Eukaryotic Gene Expression: Basics & Benefits

P N RANGARAJAN

Lecture 32

Eukaryotic protein expression systems - III

Gene expression in mammalian cells using viral vectors

Eukaryotic protein expression systems-I (lecture 30)

Protein expression in yeast and insect cells

Eukaryotic protein expression systems-II (lecture 31)

Protein expression in mammalian cells (non viral vectors)

Cell-free protein expression systems

Eukaryotic protein expression systems-III (lecture 32)

Protein expression in mammalian cells (viral vectors)

Human gene therapy (lecture 33)

Eukaryotic protein expression systems-IV (lecture 34)

Protein expression in plant cells and generation of transgenic plants

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Transgenic animals (lecture 35)

Introduction of DNA into animal cells

Calcium Phosphate

Cationic Lipids,

Liposomes

Electroporation

DEAE dextran

Direct DNA Injections

Non viral gene delivery techniques

Non viral gene delivery

1. **Efficiency of transfection**
2. **Nuclear localization of the vector**

The nuclear envelope is a major barrier for successful nonviral transfection of exogenous genes and their subsequent expression both in vitro and in vivo.

The nuclear envelope poses a major problem for transfections in which exogenous DNA is delivered into the cytoplasm.

After delivery to the cytoplasm, nucleic acids rapidly become complexed with cellular proteins that mediate interactions with the cellular machinery for trafficking.

A P Lam and D A Dean
Progress and prospects: nuclear import of nonviral vectors
Gene Therapy **17**, 439-447 (April 2010)

Viruses as gene delivery vehicles

Papova (SV40, Polyoma)

Papilloma (BPV)

Parvo (Adeno associated , AAV)

Adeno

Herpes/Vaccinia

Retroviruses (MMTV)

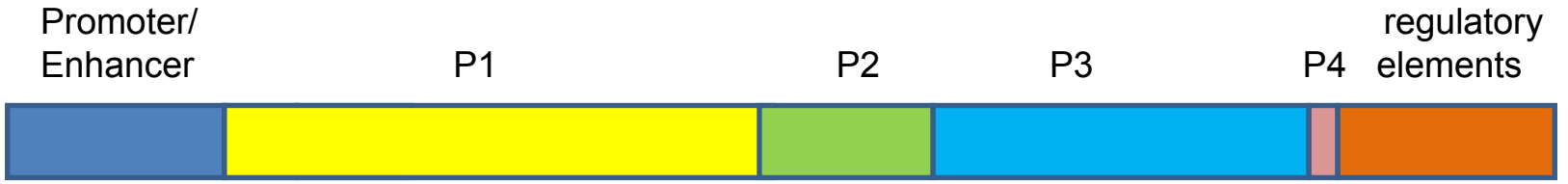
Lentiviruses (HIV)

Viruses have evolved specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect.

Construction of a viral vector

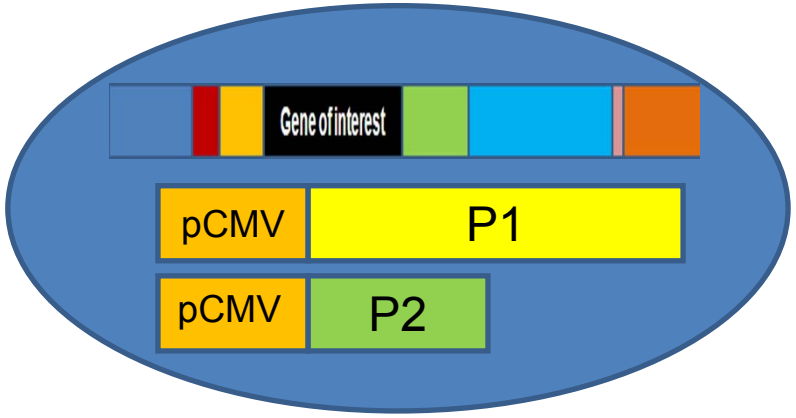
Generation of a recombinant virus

Construction of a viral vector and generation of a recombinant virus

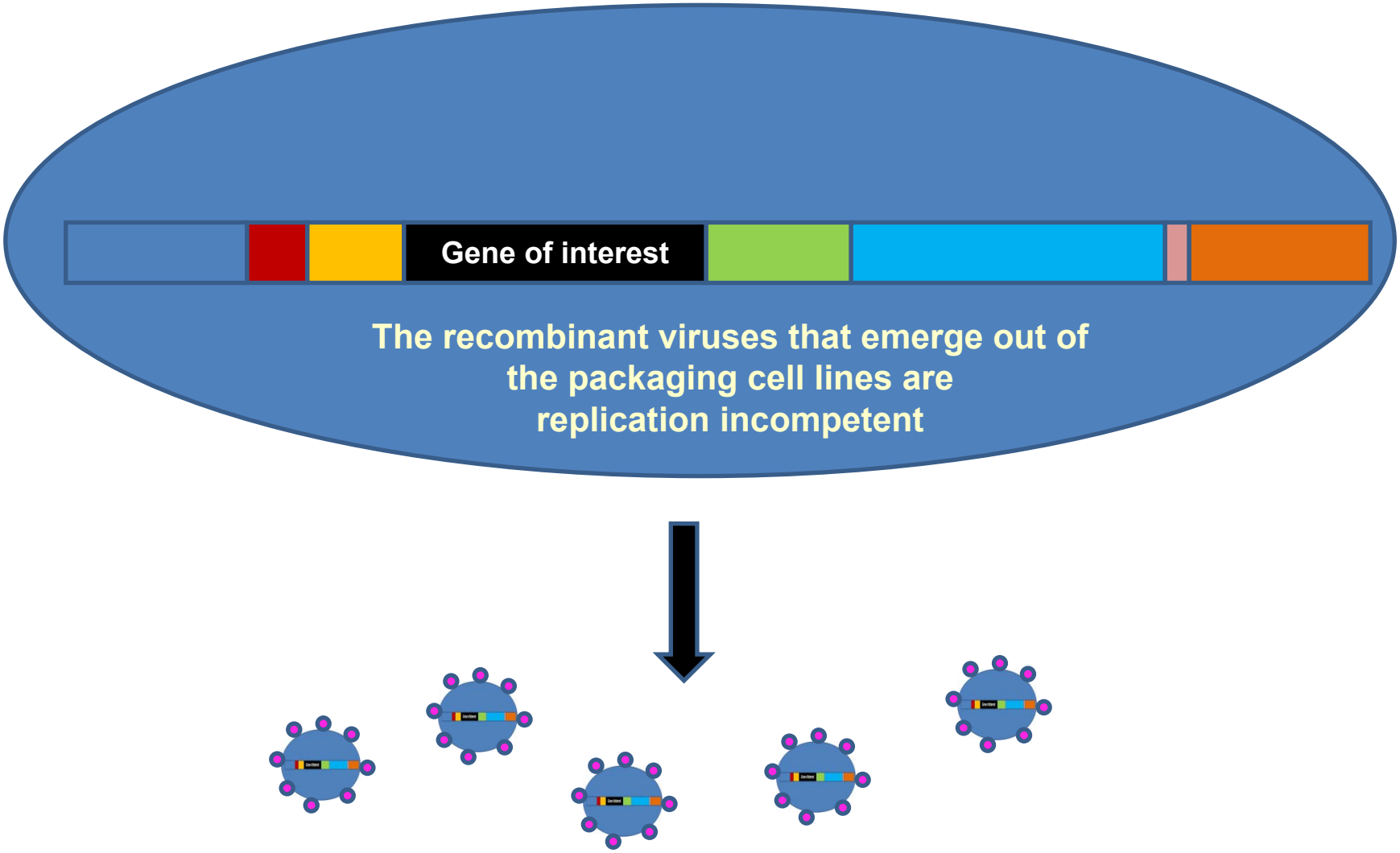


GOI: gene of interest

Packaging cell lines
cell lines that package recombinant retroviral RNAs into infectious, replication-incompetent particles



RECOMBINANT VIRUS

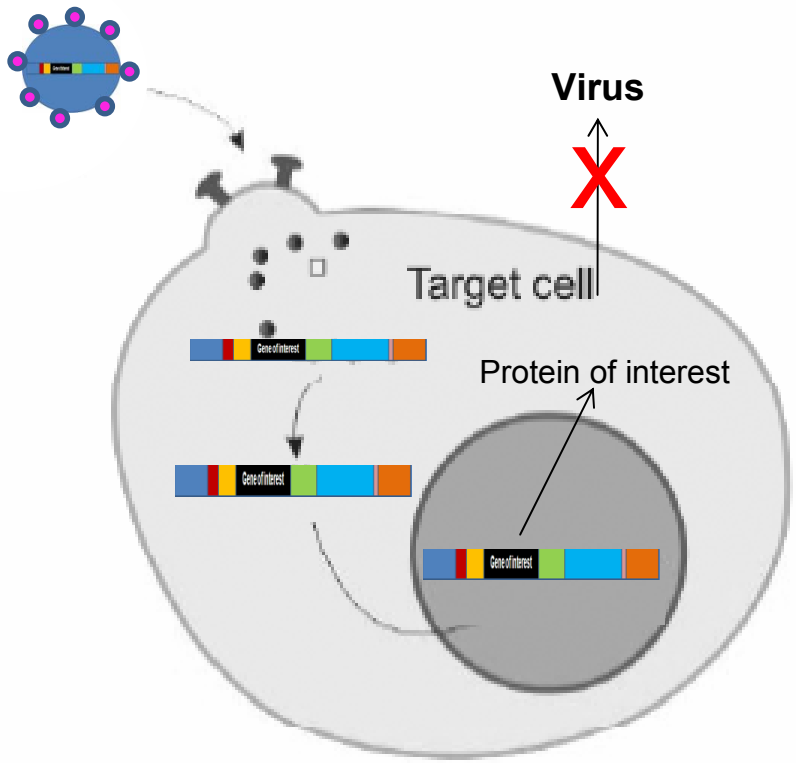


REPLICATION-DEFECTIVE RECOMBINANT VIRUSES

REPLICATION-DEFECTIVE RECOMBINANT VIRUSES

TRANSGENE EXPRESSION

PROTEIN OF INTEREST



Gene expression in mammalian cells using recombinant viruses

Viral genome

Recombinant viral vector

Viruses

Recombinant viruses

Recombinant adenoviral vector	Recombinant adenovirus
Recombinant adeno associated viral (AAV) vector	Recombinant AAV
Recombinant retroviral vector	Recombinant retrovirus
Recombinant lentiviral vector	Recombinant lentivirus

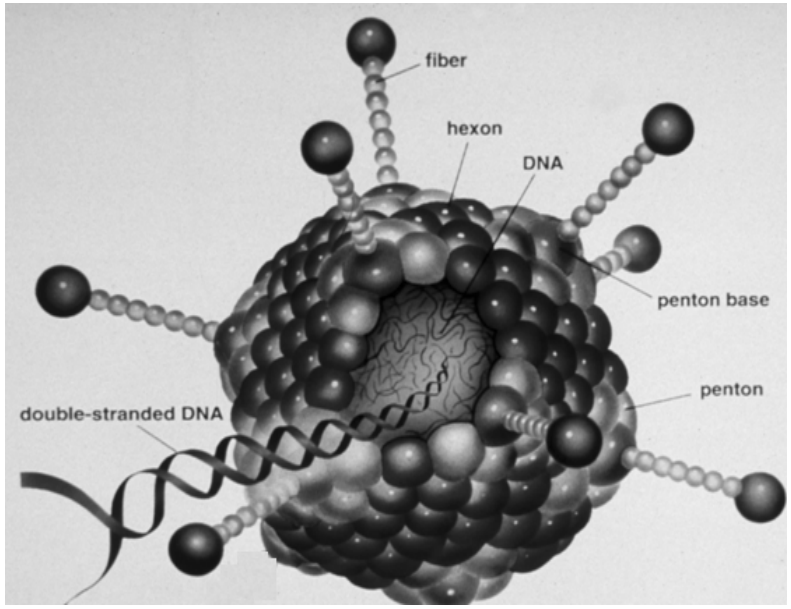
Gene transfer and expression using DNA viruses

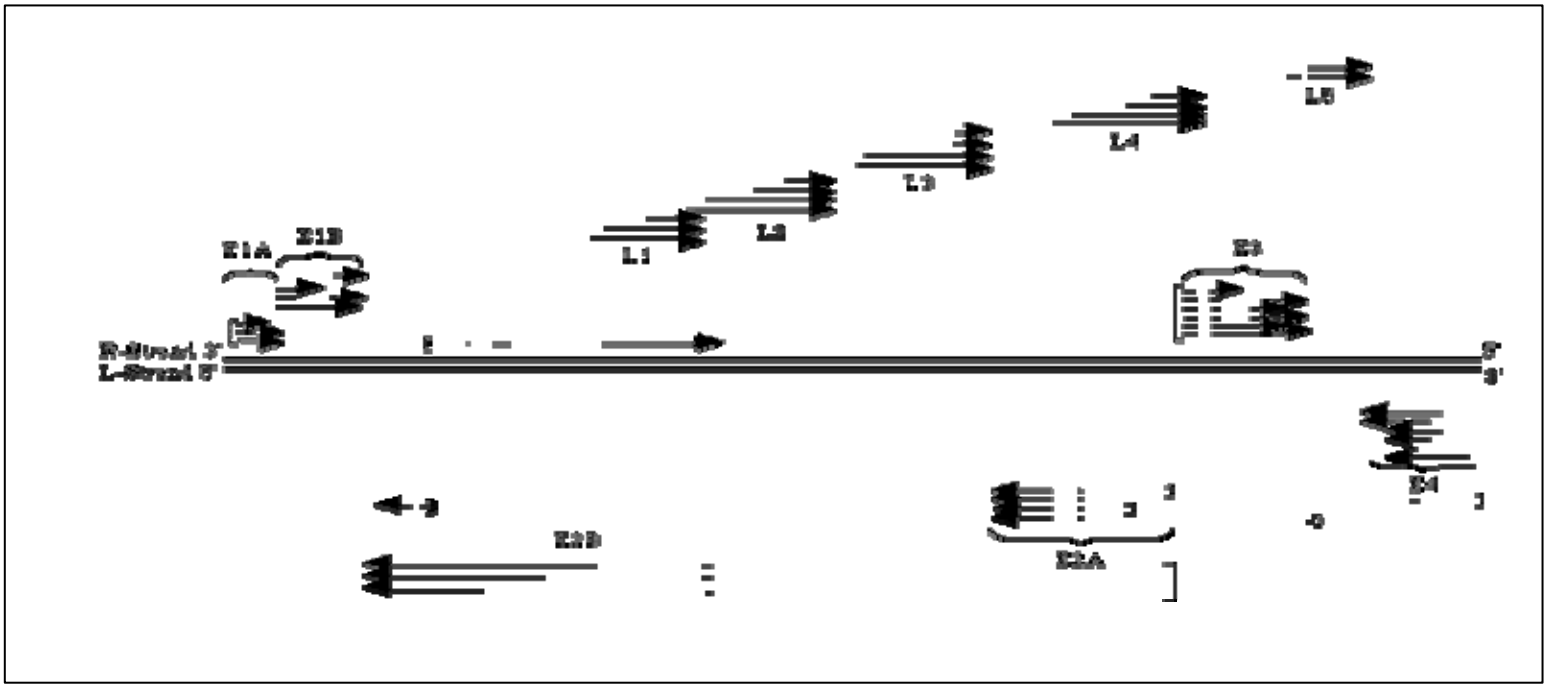
ADENOVIRUSES

ADENO ASSOCIATED VIRUSES

ADENOVIRUS
ADENOVIRAL VECTOR
RECOMBINANT ADENOVIRUS

- Nonenveloped particle
- Contains linear double stranded DNA
- Does not integrate into the host genome
- Replicates as an episomal element in the nucleus





Ad Vector	Gene(s) Deleted	Packaging Line	Cloning Capacity
ΔE1	E1	E1-expressing cells	3 - 5 kb
ΔE1	E1 and E3	E1-expressing cells	7.5 kb
ΔE1ΔE4	E1, E3 and E4	E1/E4-expressing cells	10 kb
ΔE1ΔE2	E1, E2a/E2b & E3	E1/E2-expressing cells	up to 9 kb

Adenoviral Vector: ΔE1/ΔE3

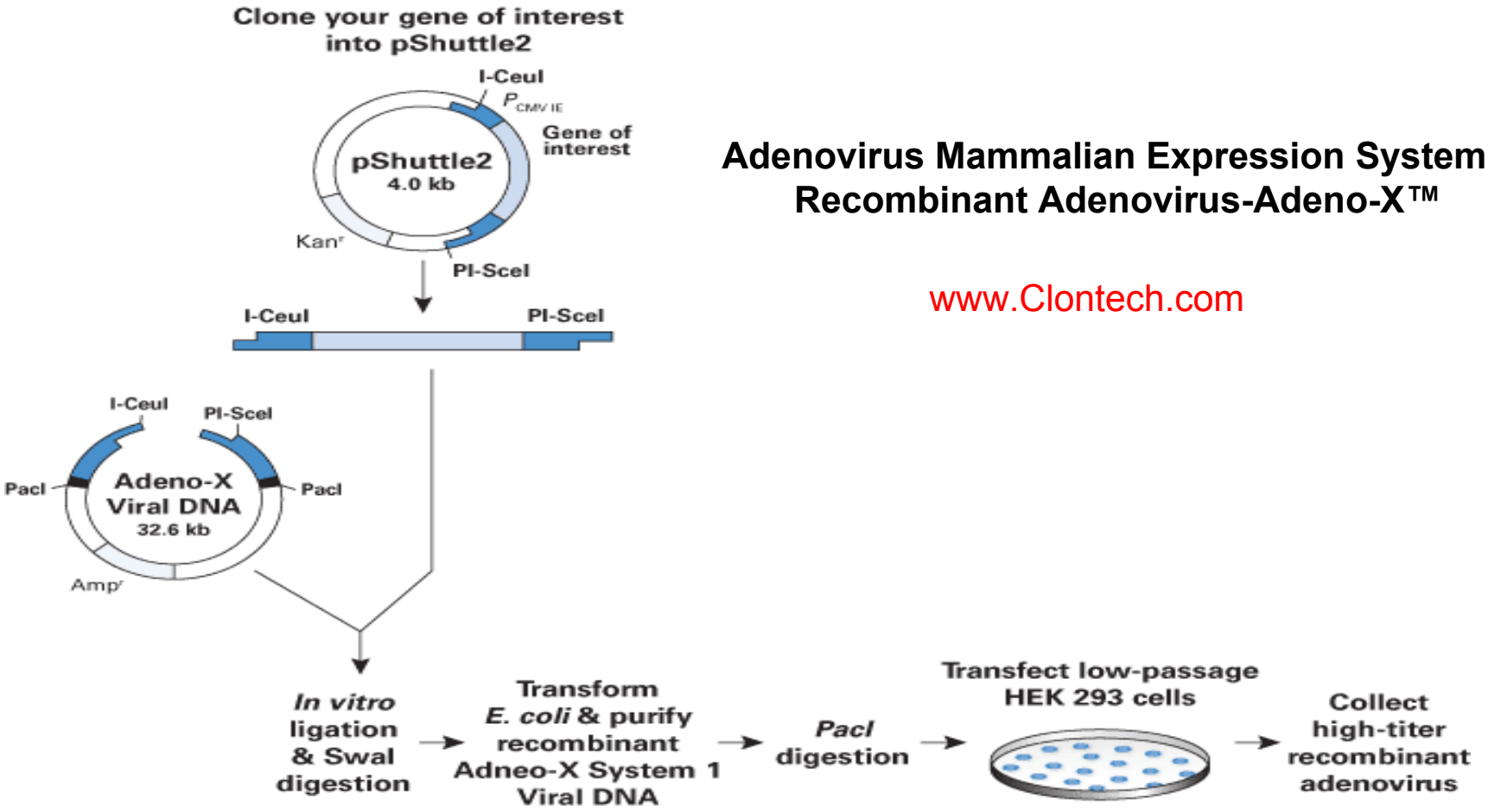
1. Human adenoviral type 5 (Ad5) with ΔE1/ΔE3.
2. Replication-incompetent (replication-deficit).
3. Insert up to 8 kb of foreign DNA.
4. Transient non-cytopathic (non-lytic) infection.

Packaging cells: HEK 293 cells stably expressing the Ad5 E1.

Replication-incompetent adenovirus propagates only in those cell types that express the E1-encoded transcomplementing factors.

MANY ADENOVIRAL EXPRESSION SYSTEMS ARE AVAILABLE COMMERCIALLY

http://www.clontech.com/products/detail.asp?product_id=152613&tabno=2



Adenovirus Mammalian Expression System
Recombinant Adenovirus-Adeno-X™

www.Clontech.com

Adeno-X Method

To produce recombinant adenovirus, insert your gene of interest into the multiple cloning site of the pShuttle2 vector's expression cassette. Then, transfer the cassette into the ligation-ready Adeno-X Viral DNA using the two extremely rare restriction enzyme sites, PI-*Sce* I and I-*Ceu* I. Subsequent digestion with Swa I removes self-ligated or nonrecombinant adenoviral DNA. Transform *E. coli* strain with the ligation mixture and identify recombinant clones by restriction enzyme digests. Then transfect a low-passage HEK 293 cell line with linearized recombinant adenoviral DNA and harvest recombinant adenovirus several days later

ADENOVIRAL VECTORS

Cell-cycle independent.

Primary cells as well as transformed cell lines.

Host tropism: many different animal species including

humans, non-human primates, pigs, rodents, mice, & rabbits.

Tissue tropism: all human cell types - including skin, muscle, bone, nerve, and liver cells - are susceptible.

Advantages:

Higher titer

Efficient transduction of nondividing cells in vitro and in vivo

Disadvantages:

Toxicity

Immunological response

Prior exposure

ADENO ASSOCIATED VIRUS (AAV)
AAV VECTOR
RECOMBINANT AAV

- AAV is a simple, non-pathogenic, single stranded DNA virus dependent on the helper virus (usually adenovirus) to replicate.

- It has two genes (cap and rep), sandwiched between inverted terminal repeats that define the beginning and the end of the virus and contain the packaging sequence.

- The cap gene encodes viral capsid proteins and the rep gene product is involved in viral replication and integration.

- It can infect a variety of cell types and in the presence of the rep gene product, the viral DNA can integrate preferentially into human chromosome 19.

- AAV is a defective virus and needs a helper virus (adenovirus) for its replication.

- AAV is not known to cause disease.

AAV vectors

- To produce an AAV vector, the rep and cap genes are replaced with a transgene.
- The total length of the insert cannot exceed 4.7 kb, the length of the wild type genome.

- Production of the recombinant vector requires that rep and cap are provided in trans along with the helper virus gene products.

Adeno-associated virus vectors

Advantages:

All viral genes removed

Safe

Transduction of nondividing cells

Stable expression

Disadvantages:

Small genome limits size of foreign DNA

Labor intensive production

Status of genome not fully elucidated

Vaccinia virus

Recombinant Vaccinia virus Expression Vector

Recombinant vaccinia virus

Generation of recombinant vaccinia viruses

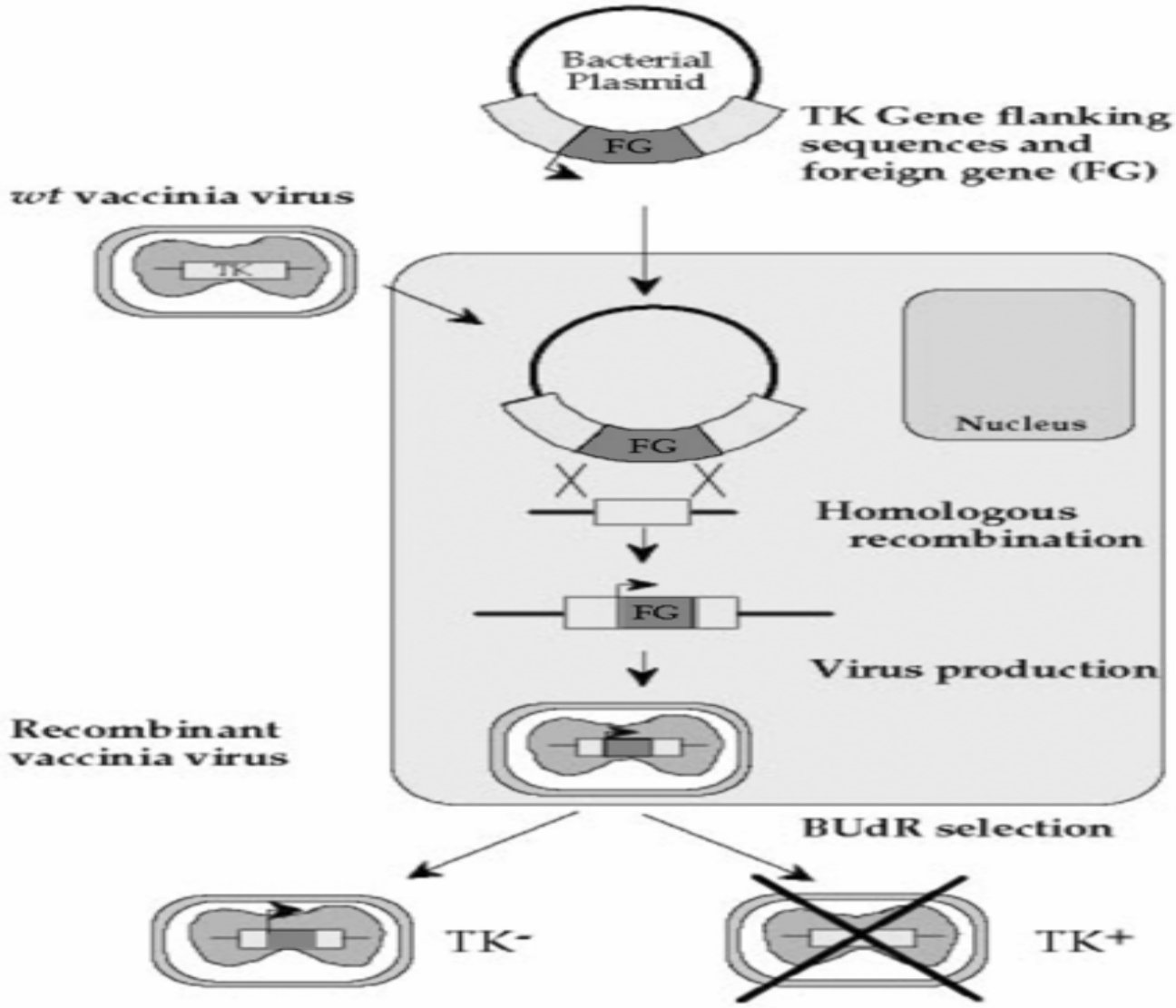
The vaccinia virus genome is about 187000 base pairs.

The large size of the vaccinia virus genome prevents construction of recombinant genomes by restriction endonuclease cleavage and re-ligation with foreign DNA.

A two-step strategy has been utilized for generating recombinant vaccinia viruses:

In the first step, a plasmid is constructed that contains the foreign gene linked to a vaccinia virus promoter.

In the next step, this chimeric gene is inserted into the vaccinia virus genome by homologous recombination between virus and plasmid DNA *in vivo*



Generation of a recombinant vaccinia virus

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10847–10851, November 1992
Biochemistry

Nonreplicating vaccinia vector efficiently expresses recombinant genes

(poxvirus/expression vector/attenuation/host restriction)

GERD SUTTER AND BERNARD MOSS

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892

Modified vaccinia Ankara (MVA)

MVA was derived from wild type vaccinia virus strain Ankara, by over 570 serial passages in chicken embryo fibroblast cells.

The resulting MVA strain lost the capacity to productively infect mammalian cells and had six major deletions of DNA totaling 31,000 base pairs (bp), including at least two host-range genes.

As a result, MVA is severely host cell restricted: it grows well in avian cells but not human and most other mammalian cells.

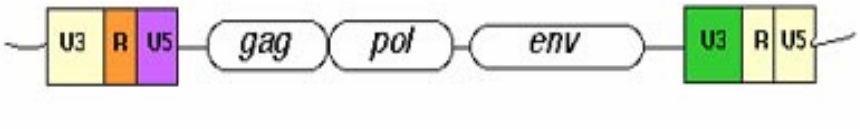
Features	Adenoviruses	Adeno-associated viruses	Herpesviruses	Vaccinia virus
Maximum Insert size	7.5 kb	4.5kb	~30kb	>25 kb
Concentrations viral particles/ml	$>10^{10}$	$>10^{12}$	$>10^{8}$	10^{7}-10^{9}
Route of gene delivery	Ex/In vivo	Ex/In vivo	Ex vivo	Ex/In vivo
Integration	No	Yes/No	No	No
Duration of expression in vivo	Short	Long	Short/ Long in CNS?	Short
Stability	Good	Good	Unknown	Good
Ease of Preparation scale up	Easy to scale up	Difficult to purify, difficult to scale up	Not yet tried	Vaccine production facilities exist
Immunological problems	Extensive	Not known	Not known	Extensive
Pre-existing host immunity	Yes	Yes	Yes	Diminishing as unvaccinated population grows
Safety	Inflammatory response, toxicity	Inflammatory response, toxicity	Neurovirulence? Insertional mutagenesis	Disseminated vaccinia in immunocompromised hosts

Gene transfer and expression using recombinant RNA viruses:

RETROVIRUSES

Retroviruses

Ex. Moloney murine leukemia virus (Mo-MLV)



Gag is a polyprotein and is an acronym for Group Antigens (ag).

Pol is the reverse transcriptase.

Env in the envelope protein.

Gag: processed to matrix and other core proteins that determine retroviral core.

Pol: reverse transcriptase, RNase H and integrase functions.

Env: envelope protein, resides in lipid layer; determine viral tropism.

Transcription proceeds through the genome and mRNA is polyadenylated and processed using signals in transcribed regions from the 3' LTR at the end of the transcribed region.

The full-length message is spliced to lead to production of envelope proteins

Unspliced full-length mRNA can give rise to gag-pol proteins.

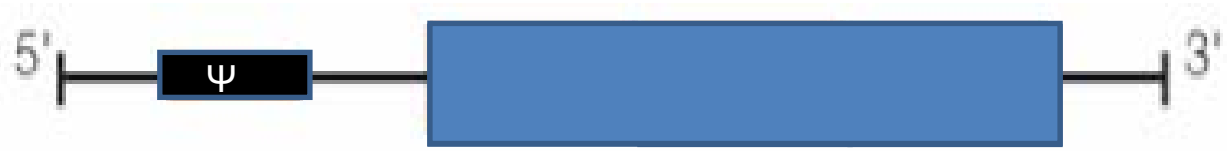
Gag and Pol are made as either Gag protein or a Gag-Pol precursor. A viral protease cleaves the precursor into multiple subunits with varying functions.

The Env protein is also translated as a precursor which is cleaved by endogenous proteases to yield the mature surface glycoprotein.

Translated proteins assemble a retroviral particle at the cell surface.

Full-length genomic unspliced mRNA (containing a packaging signal termed Psi) is bound by gag-derived proteins and incorporated into the budding particle.

Production of a recombinant retrovirus



Gene of interest

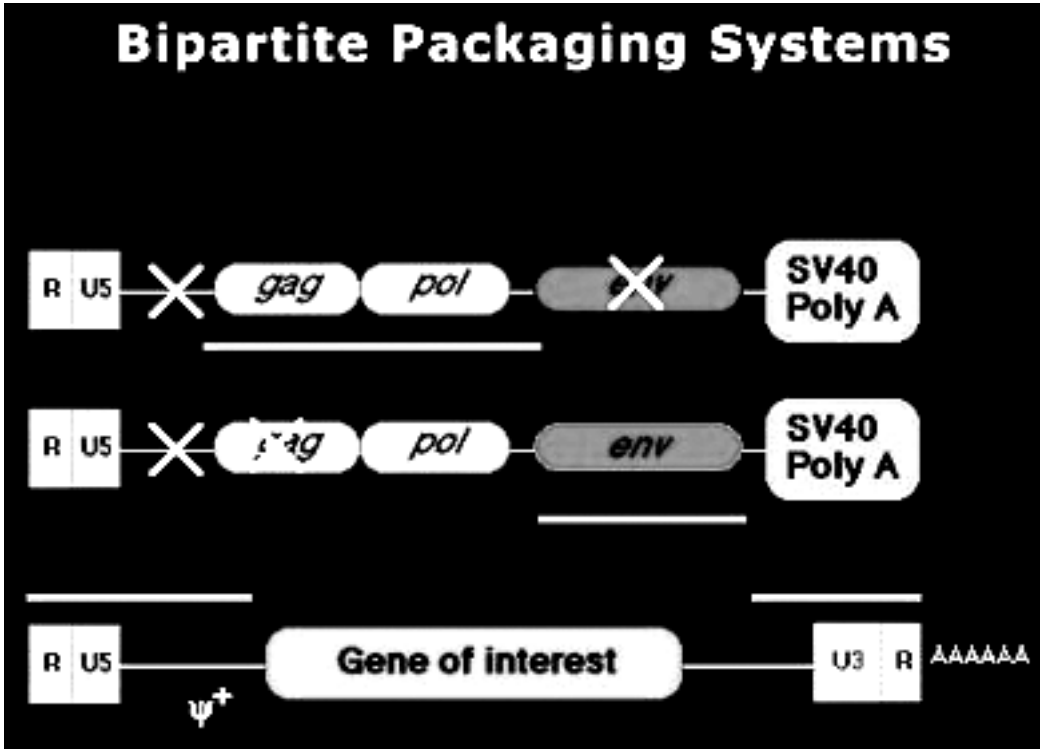
1. Introduce the vector into packaging a cell line that expresses the viral gag, pol, and env genes - necessary for particle formation and replication.

2. Retroviral expression vectors provide the packaging signal Ψ, transcription and processing elements, and a target gene.

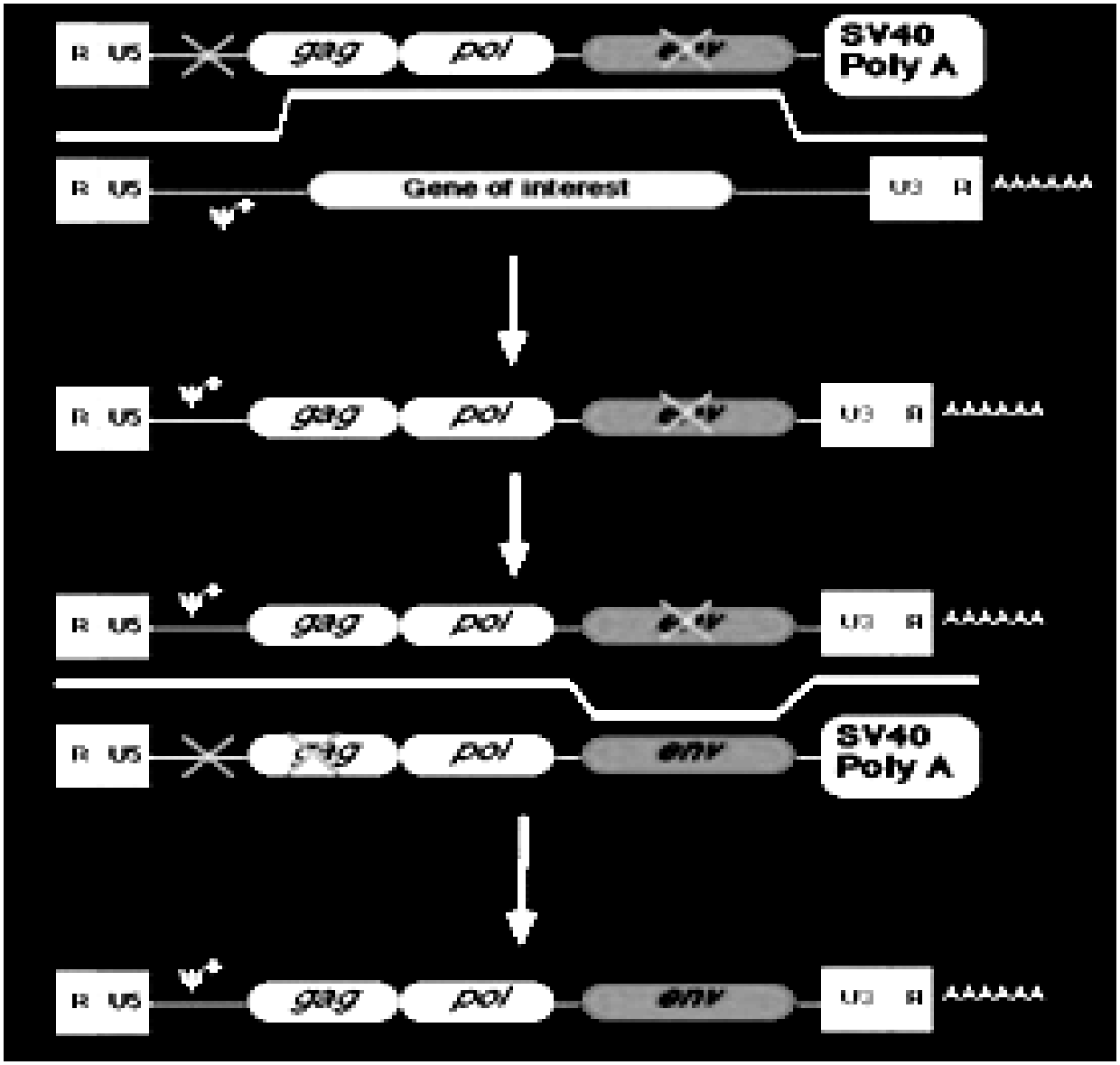
3. Inserts of up to 6.5 kb can be efficiently packaged.

4. The viral env gene, expressed by the packaging cell line, encodes the envelope protein, which determines the range of infectivity (tropism) of the packaged virus.

Unigenomic packaging cell lines



Multiple recombination events are required for the emergence of wild type virus



Altering the tropism of a recombinant retrovirus

The viral env gene, expressed by the packaging cell line, encodes the envelope protein, which determines the range of infectivity (tropism) of the packaged virus.

Viral envelopes are classified according to the receptors used to enter host cells:

Ecotropic virus can recognize a receptor found on only mouse and rat cells.

Amphotropic virus recognizes a receptor found on a broad range of mammalian cell types.

Dualtropic virus recognizes two different receptors found on a broad range of mammalian cell types.

A pantropic packaging cell produces virus that can infect both mammalian and non-mammalian cells. Using this cell line, virions are pseudo-typed with the envelope glycoprotein from the vesicular stomatitis virus (VSV-G) which mediates viral entry through lipid binding and plasma membrane fusion.

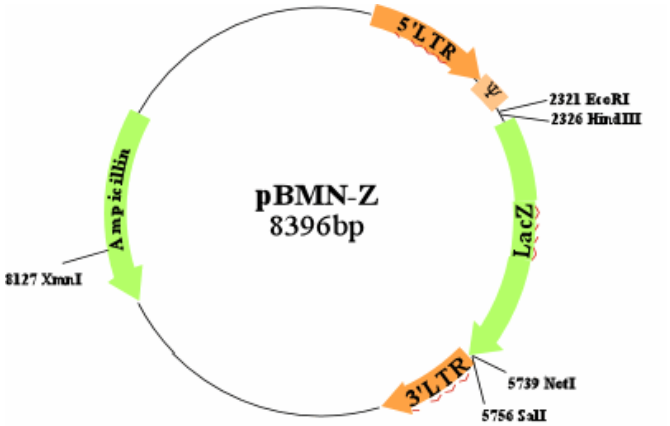
Stable expression of the VSV-G envelope protein is toxic. Thus, the packaging cell line only contains the viral gag and pol genes. Virus is produced by transiently co-transfecting a retroviral expression vector and pVSV-G into a pantropic packaging cell line.

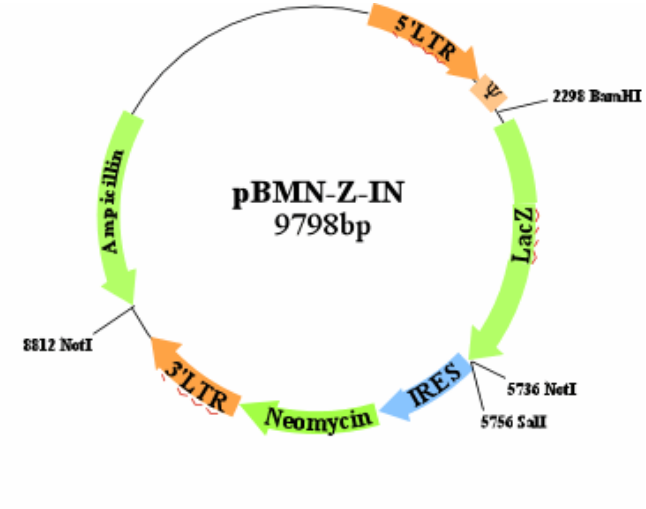
HOST RANGE OF PACKAGING CELL LINES EXPRESSING DIFFERENT ENVELOPES

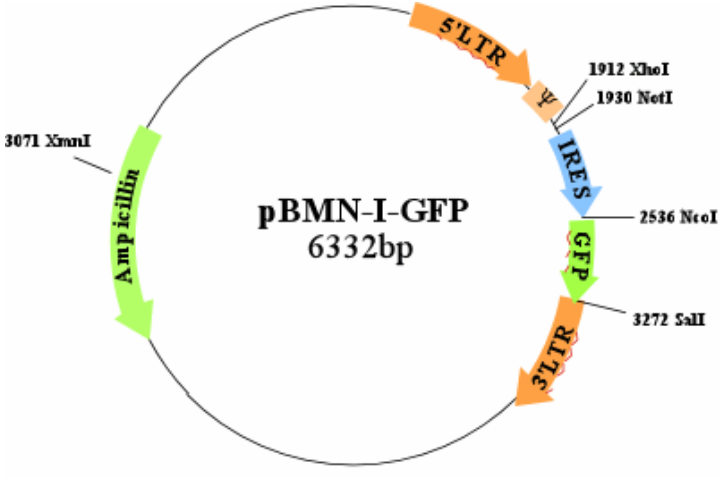
Target Cells	Envelopes			
	Dualtropic	Amphotropic	Ecotropic	Pantropic
Mouse	+	+	+	+
Rat	+	+	+	+
Hamster	+	+/–	–	+
Mink	+	+	–	+
Cat	+	+	–	+
Dog	+	+	–	+
Monkey	+	+	–	+
Human	+	+	–	+

Table I: Retroviral Packaging Cell Lines

Product	Cell Type	Tropism	Envelope	Receptors	Integrated Markers		Host Cell
					gag-pol	*env*	
EcoPack 2-293 Cell Line	HEK 293	Ecotropic	gp70	mCAT1	Bleo	Hyg	Rat and mouse
AmphoPack 293 Cell Line	HEK 293	Amphotropic	4070A	Ram-1 (rPit-2)	Bleo	Puro	Many mammalian cell types
RetroPack PT67 Cell Line	NIH 3T3	Dualtropic	10A1	GALV, RAM	TK	DHFR	Many mammalian cell types
GP2-293 Cell Line*	HEK 293	**	**	**	DHFR	n/a	All cell types







http://www.stanford.edu/group/nolan/plasmid_maps/pmaps.html

Retroviral vectors- Limitations

- A critical limitation of retroviral vectors is their inability to infect nondividing cells.
- Random integration of vector DNA into the host chromosome.

Gene transfer and expression using RNA viruses:

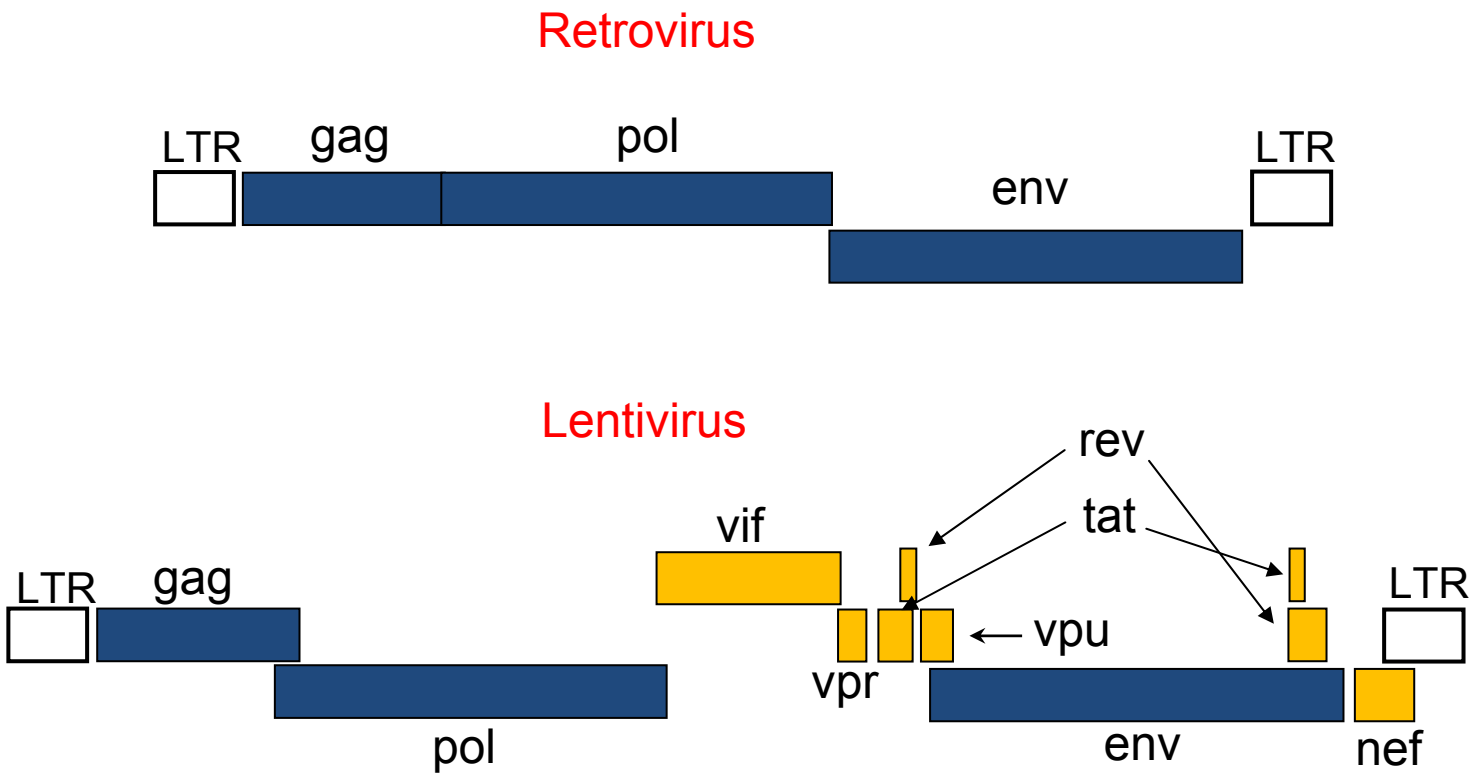
LENTIVIRUSES

Lentiviruses

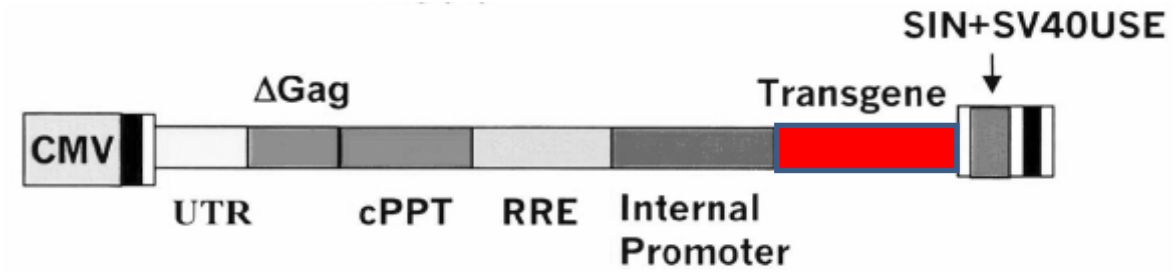
- Belong to the retrovirus family but can infect both dividing and non-dividing cells.

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

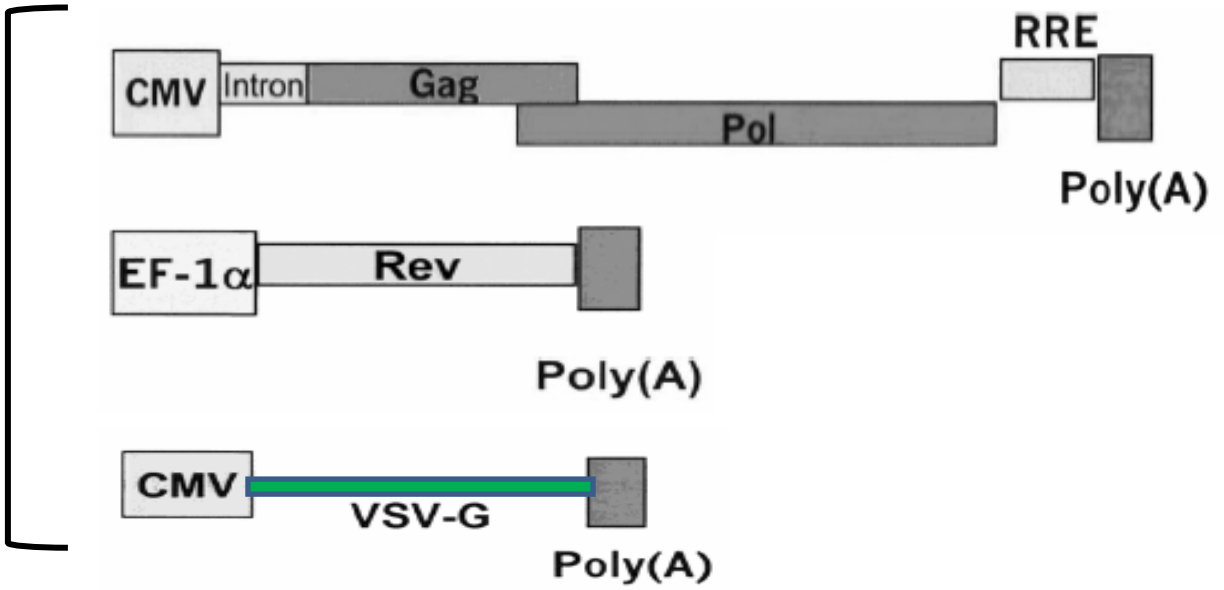
- Human immunodeficiency virus (HIV) has been disabled and developed as a vector for in vivo gene delivery.



Lentiviral vector



Packaging cells



The HIV-1 *env* glycoprotein has a highly restricted host range in that it infects cells containing CD4 and coreceptors. To broaden the host range of lentiviral vectors, they are pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) *env.*

1st Generation Vectors

- Limited homology between vector and helper sequences
- Separation of helper plasmids
- Still retains HIV accessory genes in the packaging plasmid

2nd Generation Vectors

Elimination of accessory genes from packaging plasmid

- No effect on vector titer
- Retains property of transduction of many dividing and non-dividing cells
- Increased safety margin

3rd Generation Vectors

Self-inactivating (SIN) vectors

- Deletion in the enhancer region of the 3' U3 of the long terminal repeat (LTR)
- Results in a transcriptionally inactive vector that can not be converted into a full length RNA
- Reduces likelihood of replication competent lentivirus regeneration
- Hampers mobilization by wild-type HIV
- May reduce risk of tumorigenesis via promoter insertion

http://www.clontech.com

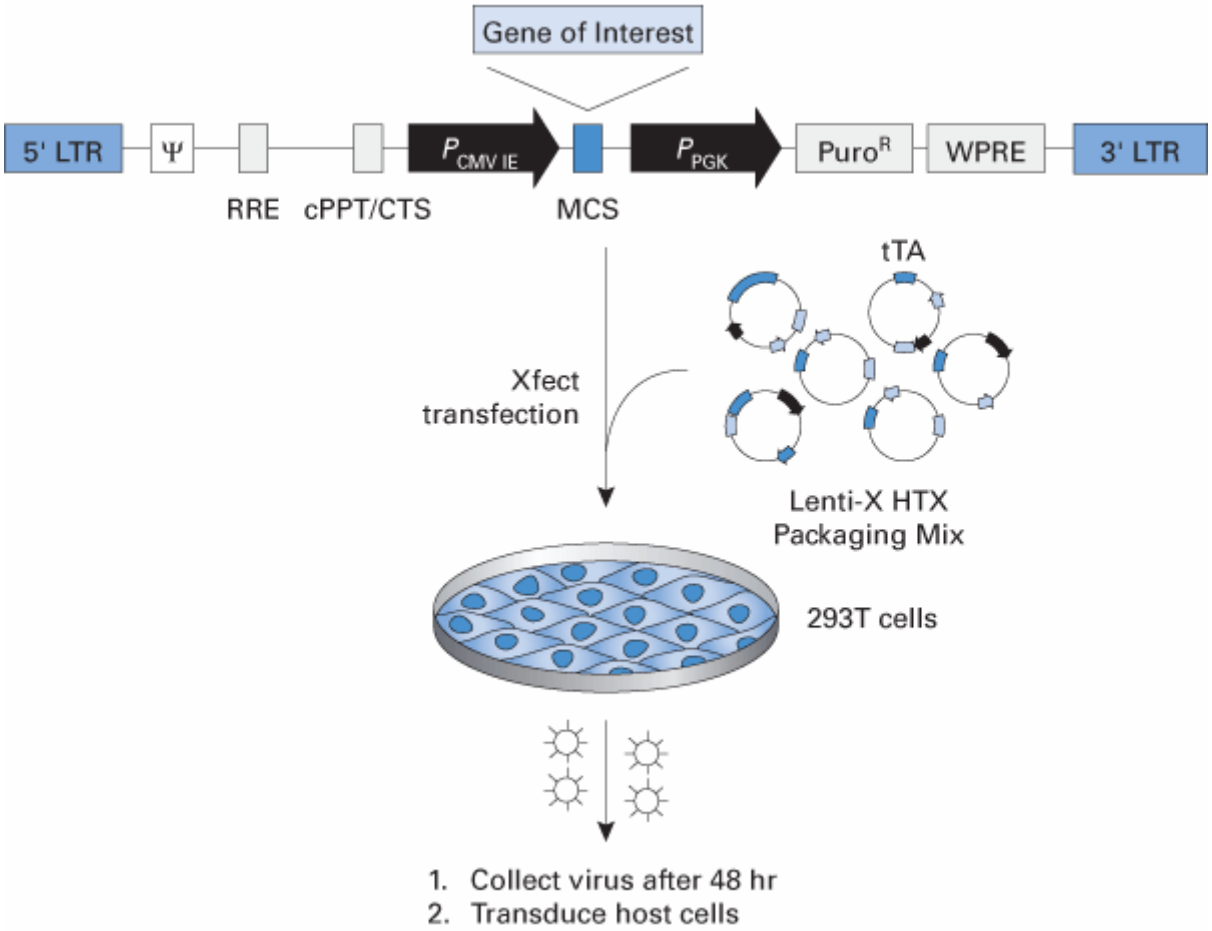
VSV-G lentiviral packaging

The standard **Lenti-X HTX Packaging System** produces VSV-G pseudotyped lentivirus, which readily infects virtually all types of cells.

Ecotropic Lentiviral packaging

The **Lenti-X HTX Ecotropic Packaging System** produces lentivirus pseudotyped with the MLV ecotropic envelope glycoprotein, which allows you to limit transduction to mouse and rat cells

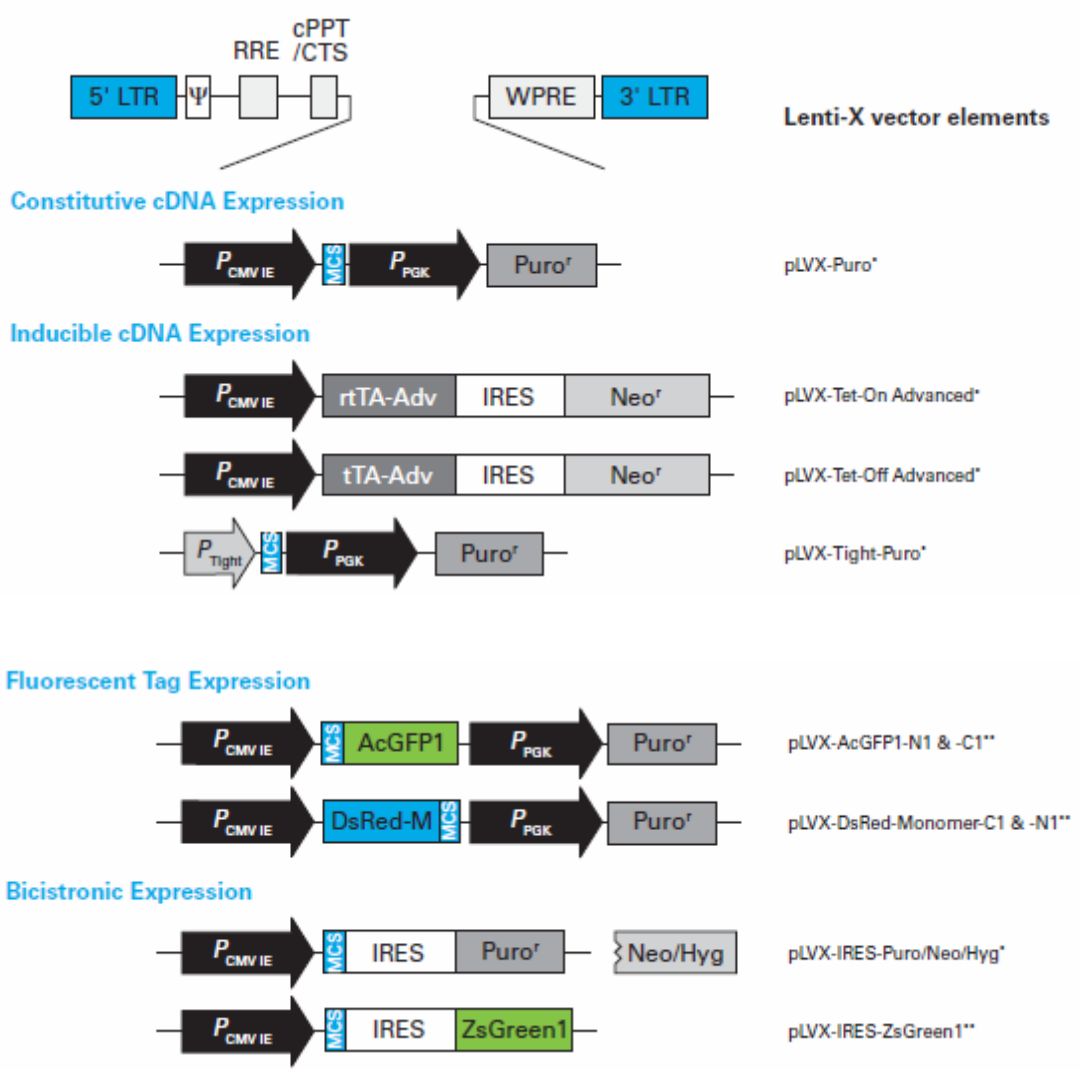
http://www.clontech.com



The Lenti-X HTX Packaging System. A lentiviral vector and the Lenti-X HTX Packaging Mix are cotransfected into 293T cells using the Xfect transfection system.

High titer virus supernatants can be obtained within 48 hr after transfection

Recombinant lentiviruses represent the latest generation of powerful, multipurpose vectors for delivering genes into almost any mammalian cell type, including primary cultures, nondividing cells, and stem cells.



Lenti-X vectors contain sequence elements that facilitate lentiviral packaging, boost transgene expression, or both.

Among them are the HIV-1 LTRs and packaging signal (Y), a Rev response element (RRE), the central polypurine tract/central termination sequence (cPPT/CTS), and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE).

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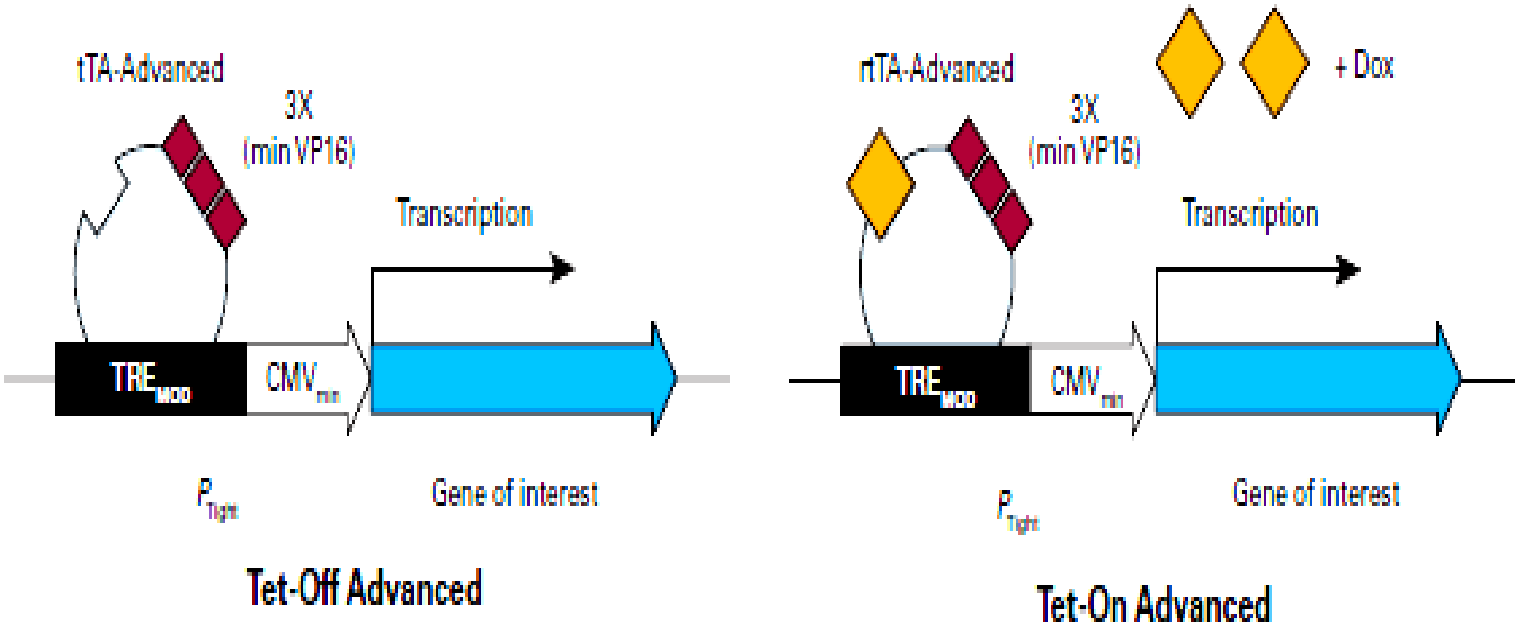


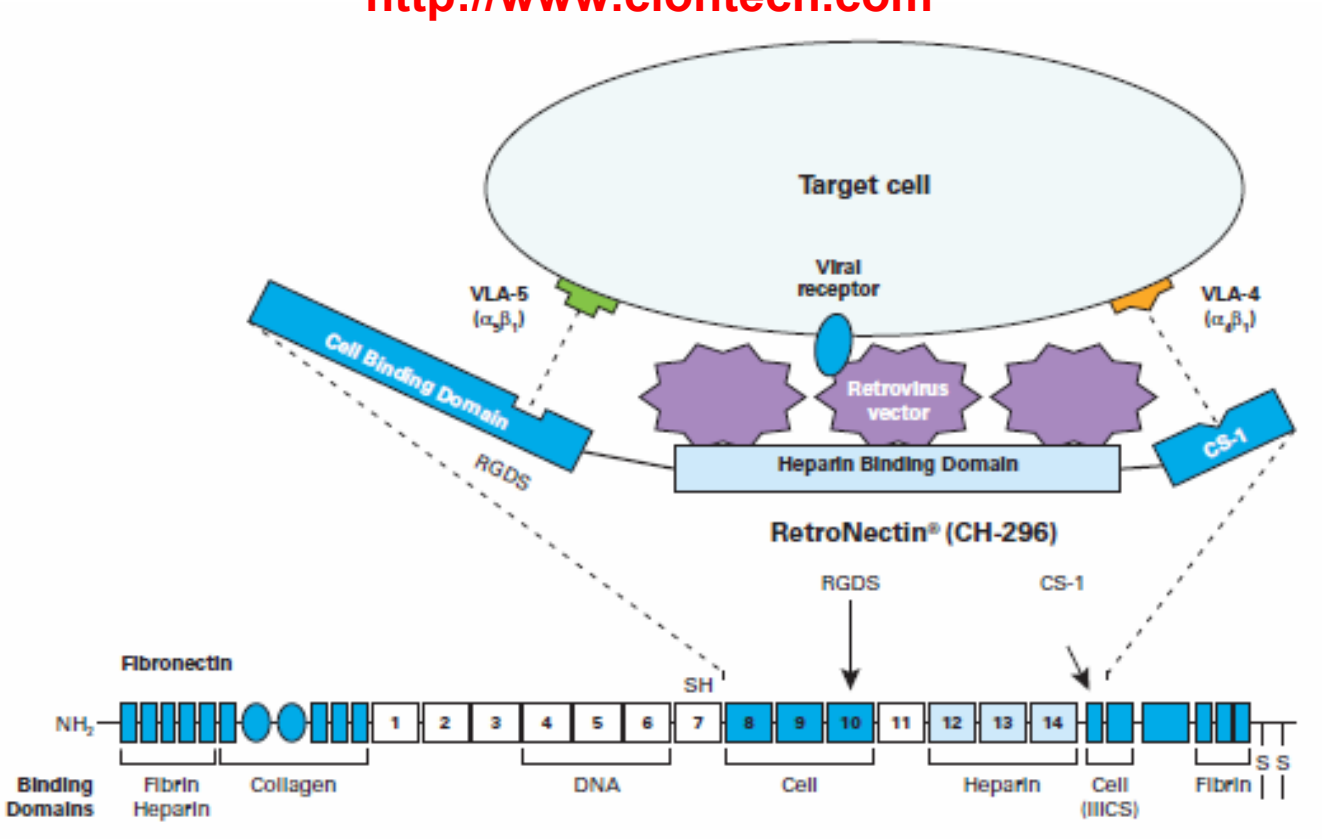
Induced expression in the Tet-Off Advanced and Tet-On Advanced Systems.

The Tet-controlled transactivators are fusion proteins that contain a DNA-binding TetR domain joined to three minimal transcription activation domains from HSV VP16.

In Tet-Off Advanced Systems, the basal state is maintained in the presence of Dox, whereas the withdrawal of Dox triggers the activated state.

In contrast, Tet-On Advanced Systems are activated in the presence of Dox. Induction in either system produces high-level transcription of your gene from *PTight.*

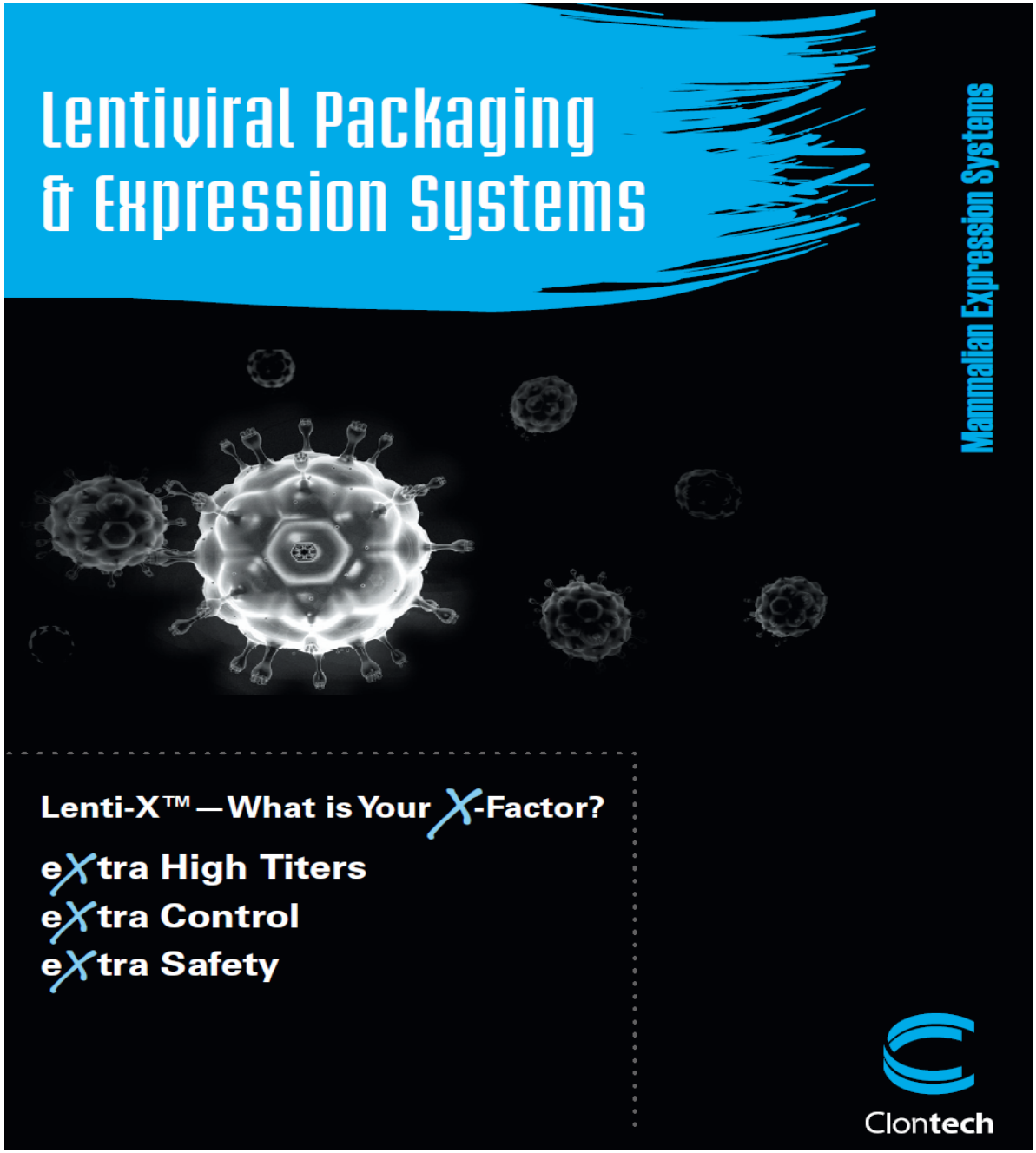
http://www.clontech.com



RetroNectin greatly enhances virus-mediated gene transduction.

RetroNectin is a chimeric recombinant peptide consisting of 3 functional domains derived from human fibronectin, that bind either cell surface proteinsor virus particles.

The enhancement is likely due to the colocalization of virus and cells on RetroNectin molecules.



http://www.clontech.com/images/brochures/BR8Y2848_LentiX_IN.pdf

User Manual

Retroviral Gene Transfer and Expression User Manual

http://www.clontech.com/images/pt/PT3132-1.pdf

GENE THERAPY: Twenty-First Century Medicine

Inder M. Verma and Matthew D. Weitzman

Annu. Rev. Biochem (2005) 74:711–38