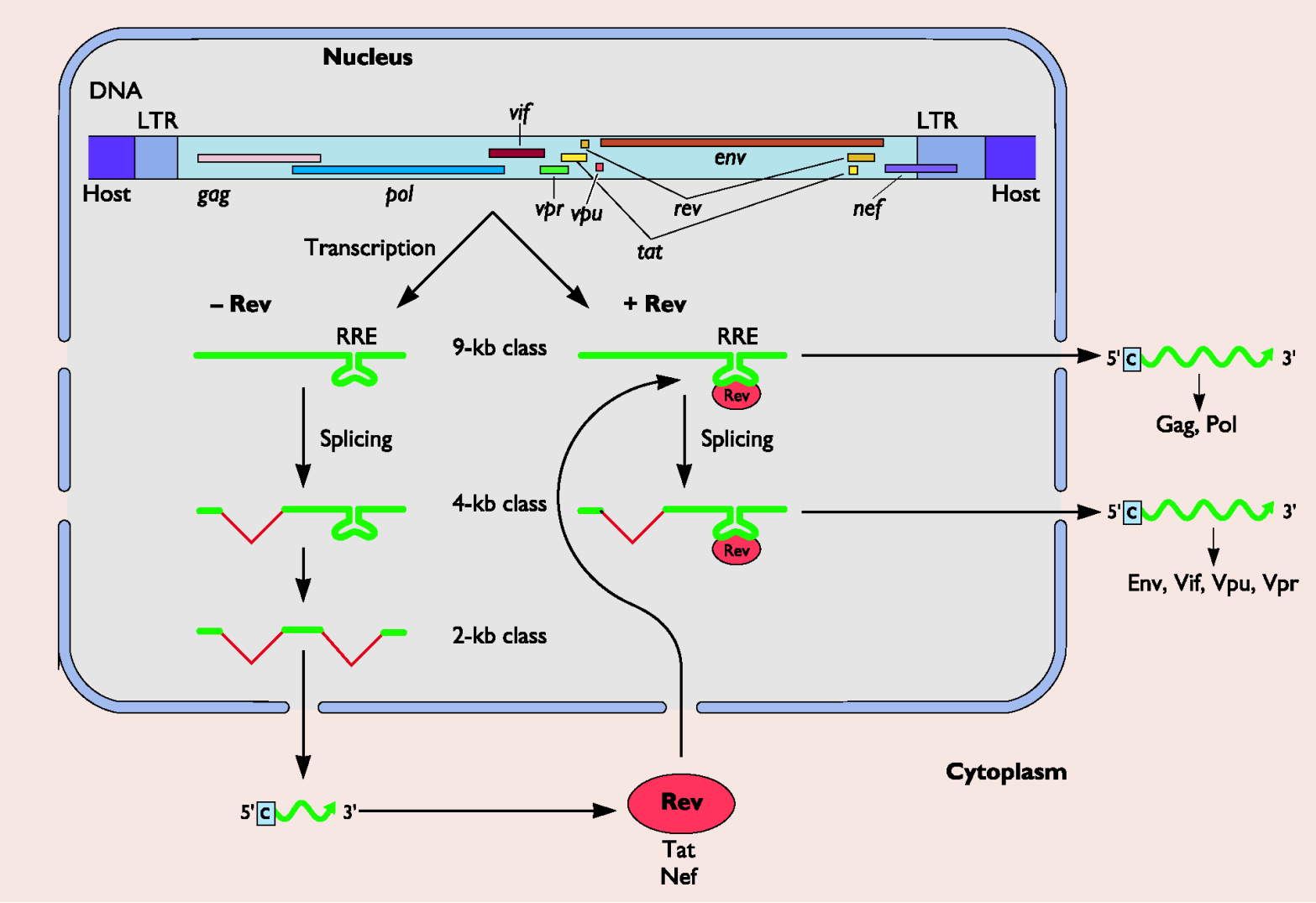
Multiply spliced mRNAs: Tat, Rev, Nef



HIV Rev: features and functions

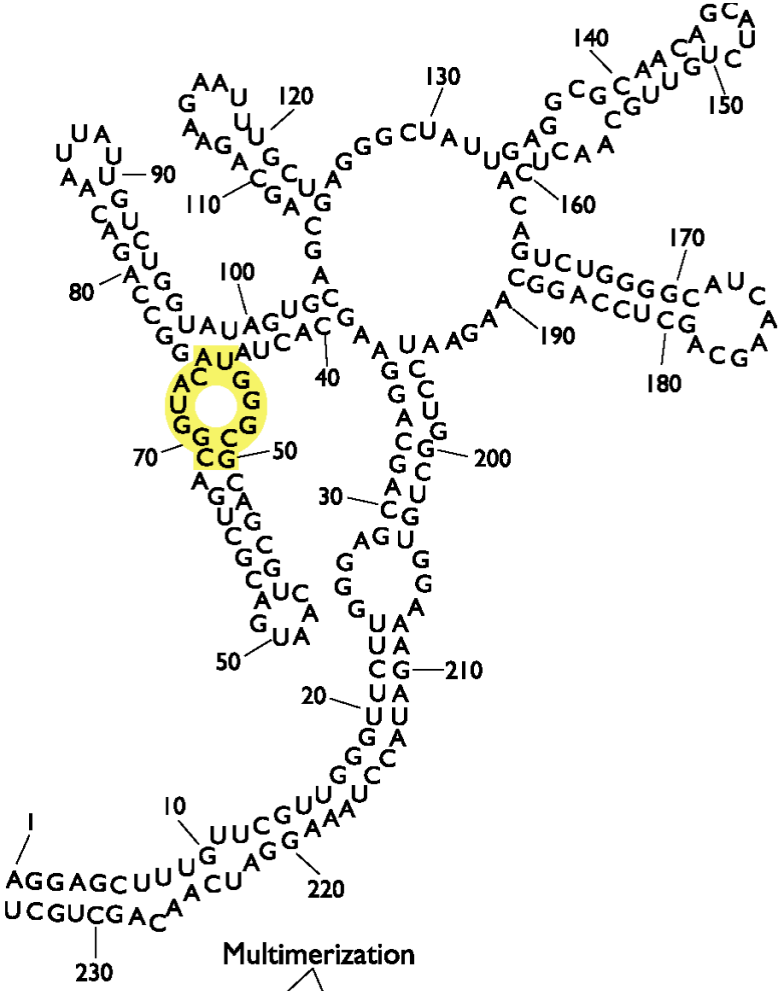
- 19 kDa protein encodes by a multispliced mRNA
- Binds the RRE element of HIV mRNAs
- Allows export to cytoplasm of RRE-containing mRNAS from which virion structural proteins and enzymes are made. It interacts with exportin-1 and Ran-GTP
- Allows expression of proteins encoded by unspliced mRNAs (gag-pol)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

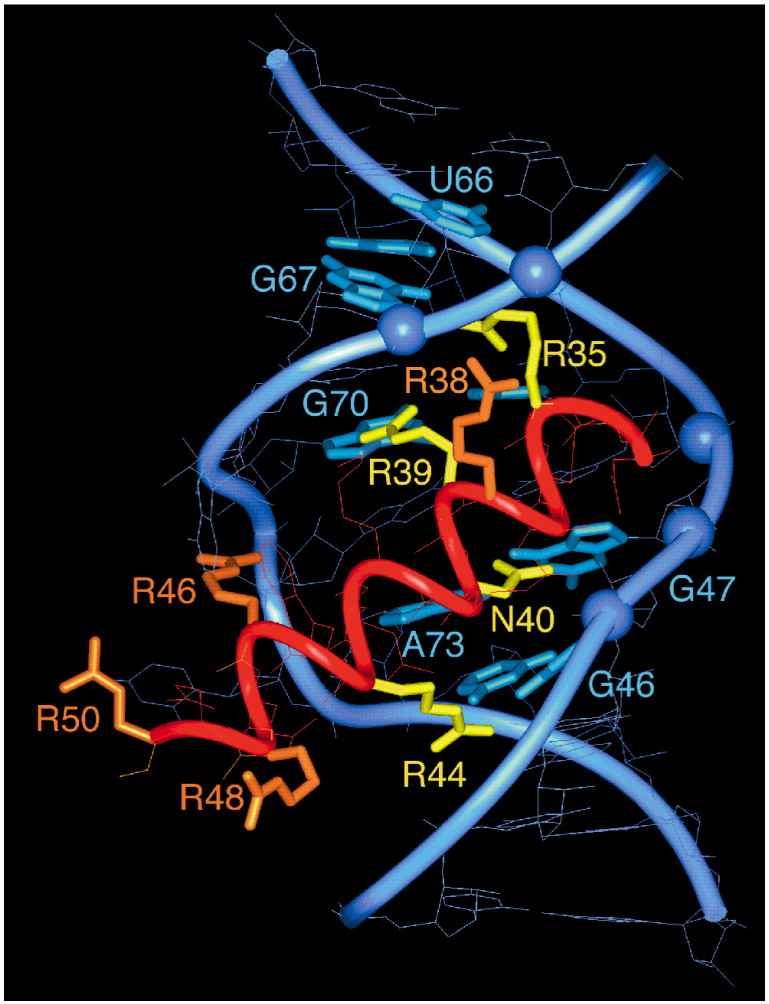
Regulation of export of HIV-1 mRNAs by the viral Rev protein



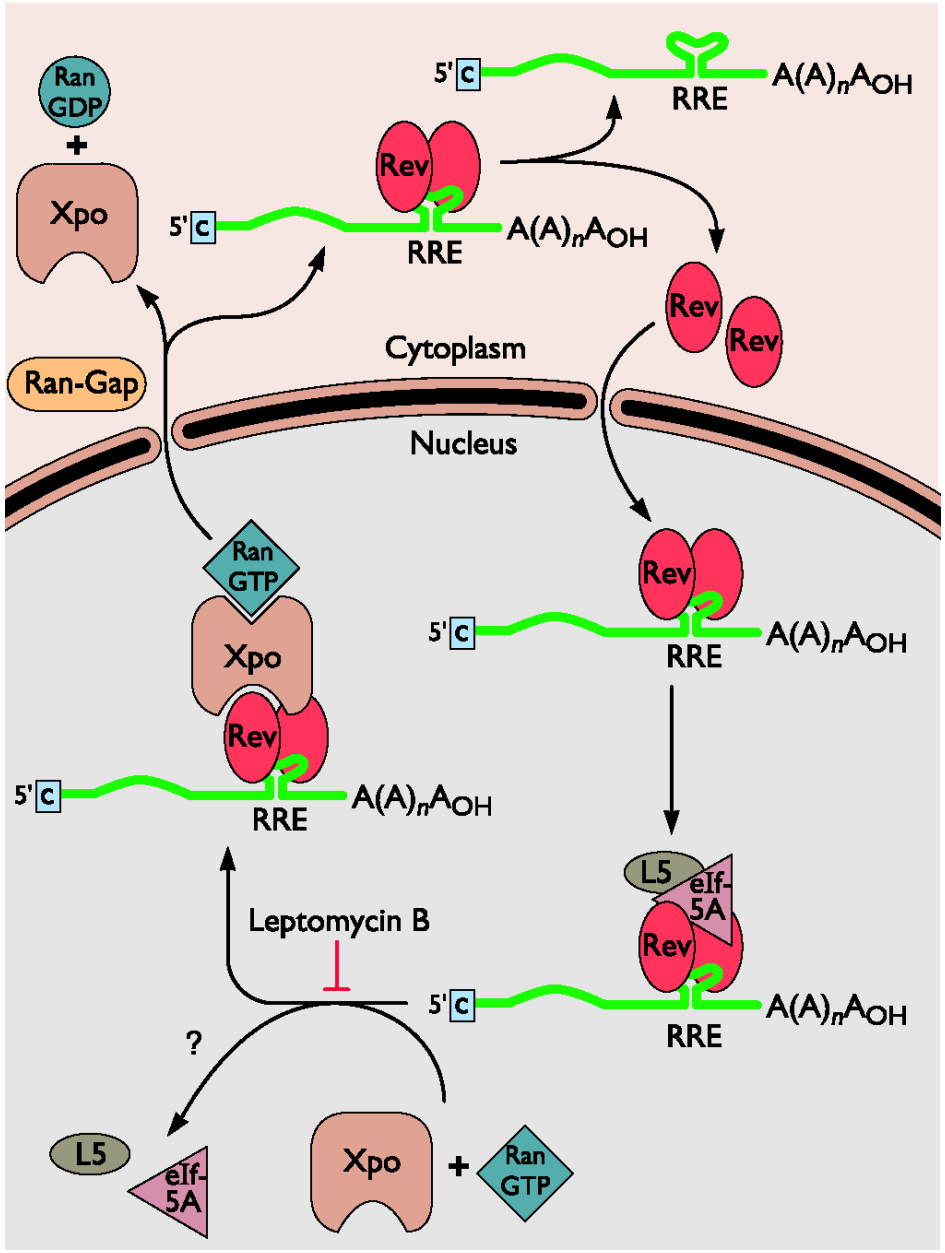
Prior to Rev synthesis only fully spliced mRNA (2-kb class) are exported in the cytoplasm.
When Rev is made it enters the nucleus and binds the RRE in unspliced (9-kb class) and singly-spliced (4-kb class) viral mRNAs. This interaction induces export to cytoplasm of RRE-containing mRNAS, from which virion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

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



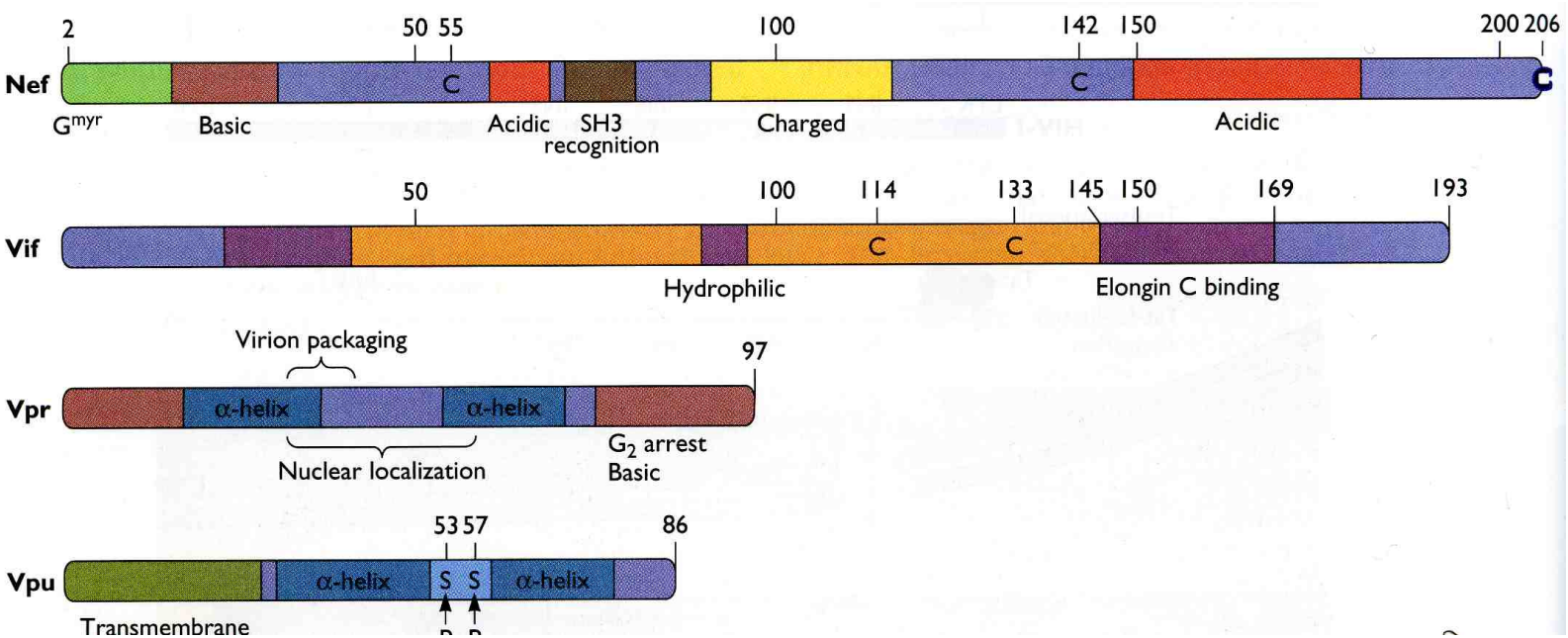


Model of export HIV-1 mRNAs containing introns by the viral Rev protein



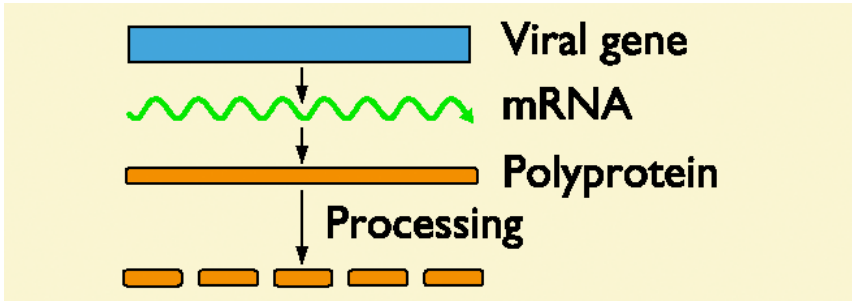
HIV auxiliary proteins

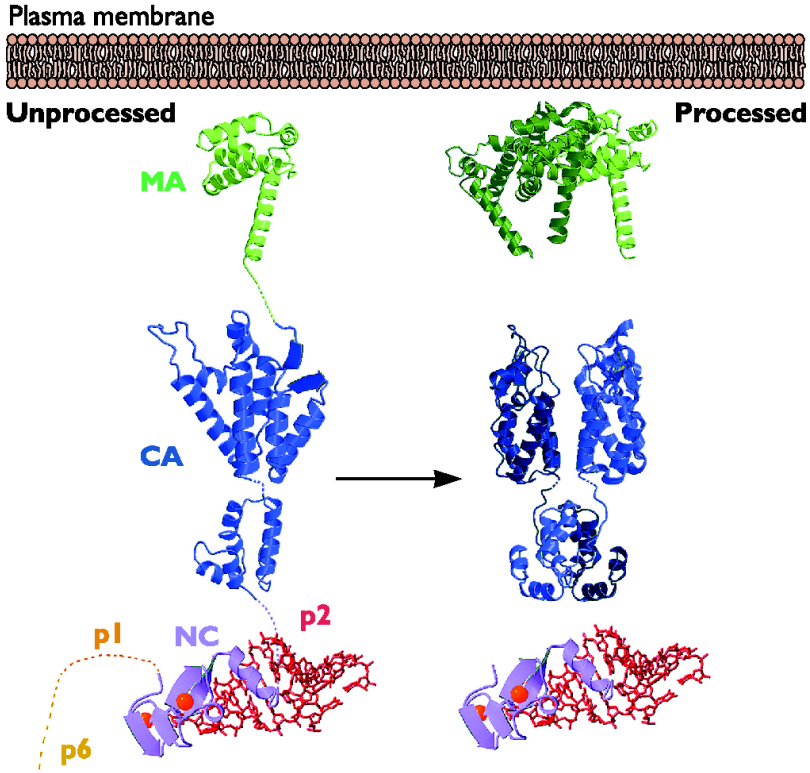
Protein[b]	Size (kDa)	Function	Location
Regulatory			
Tat	14	Stimulation of transcription; binds TAR to facilitate initiation and elongation of viral transcription	Primarily in cell nucleus
Rev	19	Regulation of viral mRNA production; binds RRE and facilitates nuclear export of unspliced or singly spliced 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



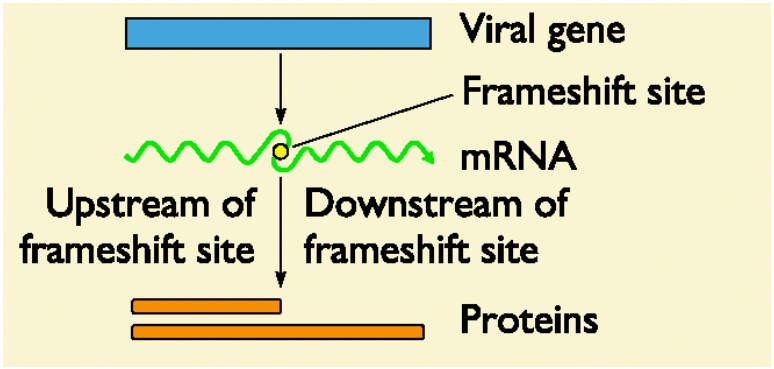
Translation strategies of HIV

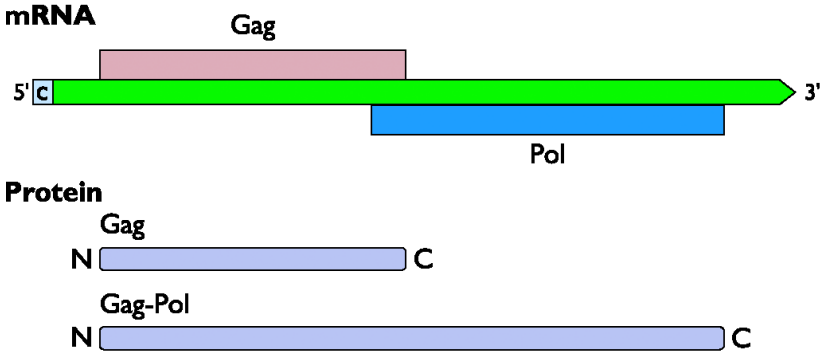
The synthesis of multiple proteins





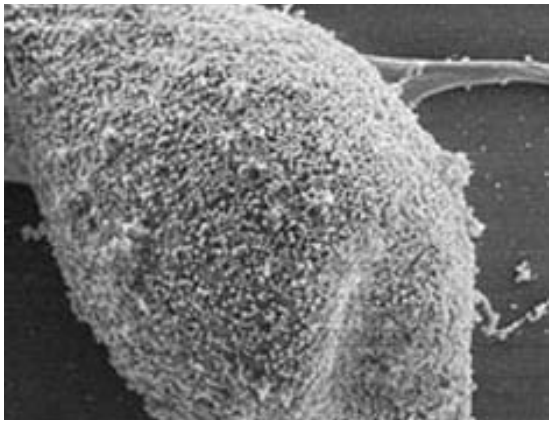
Polyprotein synthesis: the Gag polyprotein

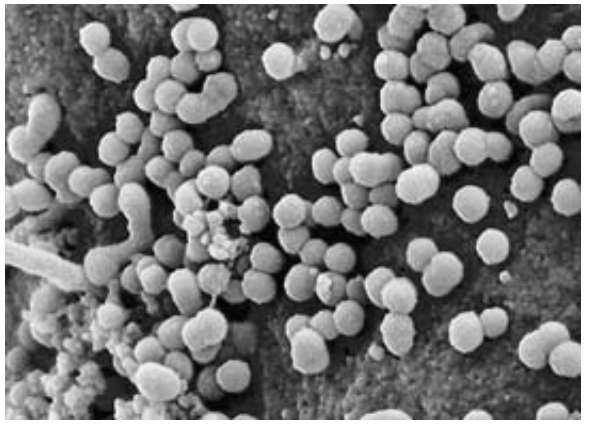


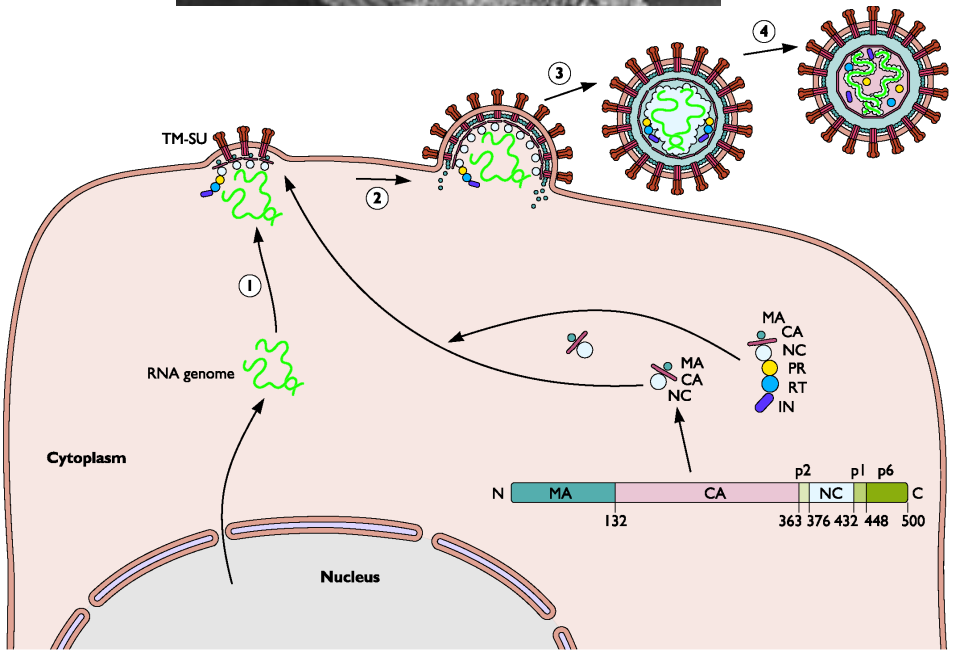


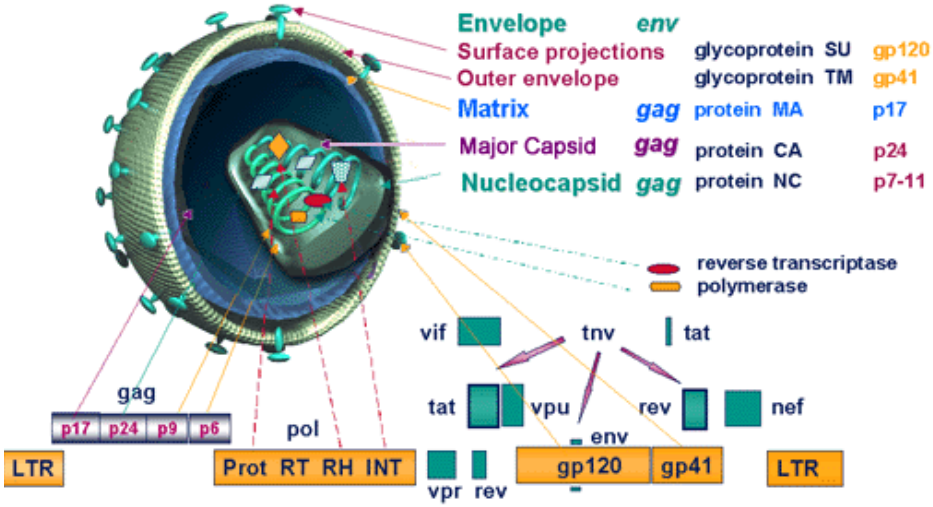
Ribosomal frameshifting:the Gag-Pol fusion

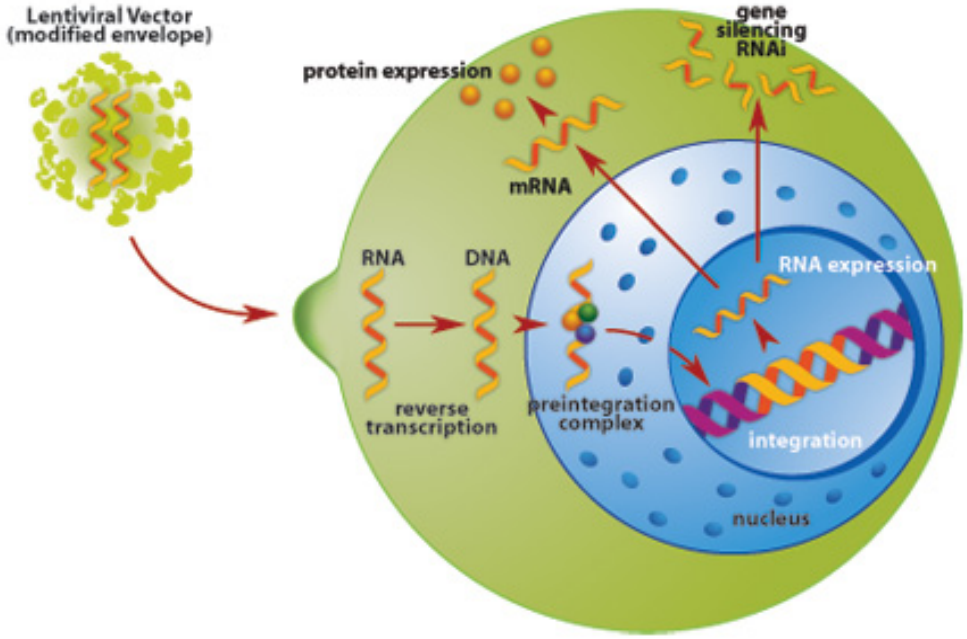
HIV egress by budding at the plasma membrane



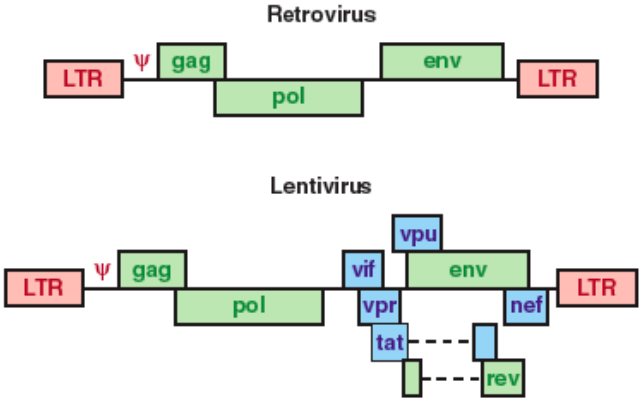


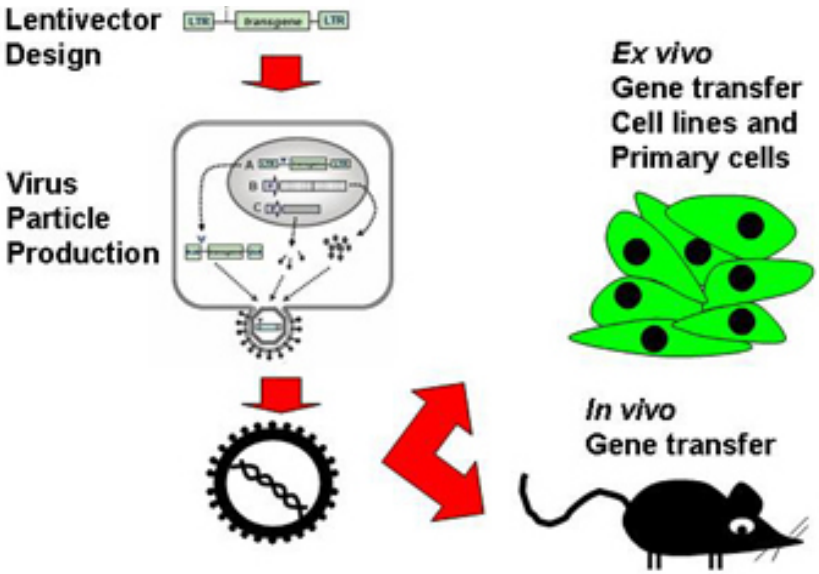






Lentiviral vectors





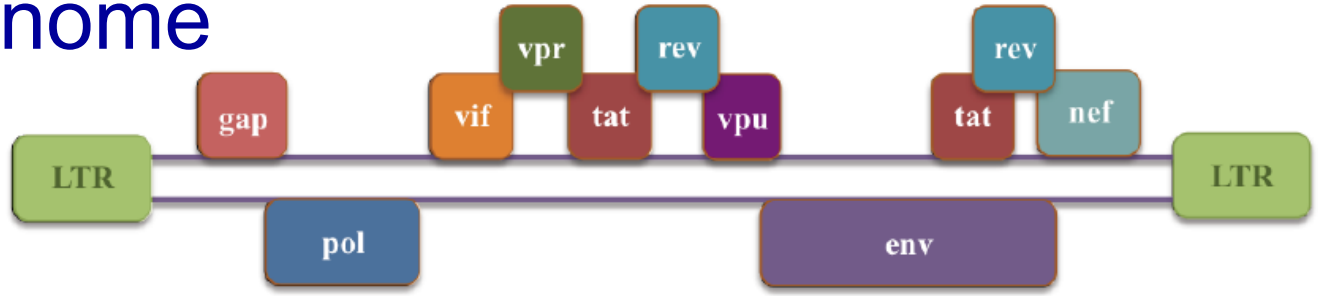
Favorable Features of Lentivirus Vectors

- HIV-1 integrates its DNA and completes a replication cycle in fully differentiated, non dividing cells (macrophages).
- So, this virus must have a mechanims 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	•	•	•	•	•	•

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

Lentiviral Vector Construction

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:

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

Thus, multiple recombination events would be necessary to reconstitute a replicationcompetent lentivirus (RCL).

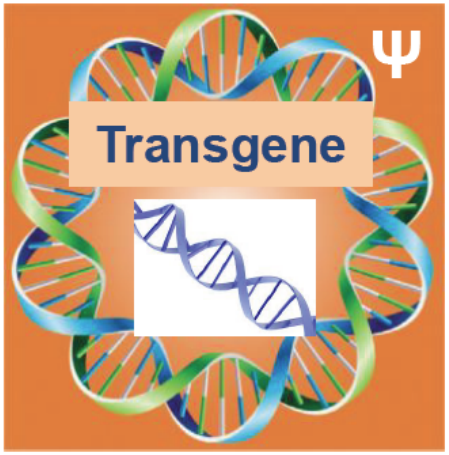
Several components are essential to generate a lentiviral vector, including:

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.

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 absent	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

+

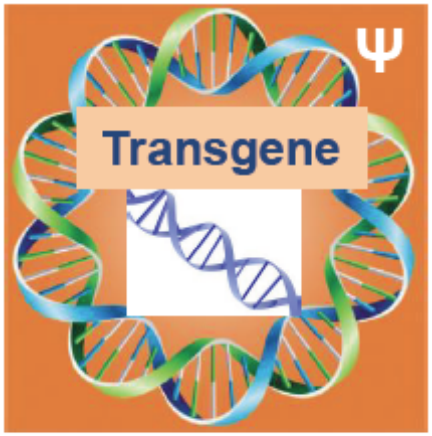
Packaging plasmid

+

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

+

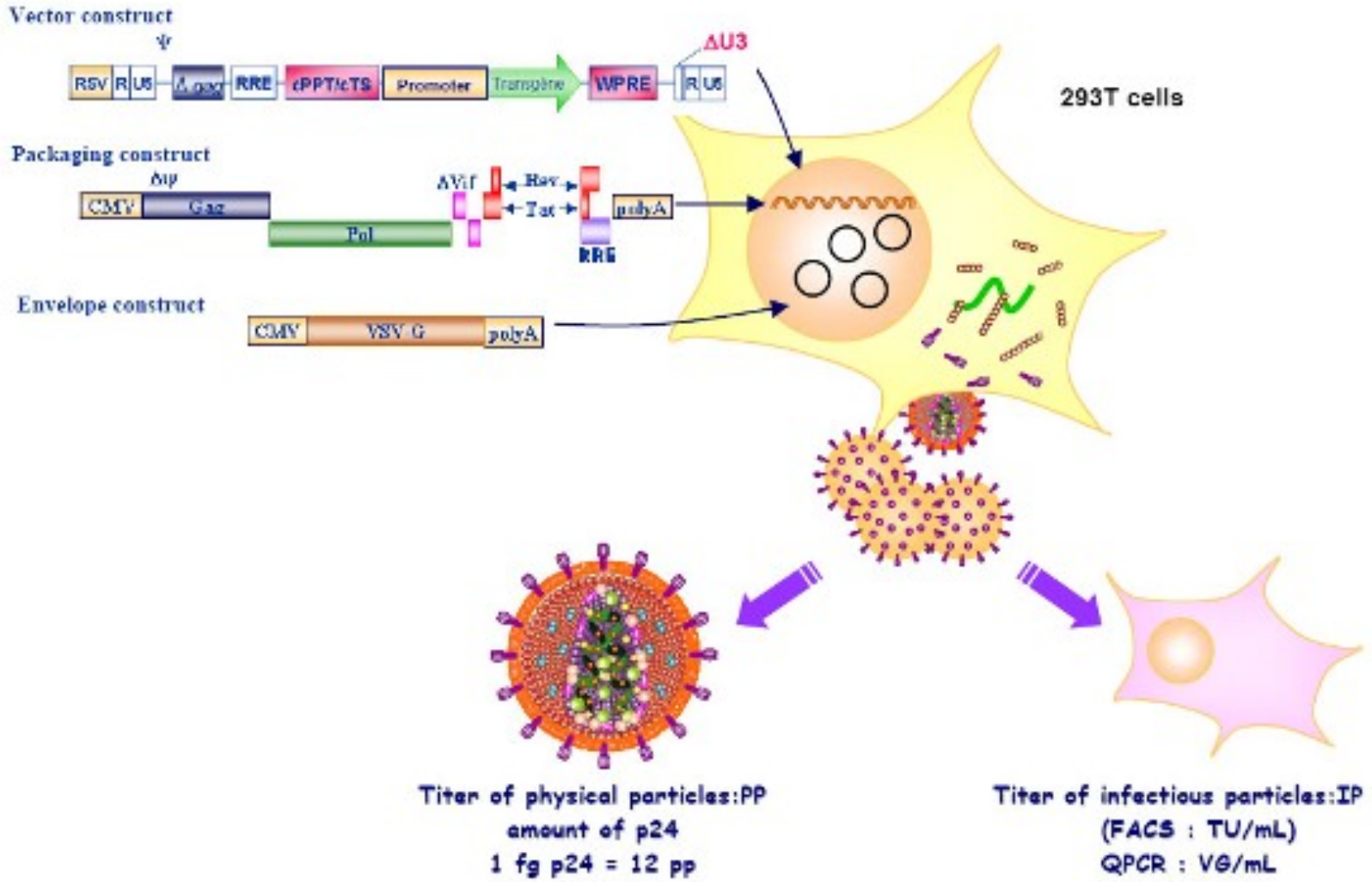
Packaging plasmid

+

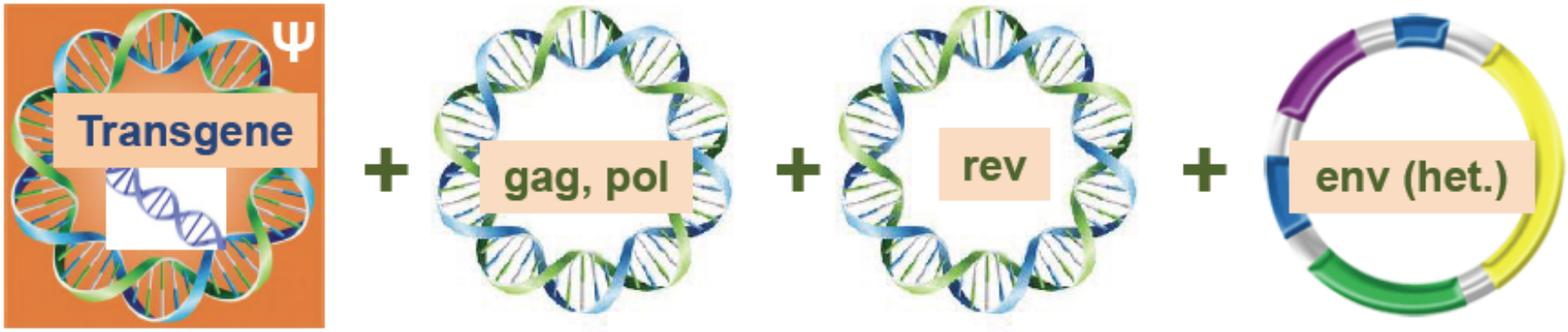
Envelope plasmid

* 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.

Production of a 2nd generation Lentiviral SIN vector



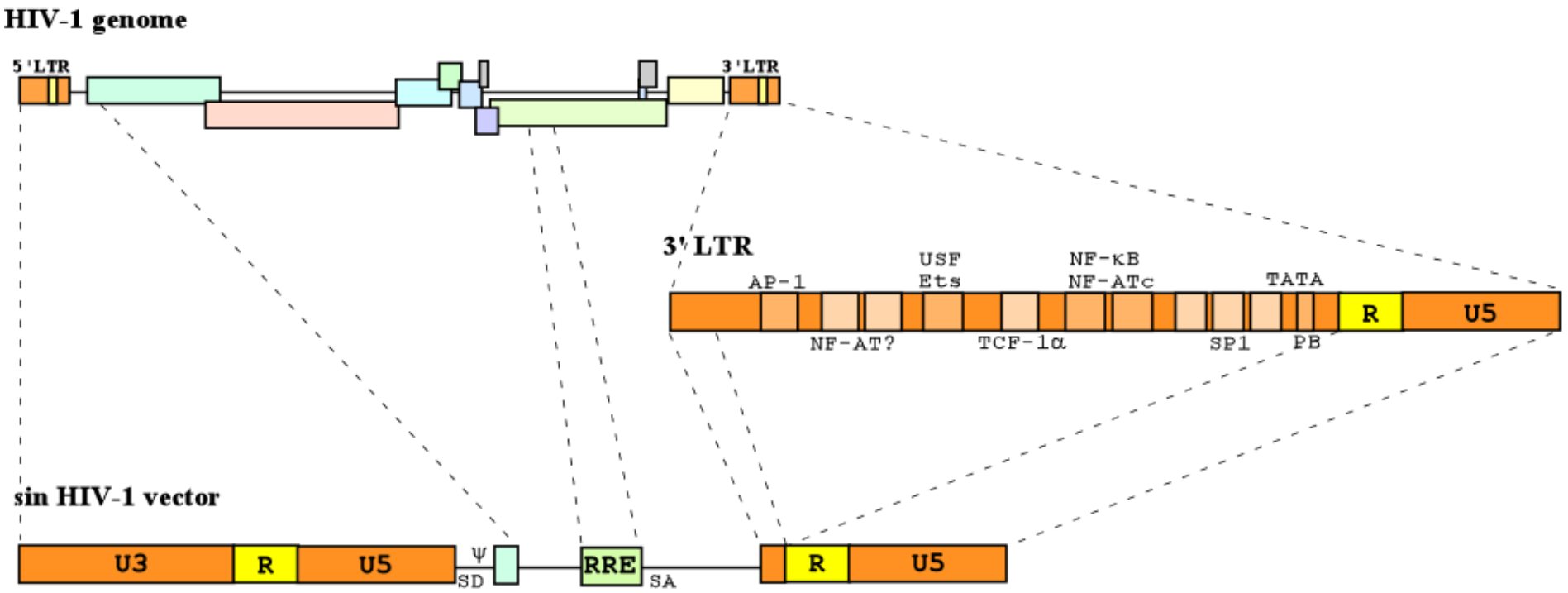
Third-generation / Self-Inactivating (SIN): In a third-generation vector, the 3' LTR is modified, with tat being eliminated and rev provided in a separate plasmid. Since the HIV promoter in the 5' LTR depends on tat, a vector without tat needs to have its wild-type promoter replaced with a heterologous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



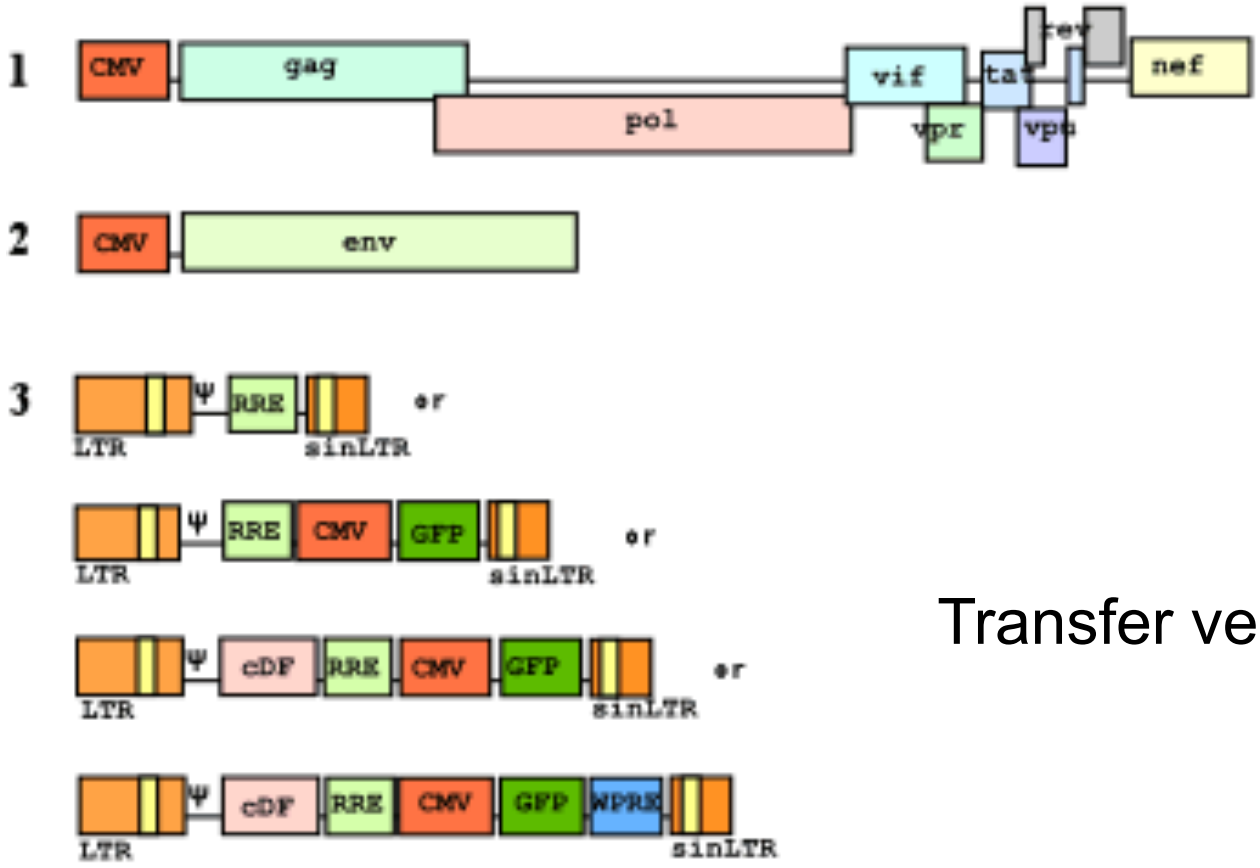
Transfer vector **Packaging plasmids** **Envelope plasmid**

* 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 non-RCR (SIN) HIV-1 based vector



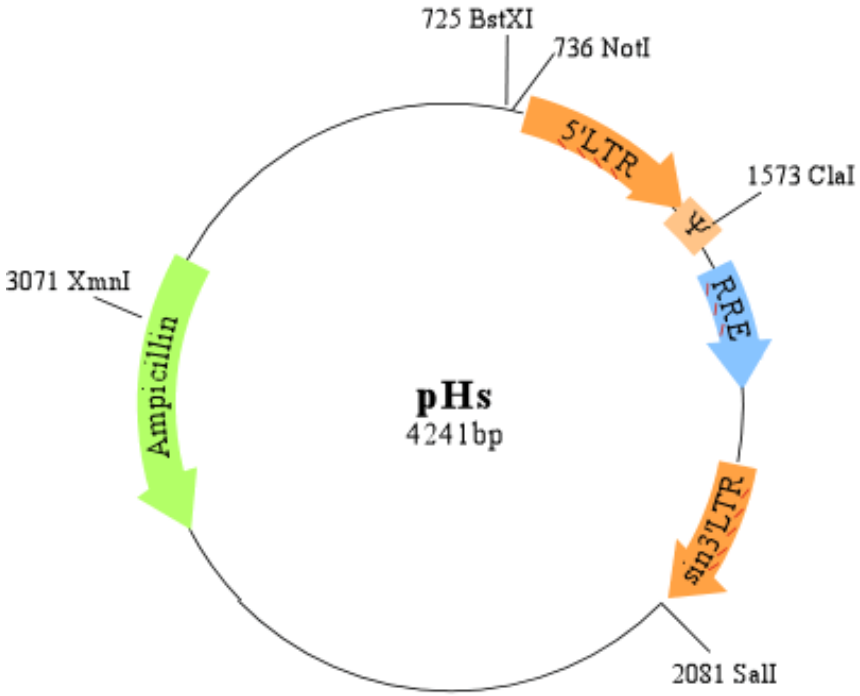
Development of self-inactivating vectors or SIN vectors



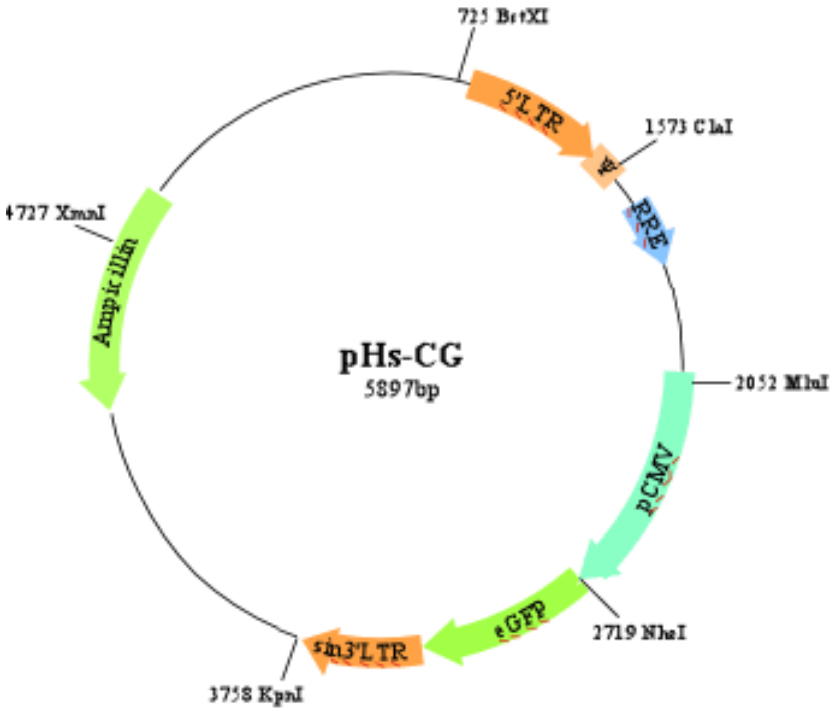
Packaging plasmids

Transfer vectors

Lentiviral SIN vectors

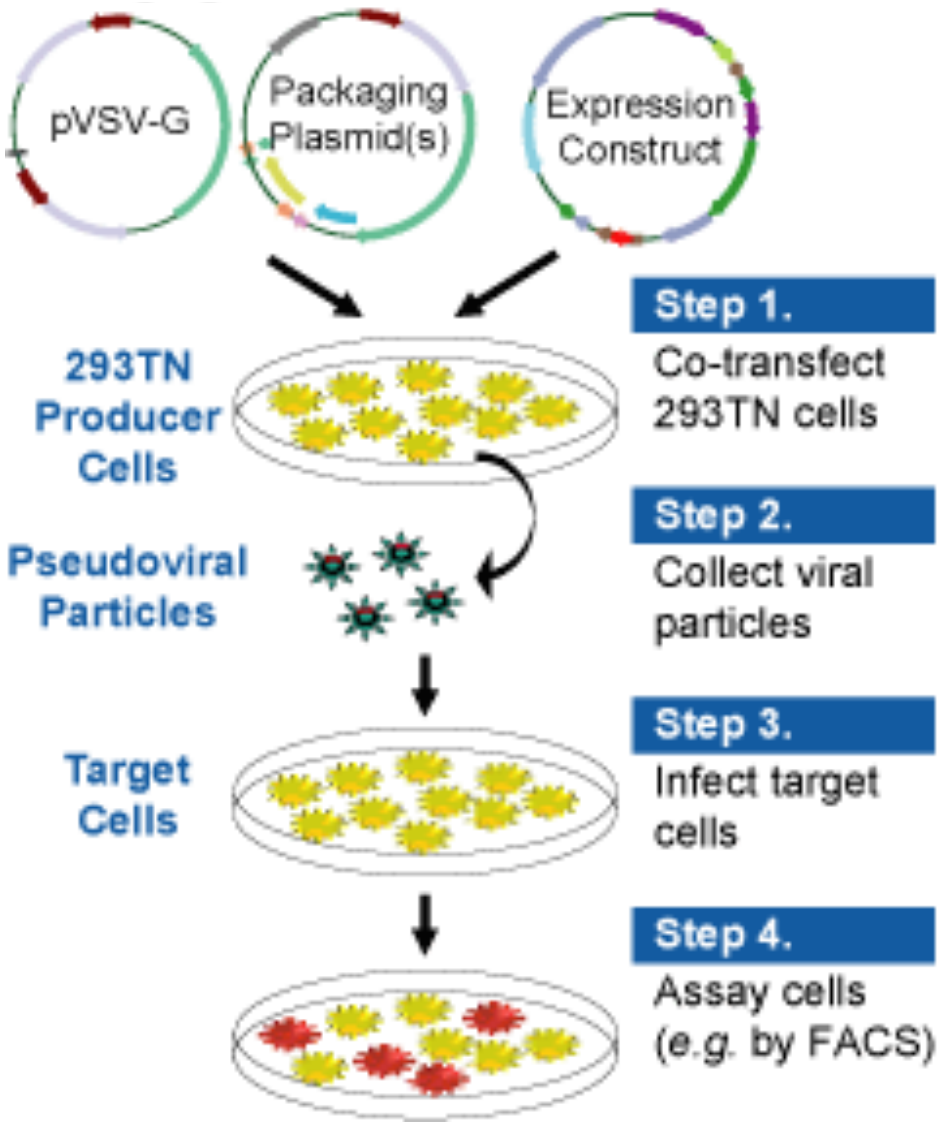


Basic HIV-1 based sin vector.

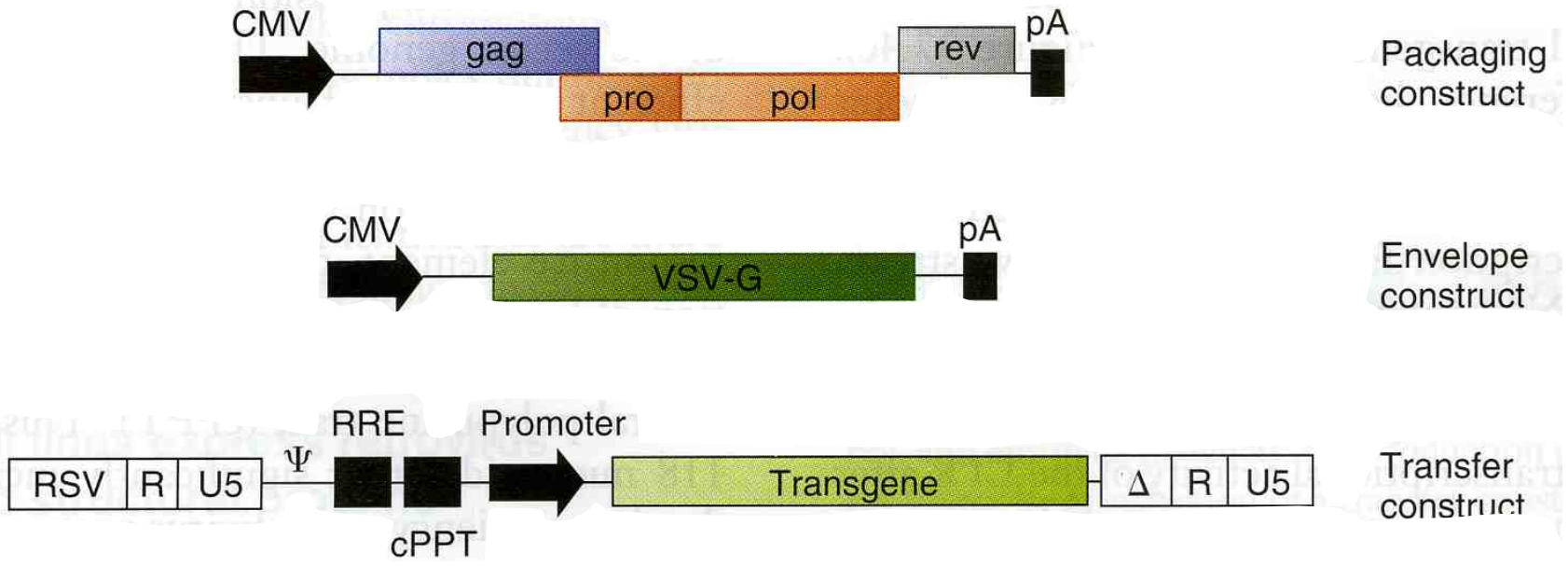


Basic HIV-1 based sin vector with the GFP marker driven by pCMV.

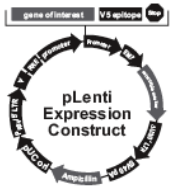
Flow-chart production of a recombinant lentiviral vector



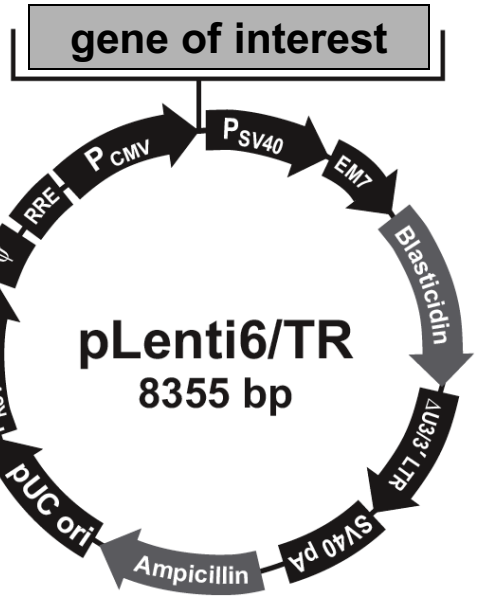
A 3rd generation Lentiviral vector system



A 3rd generation Lentiviral vector system



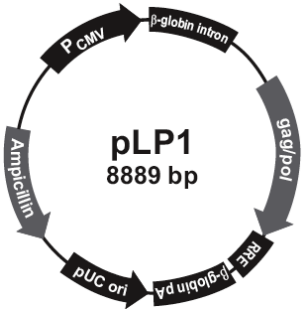
1. Generate the pLenti expression construct containing your gene of interest.



ViraPower™ Packaging Mix

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

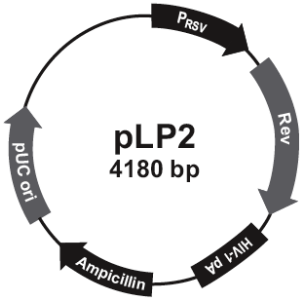
gag, pol



293FT Producer Cell Line

3. Harvest viral supernatant and determine the titer.

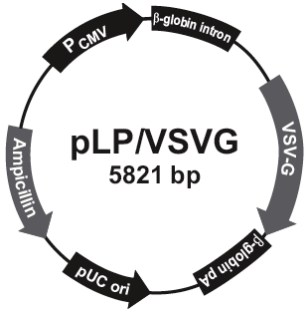
rev



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

Your Mammalian Cell Line of Interest

VSV G



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

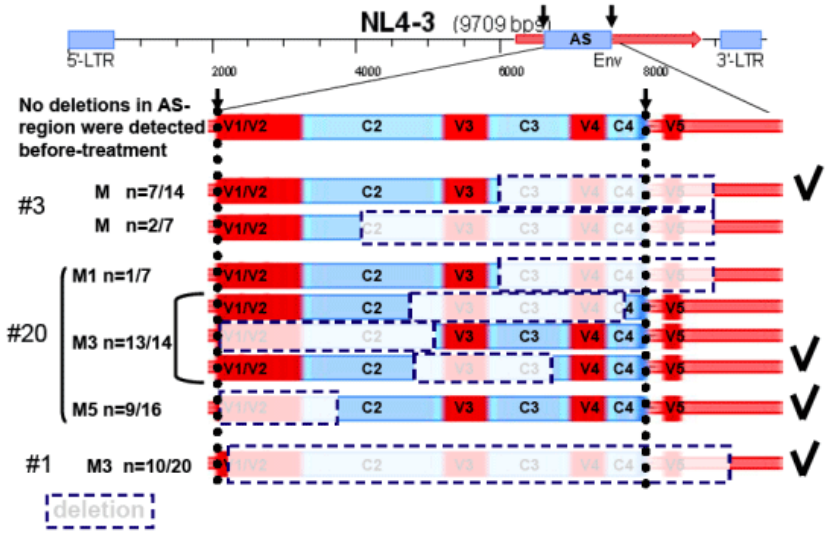
ADVANTAGES	DISADVANTAGES
Can carry large transgenes (up to 8 Kb)	Potential for generation of RCL
Efficient gene transfer	Potential for insertional mutagenesis: Even replication-incompetent lentiviruses with human tropism are able to infect human cells and integrate their genome into the host cells → 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 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)

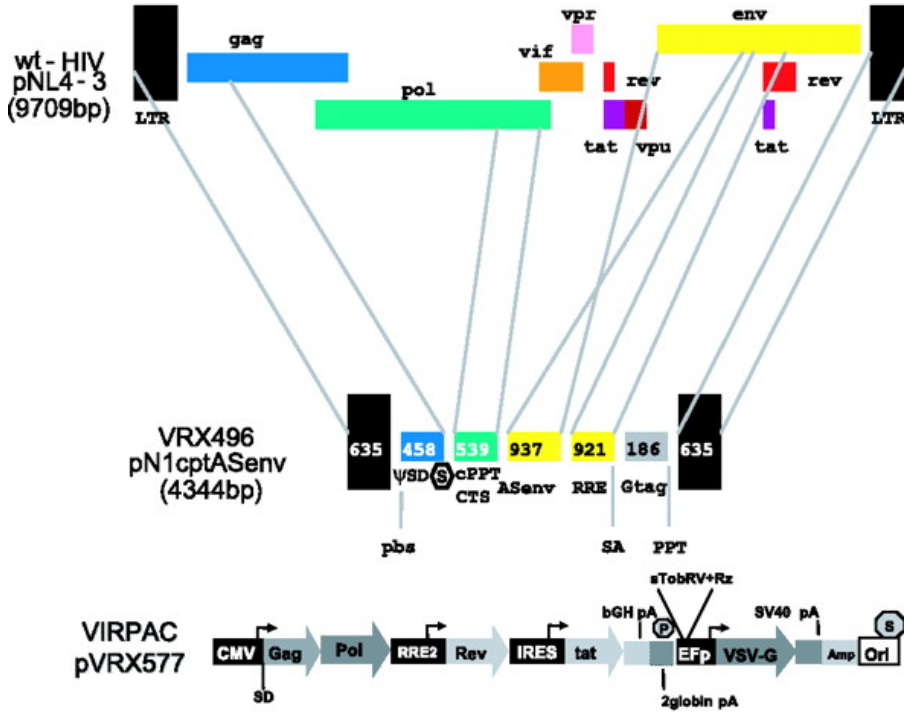
VRX496 Anti-sense Mechanism Deletes Env

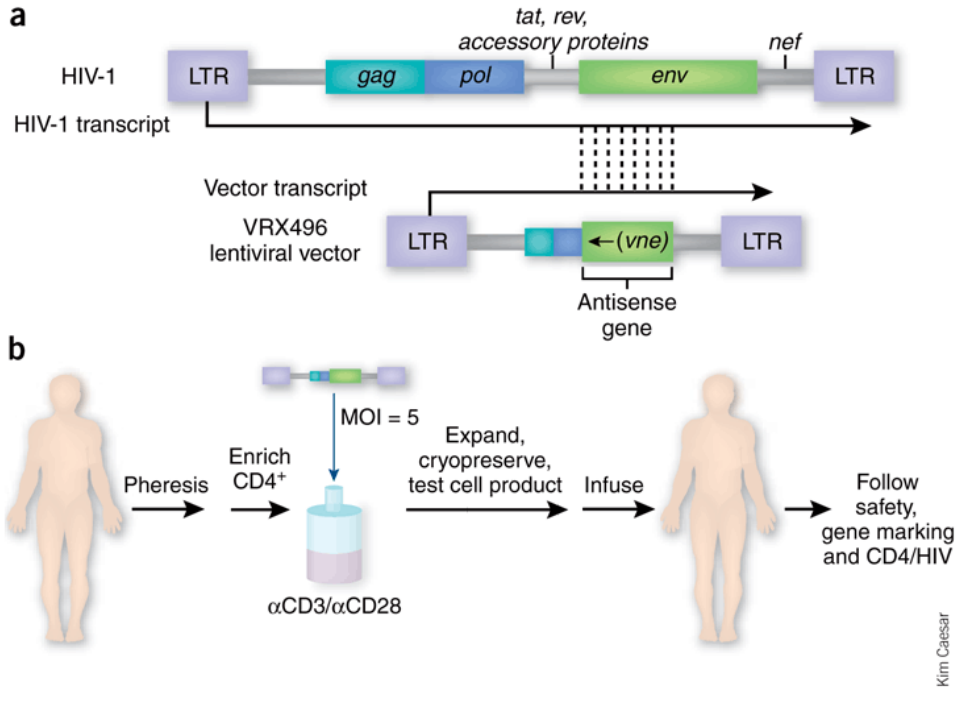


The first clinical trial of a lentiviral vector highlights the promise of this new class of gene-therapy vector

VIRxSYS

Delivering the promise of genetic medicine





Kim Caesar

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

- 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
Phase I Failed ≥2 HAART CD4 ≥150; VL ≥ 5000	Single dose	~10 billion	Completed**
Phase II 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
Phase I/II 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