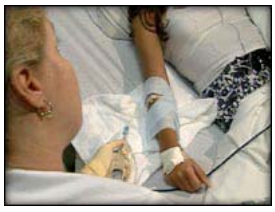
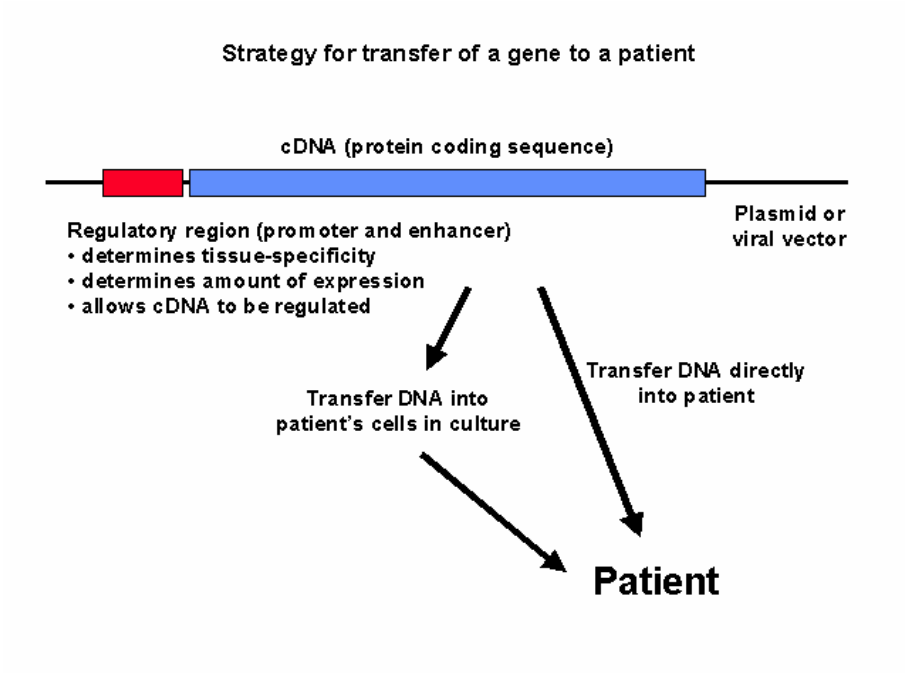
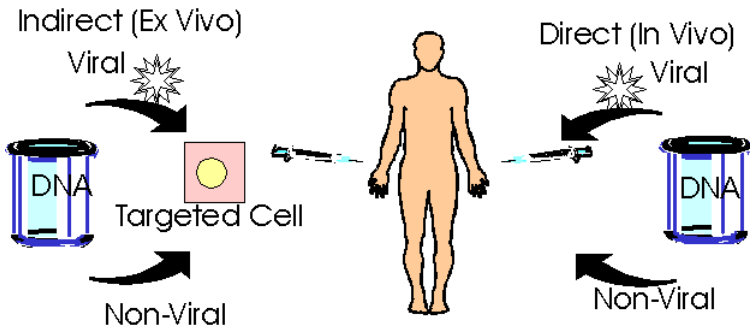
Gene Therapy




Strategy for transfer of a gene to a patient
cDNA (protein coding sequence)
Plasmid or viral vector
Regulatory region (promoter and enhancer)
• determines tissue-specificity
• determines amount of expression
• allows cDNA to be regulated
Transfer DNA into patient's cells in culture
Transfer DNA directly into patient
Patient

Three categories of somatic cell gene therapy:

1. *Ex vivo* – cells removed from body, incubated with vector and gene-engineered cells returned to body.
2. *In situ* – vector is placed directly into the affected tissues.
3. *In vivo* – vector injected directly into the blood stream.



Example of *ex vivo* somatic cell gene therapy

- Usually done with blood cells because they are easiest to remove and return.
- Sickle cell anemia

Example of *in situ* somatic cell gene therapy

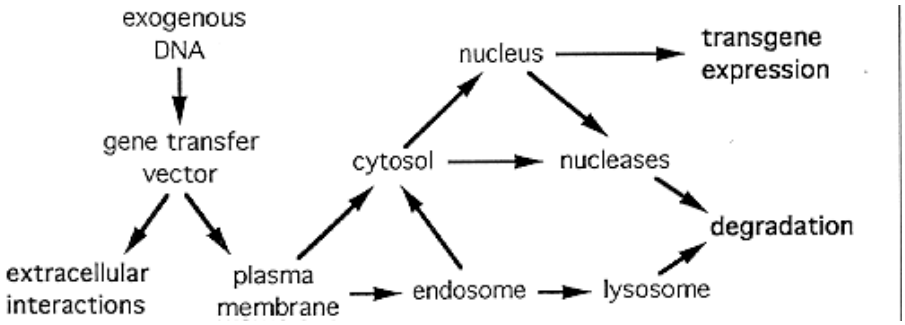
- Infusion of adenoviral vectors into the trachea and bronchi of cystic fibrosis patients.
- Injection of a tumor mass with a vector carrying the gene for a cytokine or toxin.
- Injection of a dystrophin gene directly into the muscle of muscular dystrophy patients.

Example of *in-vivo* somatic cell gene therapy

- No clinical examples.
- In vivo injectable vectors must be developed.

Barriers to successful gene therapy:

1. Vector development
2. Corrective gene construct
3. Proliferation and maintenance of target cells
4. Efficient transfection and transport of DNA to nucleus for integration into genome
5. Expansion of engineered cells and implantation into patient



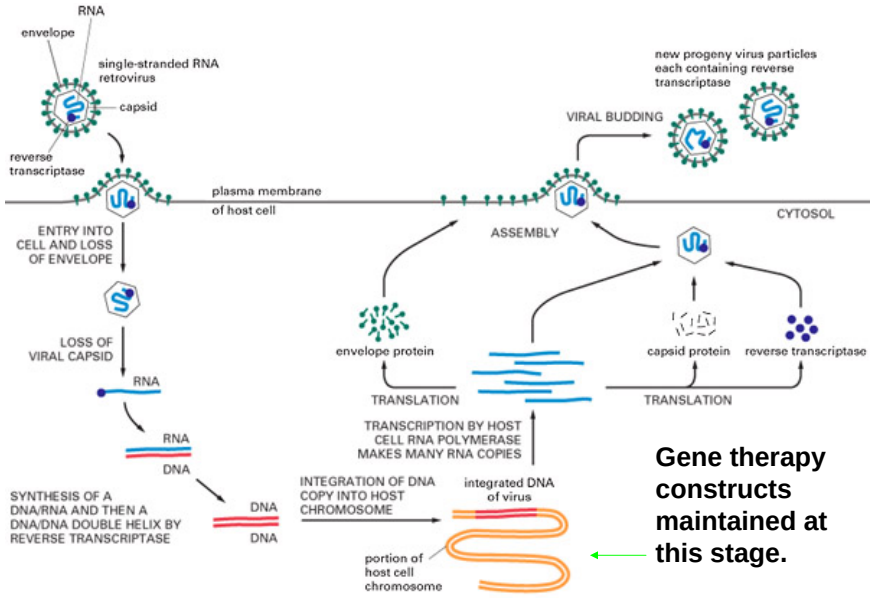
Types of vectors

- RNA viruses (Retroviruses)
  1. Murine leukemia virus (MuLV)
  2. Human immunodeficiency viruses (HIV)
  3. Human T-cell lymphotropic viruses (HTLV)
- DNA viruses
  1. Adenoviruses
  2. Adeno-associated viruses (AAV)
  3. Herpes simplex virus (HSV)
  4. Pox viruses

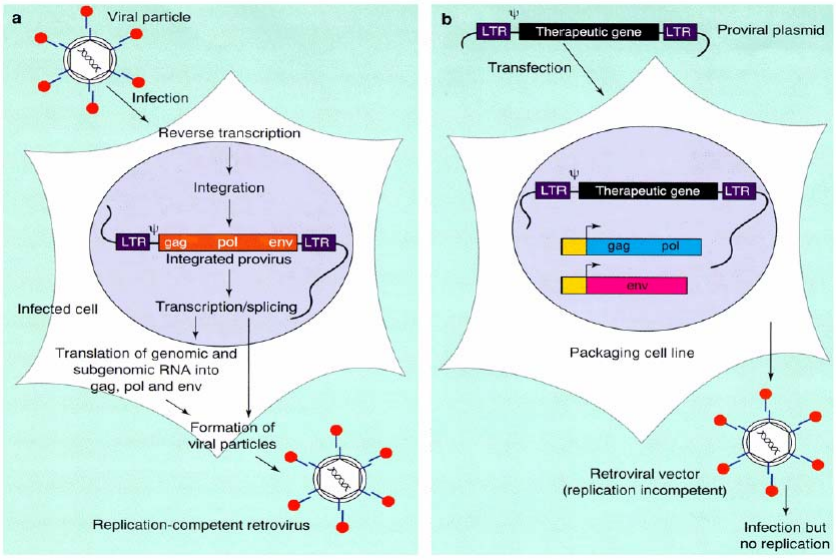
Non-viral vectors

1. Liposomes
2. Naked DNA
3. Liposome-polycation complexes
4. Peptide delivery systems

Life cycle of a retrovirus



Gene therapy constructs maintained at this stage.



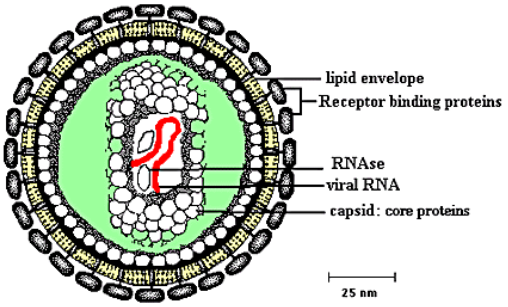


Diagram of a Retrovirus

Advantages:

1. Randomly integrates into genome
2. Wide host range
3. Long term expression of transgene

Disadvantages:

1. Capacity to carry therapeutic genes is small
2. Infectivity limited to dividing cells
3. Inactivated by complement cascade
4. Safety

A problem with retroviral vectors: they insert randomly into the genome.

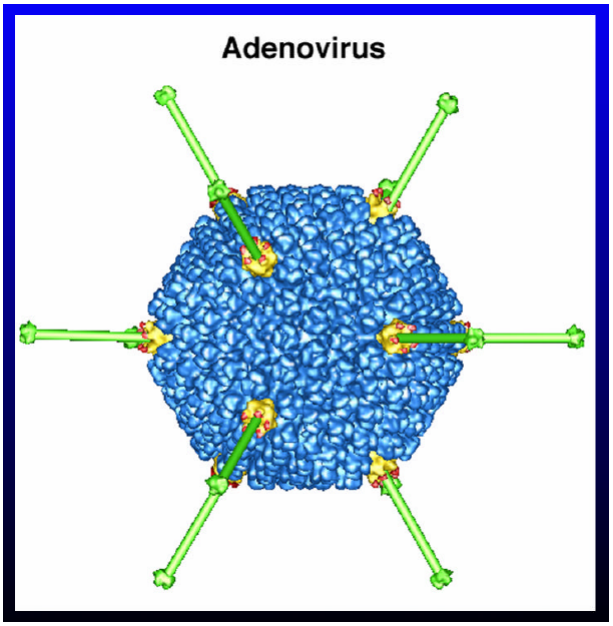
Using a murine leukemia virus vector, the γ C (constant) chain was inserted into 10 boys with SCID (no B or T cells). These are the "bubble" boys.

Removed bone marrow cells from the boys, purified CD34 cells from that population, transduced cells *ex vivo* and reintroduced cells.

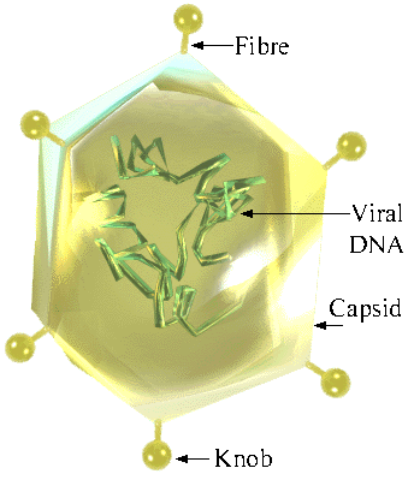
Startling success: all boys now had an adaptive immune system!!! All went home and started living more or less normal lives. Science, 2000.

Summer, 2001: two youngest boys that were given greatest numbers of virions came down with an acute T cell leukemia. Both were successfully treated for the leukemia

Analysis of the tumor cell: a clonal population of pre T cells that had the viral vector near a known T cell oncogene (that happens to be a specific pre T cell transcription factor) LMO5. Smoking gun evidence that retroviral vector in the two boys had inserted into a location that stimulated expression of LMO5 causing a selective advantage to those T cells and induction of a leukemia.



Adenovirus



Advantages:

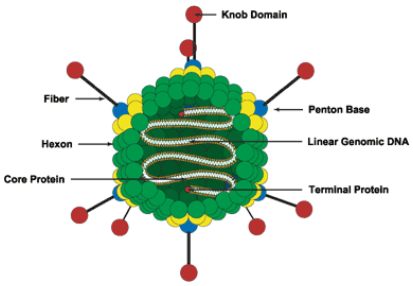
1. Efficiency of transduction is high
2. High level gene expression
3. Slightly increased capacity for exogenous DNA

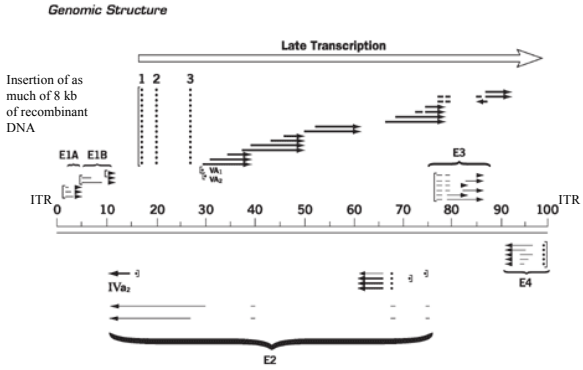
Disadvantages:

1. Expression may be transient
2. Cell-specific targeting difficult to achieve
3. Virus uptake is ubiquitous
4. Safety

Adenoviruses as delivery systems:

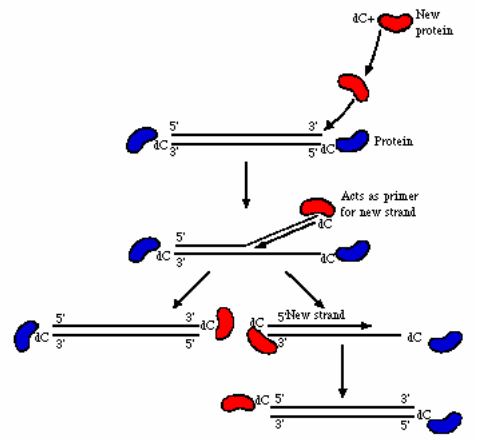
- most use adenovirus 5; infects dividing and non-dividing cells
- can enter many different cell types because it uses a relatively ubiquitous receptor for entry (CAR)
- produces very high titers of viral particles
- Non-enveloped virus – relatively stable virion





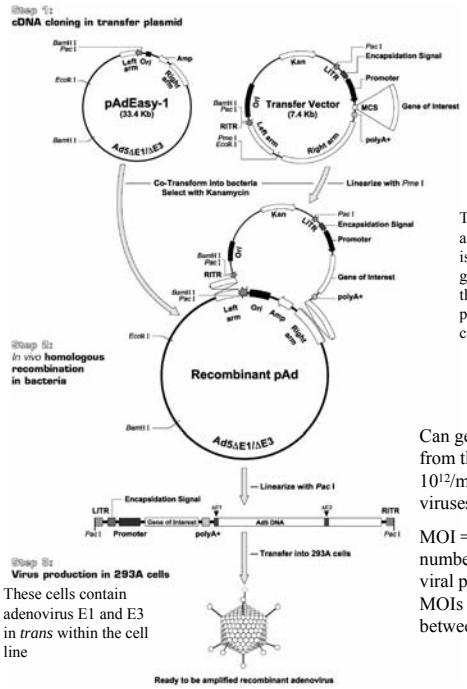
In adenovirus vectors, the essential genes E1 and/or E3 have been deleted and the proteins supplied in trans within the cell line. This prevents generation of replication competent viruses as well as wild type viruses

TP acts as a primer for initiation of synthesis.
DBP - a DNA-binding protein.
DNA Pol - 140kD DNA-dependent polymerase



- **The viral genome is coated with DBP.**
- **DBP helps binding of NFI.**
- **NFIII also binds at a specific recognition site between nucleotides 39 and 48.**
- **Protein-protein interactions, between NFI and pol, and pTP and NFIII help recruit the pTP-pol.**

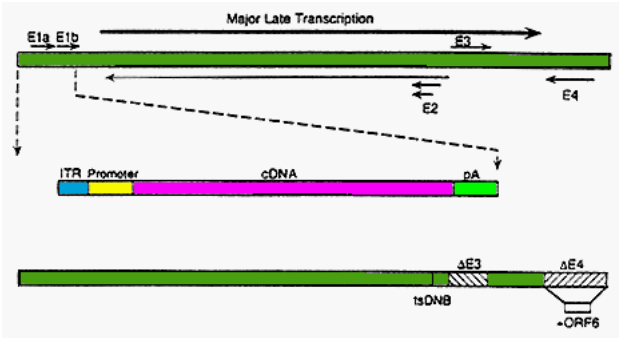
Creation of a defective, transducible virion containing the gene of interest.



Transfection of recombinant adenoviral DNA into a cell that is expressing E1 and E3 generates new viral particles that can be collected and particles can transduce new cells

Can get high titer recombinant virus from these transfections (~10^9 - 10^{12}/ml). Delivery of recombinant viruses to cells can transduce >90%.

MOI = multiplicity of infection + number of infectious or transducable viral particles/target cell. Adeno MOIs frequently are somewhere between 1 and 100 MOI



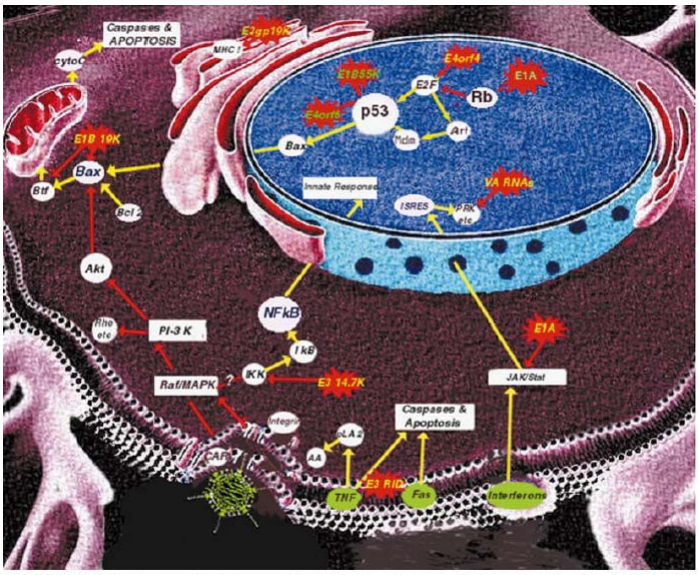


Fig. 5. A cartoon (not to scale) illustrating some of the sites of action of the virus and virus gene products (in red) on a few of the cellular pathways (in yellow). A virus particle at the receptor site is in green.

Drawbacks of using adenoviruses:

- transient expression - adenovirus is not stably maintained in cells – expression is usually lost over a several week period

- adenoviruses are highly immunogenic - cannot effectively introduce multiple rounds of the same adenovirus serotype in vivo, but there are several different serotypes of adenoviruses. Would need to put your gene of interest into each different serotype if you wanted to perform multiple transductions *in vivo*

- can be pathogenic – 1999 University of Pennsylvania gene therapy death using the largest quantity of Ad5 vector into a human – severe immune response and coagulation with no evidence of gene expression.

- productive viral replication is highly cytolytic to the infected cell, in vivo delivery system use defective virus.

Other viral vectors

- Adeno-associated virus – infects wide range of cells (both dividing and non-dividing), able to integrate into host genome, not associated with any human disease, high efficiency of transduction.
- Herpes simplex virus, vaccinia virus, syndbis virus
- Onyx virus – limited replicating adenovirus that replicates mainly in tumor cells.

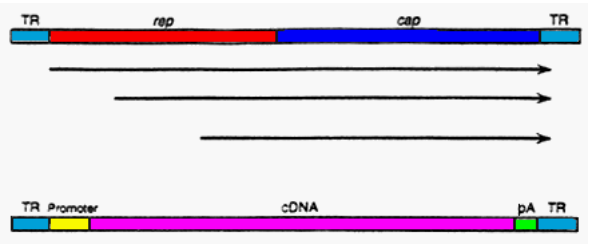
PARVOVIRIDAE BIOLOGY

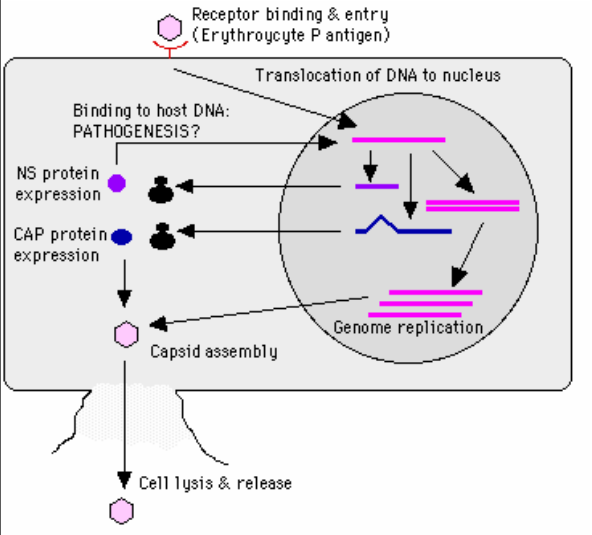
1. Small nonenveloped, icosahedral viruses with ~5 kb ssDNA genome ; some infect warm-blooded animals, others infect insects.

2. Parvoviruses are important pathogens of dogs and cats where they destroy dividing cells of the immune system; parvoviruses require actively dividing cells for replication (no host stimulation proteins).

3. Adeno-associated virus (AAV) is a well known defective parvovirus (*dependovirus* subgroup) that relies on co-infection with adenovirus or herpes virus as helper to stimulate host cell division.

4. AAV has potential as a human therapeutic vector because its genome integrates at only one specific site in the human genome (chromosome 19). (infection by itself, integration frequency > 70%)





Parvovirus replication

•Replication occurs in the nucleus & is thought to follow the scheme to the left.

• All Parvoviruses are highly dependent on cellular functions for genome replication.

•The autonomous viruses require the cell to pass through S-phase for replication to occur

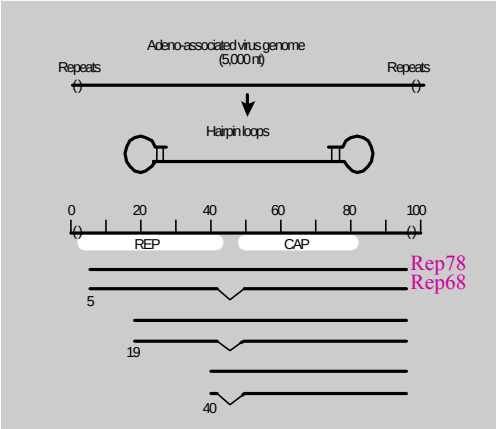
•The defective viruses are even more dependent - require host cell machinery + helper virus for replication.

PARVOVIRUS REPLICATION

1. Only two ORF's (rep and capsid protein) translated from three families of 3' co-terminal, spliced transcripts.

2. Replication uses host DNA polymerase but mechanism is unique; priming relies on hairpin loops formed by terminal inverted repeat sequences (~120-330 bases).

3. Rep protein is required for DNA synthesis (makes specific nicks in hairpin loops); Rep is also required for integration.



Rep78 & Rep68 both have DNA binding, endonuclease, & helicase activity

Source: Dr. Perrault's lecture notes

Adenovirus associated virus (AAV)

Advantages:

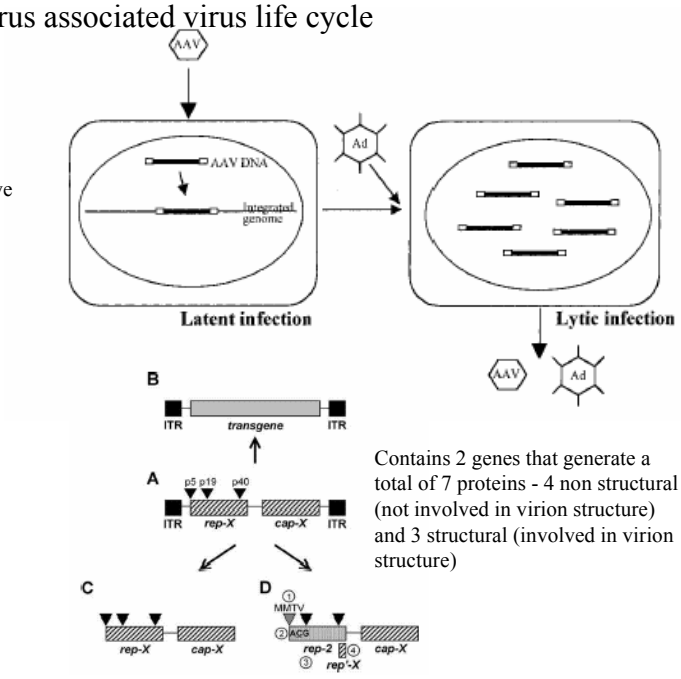
- not associated with disease
- can obtain high titered virus stocks (10^9- 10^{10}/ml)
- small genome that is easy to manipulate
- stable integration into the genome in chromosome 19
- infects both dividing and nondividing cells

Disadvantages:

- can only contain ~4.5 kb of DNA

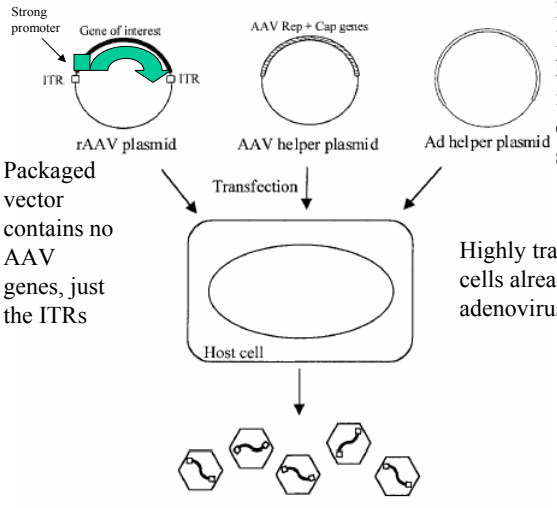
Adenovirus associated virus life cycle

6 different serotypes of virus that have differing cellular tropisms.



Contains 2 genes that generate a total of 7 proteins - 4 non structural (not involved in virion structure) and 3 structural (involved in virion structure)

Use of AAV as a gene delivery agent



Required adenovirus proteins: E1, E2a, E4, VA. Rather than using Ad helper plasmid, can use adenovirus infected cells - but can have difficulties with Ad contaminated stocks of AAV

Packaged vector contains no AAV genes, just the ITRs

Highly transfectable 293 cells already contain the adenovirus gene E1

Can generate very high titered stocks

Okada, et al (2002) Methods, 28:237 and other articles in same issue of Methods

Ability to use multiple AAV constructs to generate proteins that are encoded by genes larger than 4.5 kb

Package half of gene-of-interest in one particle and the other half in a different particle and co-transduce cells.

Approach is relatively inefficient, but does work via transplicing of RNA that is generated

HERPES SIMPLEX VECTORS

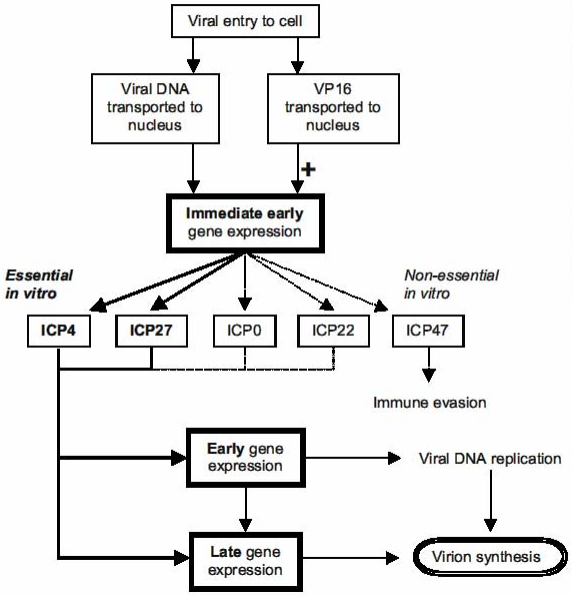
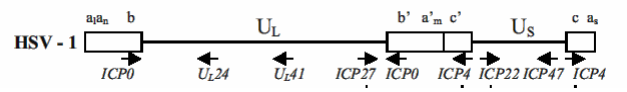
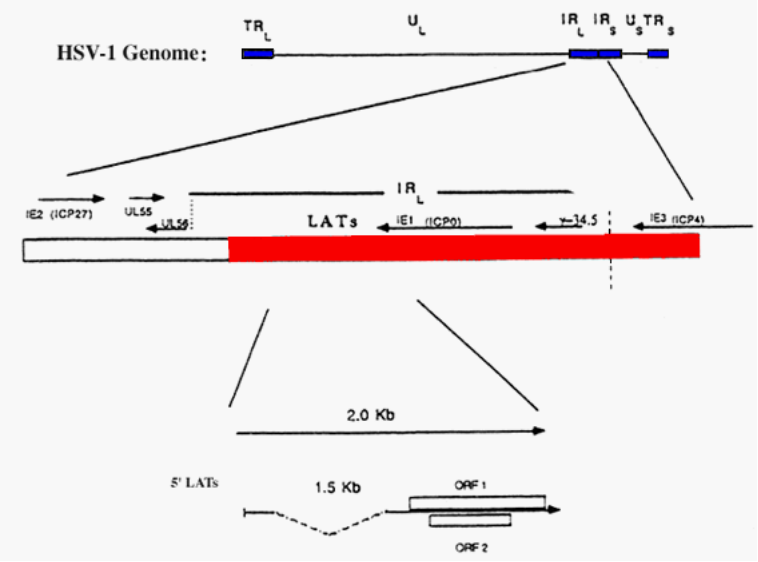


Figure 2.

Flow chart depicting the cascade of regulatory events that result in ordered sequential expression of HSV-1 genes during wild-type infection. In order to proceed to E and L gene expression from IE gene expression, both ICP4 and ICP27 must be expressed. Inactivation of either results in loss of E and L gene products and failure to produce infectious virus. Suitable ICP4 and ICP27 expressing cell lines may complement these gene products in trans. Full details of the construction of replication-deficient viruses may be found in the text and references.

HERPES SIMPLEX GENOME





TREATMENT OF CARCINOMA WITH HSV

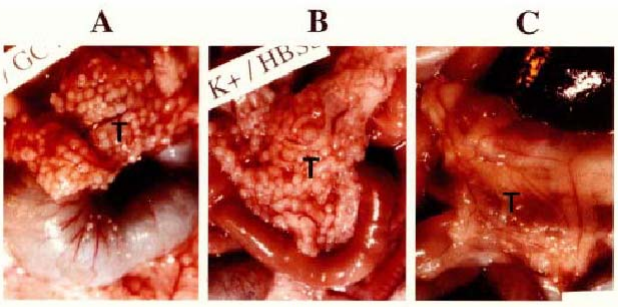


Figure 25. Eradication of peritoneal carcinomatosis with HSV-tk plus GCV. Intraperitoneal injection to rats of DHD/K12 colon carcinoma cells stably expressing the HSV-*tk* gene caused peritoneal carcinomatosis at day 21 (**A**). The animal whose intraperitoneal cavity is shown in (**B**) was treated with HBSS buffer alone and the animal shown in (C) was treated with GCV for 5 days at 150mg/Kg. The letter "T" indicates the peritoneal tumor nodes. From Lechanteur C, Princen F, Bue SL, Detroz B, Fillet G, Gielen J, Bours V, and Merville M-P (**1997**) *HSV-1* thymidine kinase gene therapy for colorectal adenocarcinoma-derived peritoneal carcinomatosis. **Gene Ther 4**, 1189-1194. Reproduced with the kind permission of the authors (Vincent Bours, University of Liège, Belgium) and of Stockton Press.

Poxvirus life cycle: early, middle and late gene expression

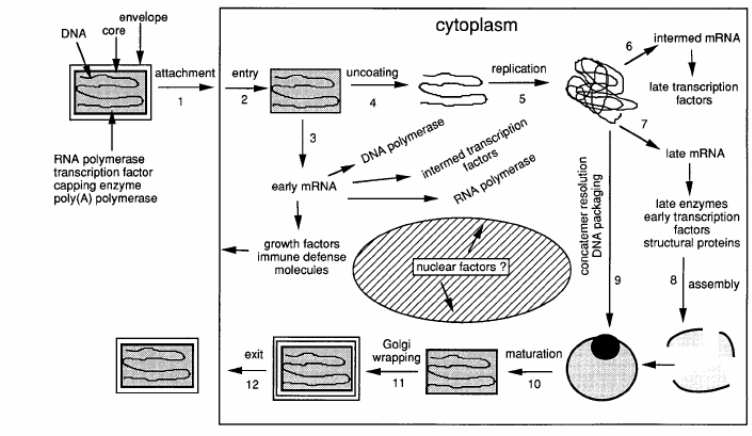


FIG. 2. Infectious cycle of vaccinia virus (from ref. 8, with permission).

Entire life cycle occurs in the cytoplasm using a virally encoded DNA dependent DNA polymerase and a DNA dependent RNA polymerase. Because the virus requires its own enzymes for replication and transcription, purified, naked viral DNA is not infectious

Poxviruses:

- family members: smallpox, cowpox, vaccinia, monkey pox, canary pox

- large, highly complex and understudied viruses

- only family of DNA viruses that replicates in cytoplasm of cell

-vaccinia is used as vaccine against smallpox

- highly immunogenic

- vaccinia has very, very, very broad tropism for different cells from different species

- through homologous recombination in vaccinia infected cells can introduce genes into the virus allowing:

    - production of large quantities of recombinant protein from infected cells

    - serve as delivery system for recombinant vaccines

Moss (1996) PNAS, 93: 11341

Homologous recombination in the vaccinia virus system:

Plasmid containing:

-Vaccinia promoter (several available depending on when you want your gene of interest expressed in the infected cells – early, intermediate or late in infection) and a portion of the vaccinia genome that will be targeted for insertion

- clone in gene of interest behind the promoter (up to 25 kb)

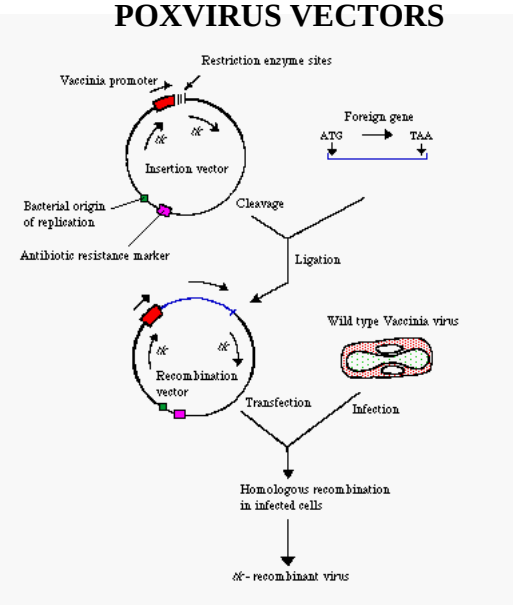
- plasmid frequently contains some sort of selectable marker

    - eukaryotic antibiotic

    - β-galactosidase, etc.

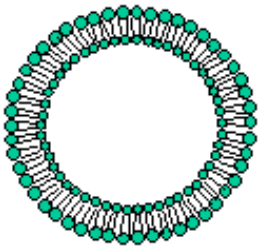
- transfect into vaccinia expressing cells and homologous recombination will occur about 0.1% of the time

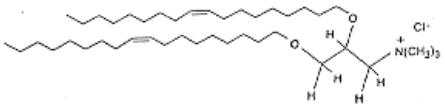
POXVIRUS VECTORS



Non-viral vectors

1. Liposome
2. Cationic polymers

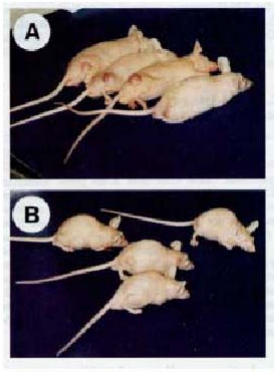




3. Naked DNA
4. Peptide-mediated gene delivery

May overcome limitations with viruses including small capacity for therapeutic DNA, difficulty in cell-type targeting and safety concerns.

Site/disease	Transgene(s)	Reference
Tumours		
Prostate	p16	Steiner *et al.* (2000)
	PKC	Fujii *et al.* (2000)
	Cytolytic virus	Rodriguez *et al.* (1997)
	Fas ligand	Hedlund *et al.* (1999)
Colon	GM–CSF/IL-2	Diaz *et al.* (1998)
	CD suicide gene	Topf *et al.* (1998)
	APC	Shih *et al.* (2000)
	Lymphotactin + suicide	Ju *et al.* (2000)
Cervix	p21	Tsao *et al.* (1999)
	Papillomavirus p21	He *et al.* (2000)
Ovary	Stomatostatin receptor	Rogers *et al.* (1999)
	Cytolytic virus/IL-6	Rancourt *et al.* (1999)
Endometrium	p53/p21	Ramondetta *et al.* (2000)
Gliomas	Caspase-3/Fas ligand	Shinoura *et al.* (2000)
Carcinoembryonic antigen-producing cancer	HSV-tk	Kijima *et al.* (1999)
Other conditions		
Autoimmune diabetes	Adenovirus E3	von Herrath *et al.* (1997)
Glycogen storage disease II	Glucosidase	Amalfitano *et al.* (1999)
CNS conditions	(via astrocytes)	Ridet *et al.* (1999)
Factor VIII deficiency	Factor VIII	Balagu *et al.* (2000)
OTC deficiency	OTC	Batshaw *et al.* (1999)
Liver graft	Bcl-2	Bilbao *et al.* (1999*a*)
Leishmania infection	IL-12	Gabaglia *et al.* (1999)
Tay–Sachs disease	Hexosaminidases	Guidotti *et al.* (1999)
Motor neurone disease	Neurotrophic factor	Haase *et al.* (1999)



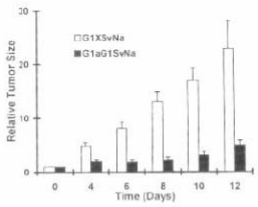
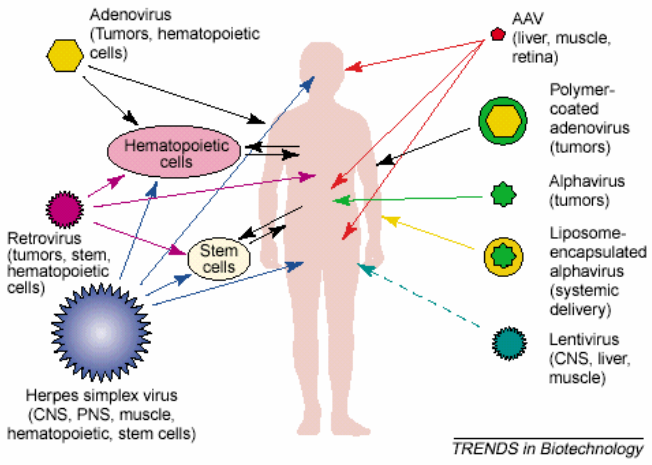


Figure 29. Photographs of nude mice treated with antisense cyclin G vector (**panel A**) have smaller tumors that animal treated with a control vector (**panel B**). **Panel C**: the relative tumor size (% of day 0 tumor size divided by 100) is plotted, on the vertical axis, as a function of time (days), plotted on the horizontal axis. From Chen DS, Zhu NL, Hung G, Skotzko MJ, Hinton DR, Tolo V, Hall FL, Anderson WF, Gordon EM (**1997**) Retroviral vector-mediated transfer of an antisense cyclin G1 construct inhibits osteosarcoma tumor growth in nude mice. **Hum Gene Ther 8**, 1667-1674. Reproduced with kind permission from the authors and Mary Ann Liebert, Inc.

Examples of Gene Therapy Trials

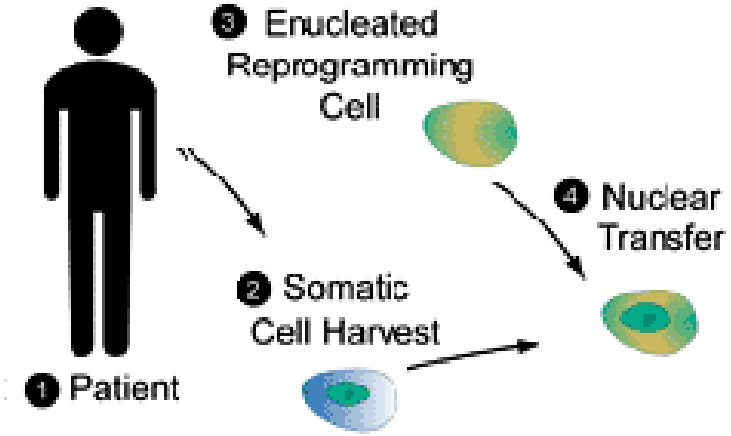
- Adenosine deaminase gene transfer to treat Severe Combined Immuno-Deficiency (SCID)
- CFTR gene transfer to treat Cystic Fibrosis (CF)
- Advanced Central Nervous System (CNS) Malignancy
- Mesothelioma
- Ornithine Transcarbamylase Deficiency
- Hemophilia
- Sickle Cell Disease

Targeting of different organs by viral vectors

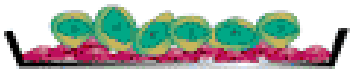


Lundstrom (2003) Trends in Biotechnology, 21: 117.

One developing technology that may be utilized for gene therapy is nuclear transfer ("cloning").



5 Laboratory Process to Derive Histocompatible Human Pluripotent Stem Cells



6 Histocompatible Human Pluripotent Stem Cells

7 Extend Replicative Lifespan with Telomerase

8 Differentiate Into Desired Cells

Ethical Considerations

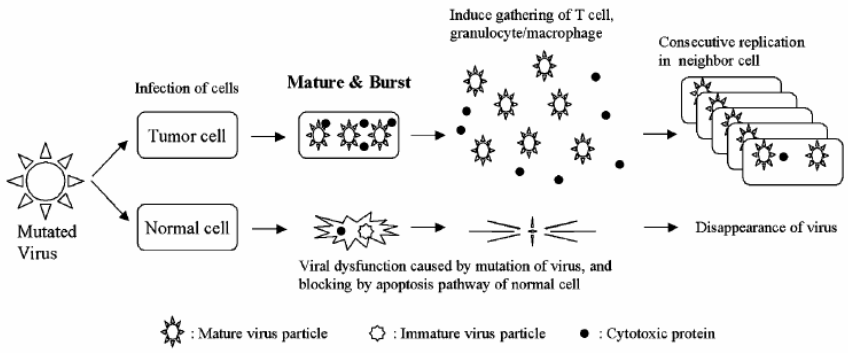
- Use of technology for non-disease conditions such as functional enhancement or "cosmetic" purposes – for example, treatment of baldness by gene transfer into follicle cells , larger size from growth hormone gene, increased muscle mass from dystrophin gene.
- In *utero* somatic gene therapy – only serious disease should be targeted and risk-benefit ratios for mother and fetus should be favorable.
- Potential for real abuse exists by combining cloning and genetic engineering.

Assigned Paper Review

Table 1 Clinical trial using oncolytic virus

Name	Genetic alterations	Disease	Phase
ONYX-015 (Adv)	E1B-55 kDa deletion	Head & neck cancer Ovarian cancer liver tumors *Pancreatic cancer*	II–III I I–II *I–II*
G207 (HSV-1)	LacZ insertion into ICP6 gene; deletion of both copies of γ34.5	Malignant glioma	I-II
NV1020 (HSV-1+2)	700 bp tk deletion +15 kb deletion across the joint region which contains an exogenous copy of tk gene under control of HSV-1α4 promoter and a 5.2 kb fragment of HSV-2 DNA	Metastatic liver tumor	I
OncoVEX GM-CSF (HSV-1)	GM-CSF insertion into ICP47 and deletion of both copies of γ34.5	Breast cancer Head & neck cancer Melanoma	I–II I–II I–II
1716 (HSV-1)	Deletion of both copies of γ34.5	Malignant glioma	I
HF10 (HSV-1)	Natural mutation of the end of UL and UL/IRL, resulting in loss of UL56	Breast cancer	I
CV706 (AdV)	Regulation of E1A under the PSA Promoter; E3 deletion	Prostate cancer	I–II
CV787 (AdV)	Regulation of E1A under the rat probasin promoter and E1B under the human PSA promoter; wild-type E3	Prostate cancer	I–II
Vaccinia-GM-CSF	Insertion of GM-CSF and LacZ genes into viral TK locus	Melanoma	I–II
PV701 (NDV)	Naturally attenuated	Advanced solid cancer	I

Mechanisms of oncolytic effect in tumor cell



1. Direct cell lysis caused by viral replication
2. Direct cytotoxicity caused by viral proteins
3. Induction of antitumoral immunity
   - Non-specific (e.g., tumor necrosis factor)
   - Specific (e.g., cytotoxic T lymphocytes)
4. High sensitization to chemotherapy and radiation therapy
5. Transgene expression
   - Enzyme-prodrug combination
   - Antiangiogenesis (e.g., endostatin)

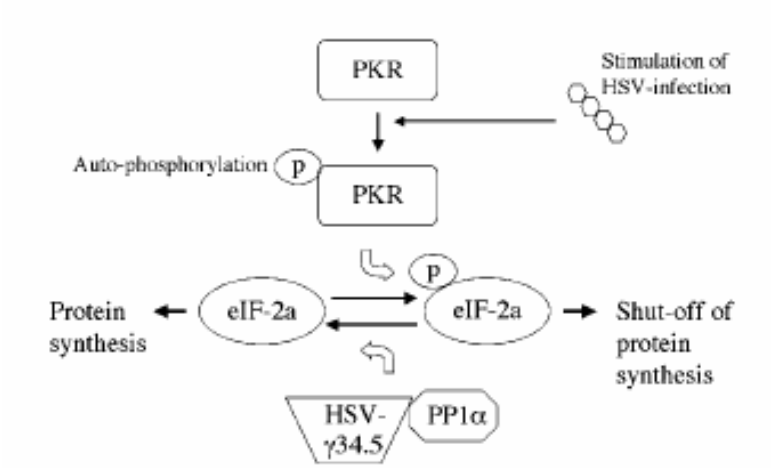


Figure 3 Cell protein kinase receptor (PKR) is stimulated by HSV-1 infection, and activated by auto-phosphorylation. Activated PKR phosphorylates eIF-2α Phosphorylated eIF-2α shut-off intra cell protein synthesis, and viral replication. HSV-1 γ34.5 with cell protein phosphatase-1α dephosphorylates eIF-2α, and allows protein synthesis.

Table 3 Promoter gene element

Gene	Tumor specificity	Oncolytic virus (reference)
Carcinoembryonic antigen (CEA)	Pancreatic, colon, and gastric carcinoma	CEAγ34.5 (78)
Muc-1	Pancreatic and breast ductal carcinoma	Ad.DF3-E1 (31), DF3γ34.5 (79)
ERBB2	Pancreatic, breast, and gastric carcinoma	
Amylase	Pancreatic acinar cell carcinoma	
Insulin	Pancreatic islet cell carcinoma	
Albumin	Albumin-producing carcinoma	G92A (80)

Table 4 Enzyme–prodrug combination

Prodrug	Product	Enzyme
Methotrexate-alanine	→ Methotrexate	Carboxypeptidase A
Benzoic acid mustard-glucuronide	→ Benzoic acid mustard	Carboxypeptidase G2
5-Deoxy-5-fluorouridine	→ 5-Fluorouracil	Thymidine phosphorylase
Etoposide phosphate	→ Etoposide	Alkaline phosphatase
Cyclophosphamide	→ Acrolein and phosphoramide-mustard	Cytochrome *P*450 2B1
5-Fluorocytosine	→ 5-Fluorouracil	Cytosine deaminase
Ganciclovir	→ Ganciclovir triphosphate	Thymidine kinase

Good reviews on the topics:

1. Baker (2004) Progress in Biophys. Mol. Biol., 84: 279 – good review on in vivo delivery systems – both non-viral and some viral (Ad and AAV only). Focus is on cardiology.
2. Lundstrom (2003) Trends in Biotechnology, 21:117. Good general review.
3. Sanders (2002) Current Opinions in Biotechnology, 13: 437. Good review on advantages and techniques of pseudotyping retroviruses.
4. Thomas, et al (2003) Nature Reviews Genetics, 4: 346. Really nice overview.