Ministry of Higher Education and Scientific Research
University of Baghdad
College of Science
Department of Biology

Gene Therapy
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ماجستير/ أحياء مجهرية
الفصل الدراسي الثاني

تدريسي المادة :

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Lec.1 Gene Therapy

What is Gene Therapy?

A- Gene therapy is a technique that uses genetic material (a piece of DNA) for the long-term treatment of genetic disorders.

B- This may involve delivering a copy of a healthy or therapeutic gene, repairing a faulty gene, and/or altering the degree to which a gene is turned 'on' and 'off'.

Trials began in 1990

Cystic fibrosis gene moderately successful

Aims of Gene Therapy?

1. To stop or slow the growth of damaged cells.

2. Make it easier for immune system to destroy damaged cells.

3. To prevent other cells and tissue from disease.

General approaches used in gene therapy?

1. Insert normal gene instead of the damaged gene.

2. Substitution of damaged gene by creating new recombinants.

3. Repair the damaged gene.

4. Regulate the function of the damaged gene.

Each of us carries about half a dozen defective genes. We remain blissfully unaware of this fact unless we, or one of our close relatives, are amongst the many millions who suffer from a genetic disease.

About one in ten people has, or will develop at some later stage, an inherited genetic disorder, and approximately 2,800 specific conditions are known to be caused by defects (mutations) in just one of the patient's genes

What vectors are used in Gene Therapy?

- Viruses eg. retro viruses, adenoviruses (commonly used)

- Direct introduction (“golden bullets”)

- Liposomes

- Endocytosis of DNA bound to cell surface receptors (low efficiency)

- Artificial chromosome (under development)

- Plasmids

- Mini genes

- Else

Lec.2 Gene Therapy

Methods are used to introduce Gene Therapy

1. ***Ex vivo*** is any procedure accomplished outside.

In gene therapy clinical trials cells are modified in a variety of ways to correct the gene. In *ex vivo* **cells** are modified outside the patient's body and the corrected version is transplanted back in to the patient.

The cells are treated with either a viral or non viral gene therapy vector carrying the corrected copy of the gene.

2. ***In VIVO*** Opposite of *ex vivo* is what we call ***in vivo*** where cells are treated inside the patient's body.

The **corrected copy of the genes** is transferred into the body of the patient.

The cells may be treated either with a viral or non viral vector carrying the corrected copy of the gene.

3. ***In-situ*** Gene therapy done in a **restricted area** or to a particular site is called ***in-situ*** .

In situ gene therapy requires the vector to be placed directly into the affected tissues.

In situ gene therapy comprises transfer of corrected copy of the gene into the targeted organ or tissue.

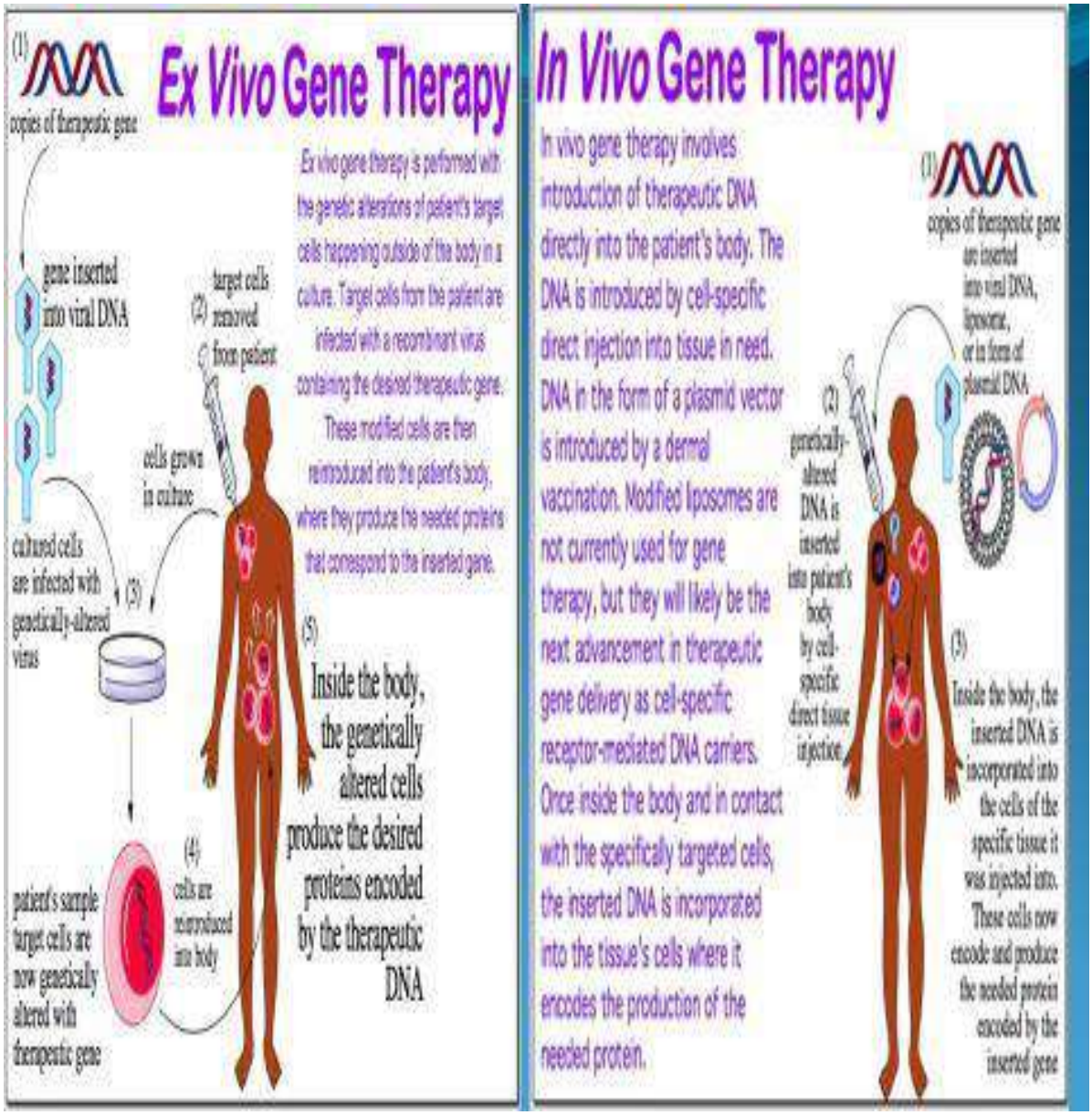
The major concern of current time gene therapy protocol is the **lack of efficient transduction of the targeted organ.**

The method is effectively used against cystic fibrosis, a disease of airway epithelium

The method is also explored for cancer gene therapy where the viral vector is engineered to contain the herpes simplex virus thymidine kinase gene. After injection of the viral vector the patient is treated with a prodrug such as Ganciclovir, which causes 75% reduction in the tumor cell population.

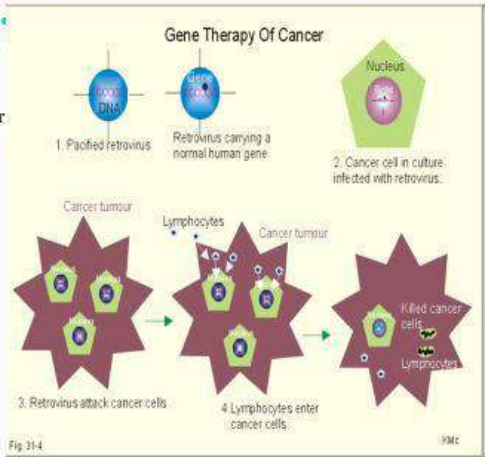
4. **Somatic cell gene therapy** involves the transfer of gene to a diseased somatic cell either within the body or outside the body with the help of a viral or non viral gene therapy vector.

- A vector delivers the therapeutic gene into a patient's target cell
- The target cells become infected with the viral vector
- The vector's genetic material is inserted into the target cell
- Functional proteins are created from the therapeutic gene causing the cell to return to a normal sta


(1)
copies of therapeutic gene
Ex Vivo Gene Therapy
gene inserted into viral DNA
(2) target cells removed from patient
cells grown in culture
cultured cells are infected with genetically-altered virus
(3)
patient's sample target cells are now genetically altered with therapeutic gene
(4) cells are reintroduced into body
Ex vivo gene therapy is performed with the genetic alterations of patient's target cells happening outside of the body in a culture. Target cells from the patient are infected with a recombinant virus containing the desired therapeutic gene. These modified cells are then reintroduced into the patient's body, where they produce the needed proteins that correspond to the inserted gene.
(5)
Inside the body, the genetically altered cells produce the desired proteins encoded by the therapeutic DNA
In Vivo Gene Therapy
In vivo gene therapy involves introduction of therapeutic DNA directly into the patient's body. The DNA is introduced by cell-specific direct injection into tissue in need. DNA in the form of a plasmid vector is introduced by a dermal vaccination. Modified liposomes are not currently used for gene therapy, but they will likely be the next advancement in therapeutic gene delivery as cell-specific receptor-mediated DNA carriers. Once inside the body and in contact with the specifically targeted cells, the inserted DNA is incorporated into the tissue's cells where it encodes the production of the needed protein.
(1)
copies of therapeutic gene are inserted into viral DNA, liposome, or in form of plasmid DNA
(2) genetically-altered DNA is inserted into patient's body by cell-specific direct tissue injection
(3)
Inside the body, the inserted DNA is incorporated into the cells of the specific tissue it was injected into. These cells now encode and produce the needed protein encoded by the inserted gene

Majority are trials

Gene therapy is being studied in clinical trials (research studies with people) for many different types of cancer and for other diseases. It is not currently available outside a clinical trials

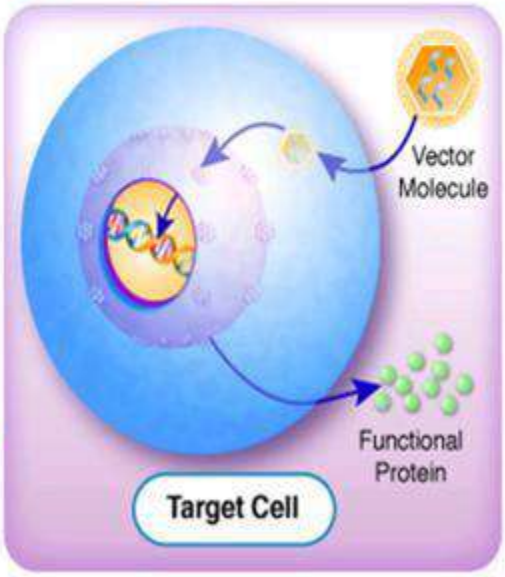


What gene therapy can achieve

Replacing a mutated gene that causes disease with a healthy copy of the gene.

Inactivating, or "knocking out," a mutated gene that is functioning improperly.

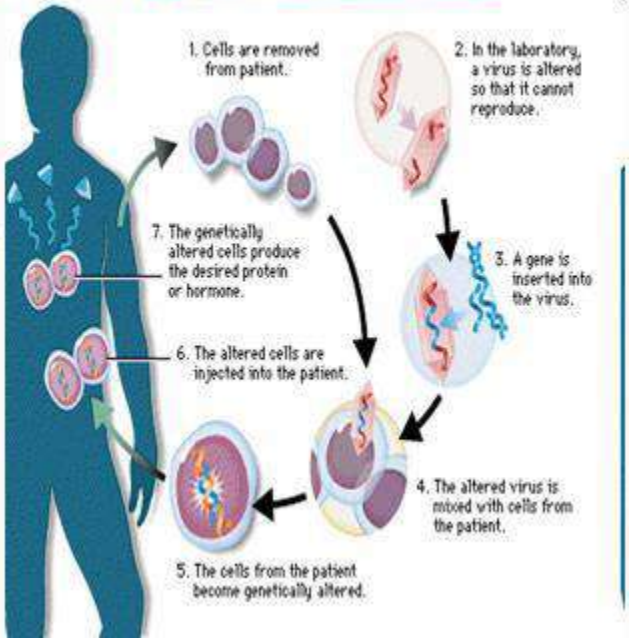
Introducing a new gene into the body to help fight a disease.



Gene therapy corrects

Gene therapy is a technique for correcting defective genes responsible for disease development.

Researchers may use one of several approaches for correcting faulty genes



Lec.3 Gene Therapy problems facing Gene Therapy?

Acute immune response to viral vectors (Consequences of the random insertion of vectors, Viral vectors problems, Viral vectors could become pathogenic, Immunological responses).

- Repeated treatment needed (short life of the therapy).
- Genes "lost" when the cell goes through mitosis
- Genes spliced at random into the genome could upset other genes
- Multigene diseases (multigene disorders too complex to treat).
-- Unsafe methods which used.
-- Not reaching enough target cells.
-- Inefficient treatment or stopping cloned gene.
-- else

Gene Therapy Technologies

1. Gene Transfer Technology.
2. Stop Target gene from Expression -attacking mRNA or Protein.

Modification by *in vivo* and *ex vivo* gene therapy

Somatic cells are modified in a number of different ways

- **1. Gene supplementation**

This method is also called as **gene augmentation**. It aims to supply a functional copy of the defective gene. The method is generally employed for a gene product that has lost its function or is showing inadequate expression of protein. The process can be used when there is **irreversible damage of the gene**. The gene supplementation can be used for cancer therapy to increase the immune response against the tumor cells. Alternatively it can be used to replace the defective tumor suppressor gene.

- **2. Gene replacement**

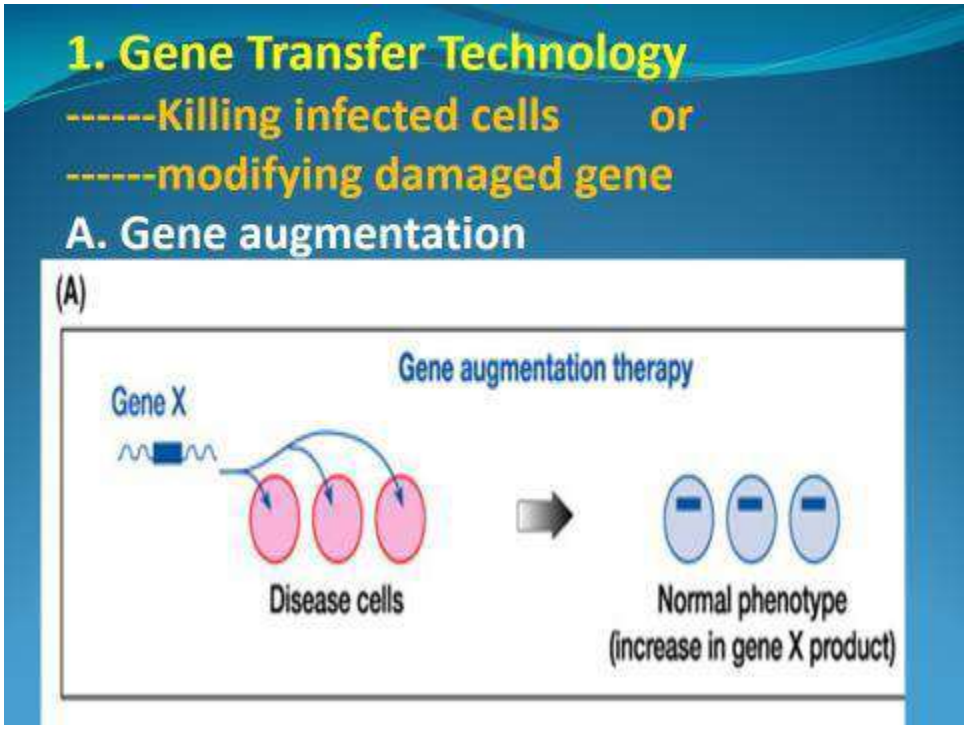
In this the mutated or nonfunctional copy of the gene is **replaced by the correct** functional copy of the same gene. The gene replacement is performed for a **mutated gene** which is harmful for the host. In general gene replacement aims for gain of function.

- **3. Targeted inhibition of gene function**

The targeted inhibition of gene function is relevant for the **infectious diseases** where specific **gene of pathogens is targeted**. The pathogen associated antigenic gene is knocked down in order to avoid the harmful effect of the protein. It also aims for targeted inhibition of tumor antigen to reduce the autoimmune response. The gene is silenced by various means including **siRNA, RNAi, etc.**

- **4. Targeted killing of the cells**

The targeted killing aims specifically for **cancer cells** where the metastatic form of the tumors are targeted and killed *in situ* . Many novel viruses called **oncolytic viruses** are targeted to kill the cancerous cells . ***Paramyxoviruses*** belong to such group of promising oncolytic viruses. Many studies using *paramyxoviruses* have shown encouraging results in reducing the cancerous condition by specifically targeting and killing the cancer cells by apoptosis.



B.Direct killing of disease cells.

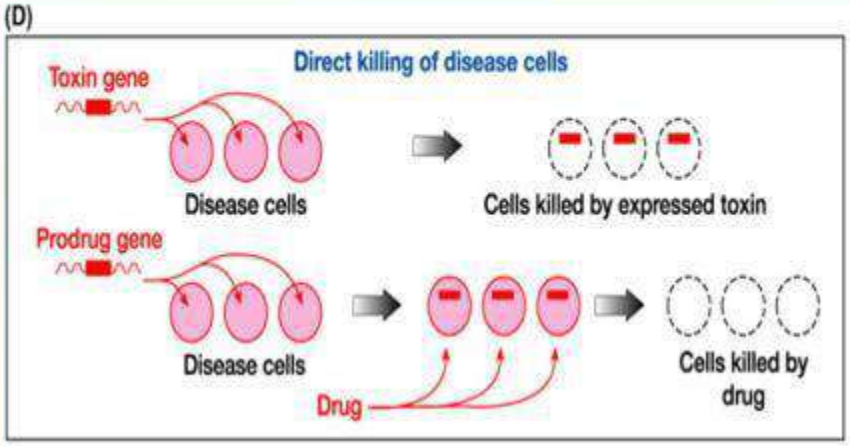


Figure 21-4 part 2 of 3 Human Molecular Genetics, 3/e. (© Garland Science 2004)

C. Assisted killing of disease cells by immune system.

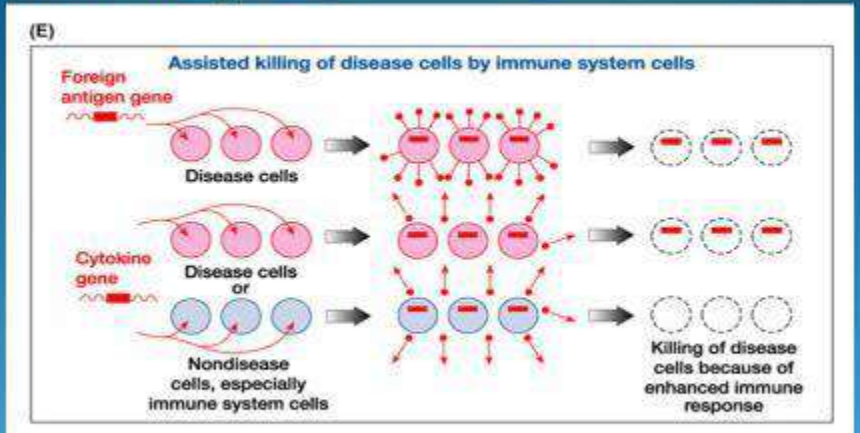
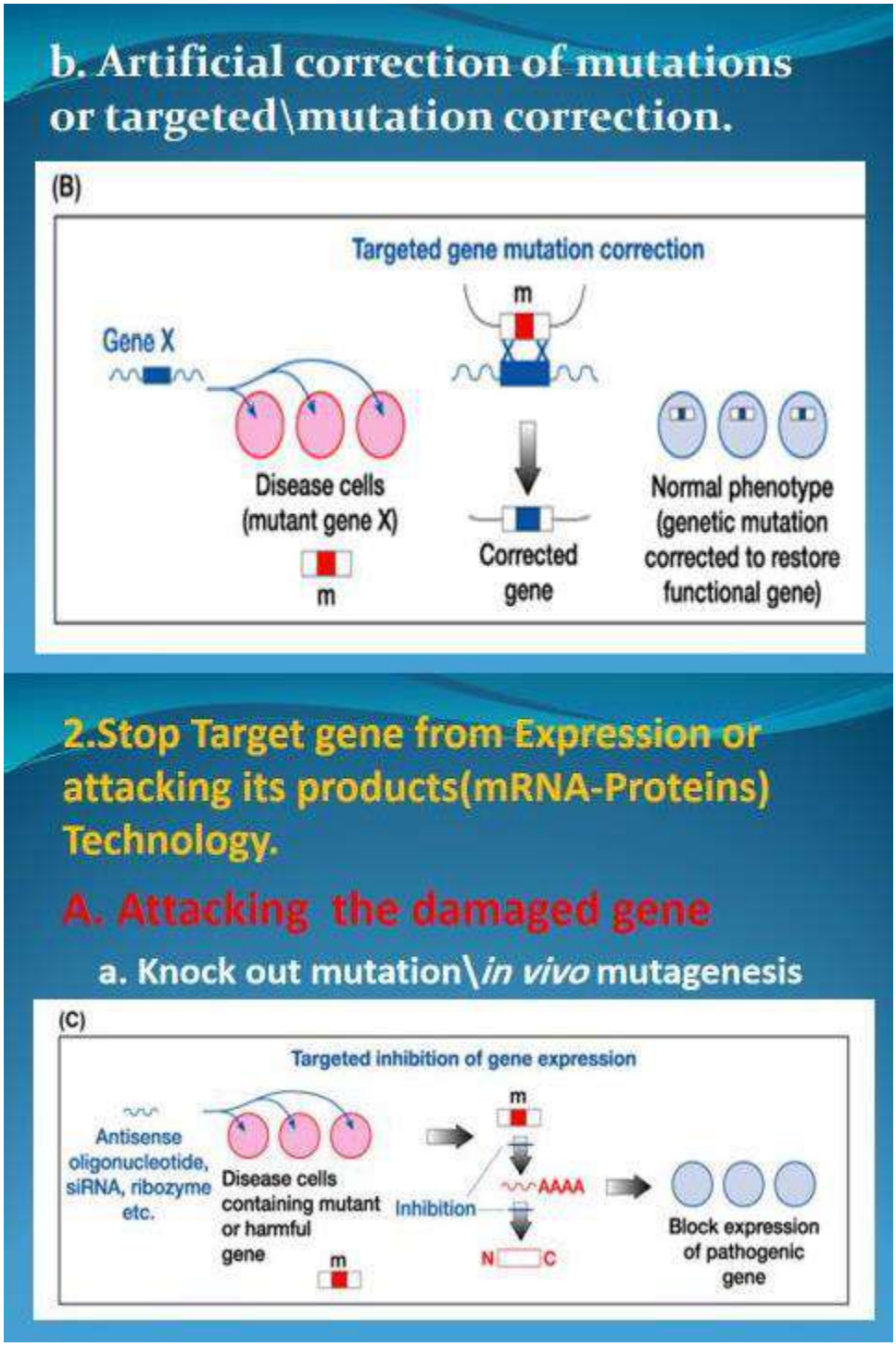


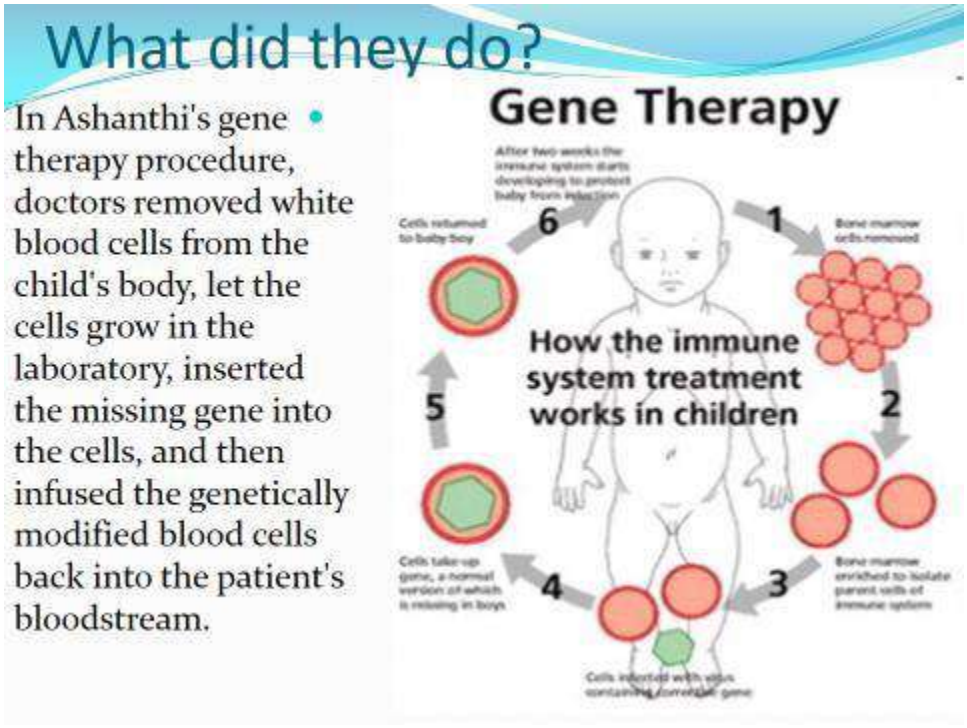
Figure 21-4 part 3 of 3 Human Molecular Genetics, 3/e. (© Garland Science 2004)



First approved gene therapy

On September 14, 1990 at the U.S. National Institutes of Health, W. French Anderson M.D. and his colleagues R. Michael Blaese, M.D., C. Bouzaid,

M.D., and Kenneth Culver, M.D., performed the first approved gene therapy procedure on four-year old Ashanthi DeSilva. Born with a rare genetic disease called severe combined immunodeficiency (SCID).



Gene Therapy trails in

acute lymphoblastic leukaemia
non-Hodgkin lymphoma (NHL) or
chronic lymphocytic leukemia (CLL)

University of Texas MD Anderson Cancer Center

Sleeping Beauty gene transfer system initially discovered at the University of Minnesota.

--- immune cells B-cells become malignant with surface molecule known as CD19.

---T-cells from the patients were treated with a harmless virus, which installed a new gene.

----Modified T-cells attack all cells bearing CD19 and Kill them all.

---the body should replenish the immune system with regular T-cells and healthy B-cells after a couple of months.

---The patients received donated bone marrow to ensure they could re grow a healthy immune system

Ethical problems

- Gene therapy for serious genetic diseases OK but for other health problems? Should people be allowed to use gene therapy to enhance basic human traits such as height, intelligence, or athletic ability?

- Somatic cell treatment stays with the individual, germ cell treatment passes down the germ line (becomes immortal)

- Very costly. Who pays? Who is eligible? Will the high costs of gene therapy make it available only to the wealthy?

- How can "good" and "bad" uses of gene therapy be distinguished?

- Who decides which traits are normal and which constitute a disability or disorder?

- Could the widespread use of gene therapy make society less accepting of people who are different?

The future of gene therapy

Current uses of gene therapy focus on treating or curing existing conditions. In the future, the focus could shift to prevention. As more of the human genome is understood, medicine will know more about which genes contribute to or cause disease. With that knowledge in hand, gene therapy could be used to head off problems before they occur.

Lec.4 Gene Therapy

Viral Vectors: Retrovirus

Lentiviruses are enveloped RNA viruses found in most of the vertebrates. The most common Lentiviruses used in gene therapy experiments are **retroviruses.**

retroviruses.

- The very first step in designing a viral vector is to know the sequences needed for the **assembly** of virus particles, **packaging** of genome into the particles, and **transduction** of gene of interest to the targeted cells
- The dispensable segment of the genome should be **deleted** in order to use the same space for the gene of interest. Moreover removal of the dispensable part reduces the **pathogenicity** and **immunogenicity** of the virus particles.
- **Viral vectors** are broadly divided into two types; namely
- **integrating (adeno-associated virus)** and
- **non-integrating (adenovirus).**
- Retroviral vectors are based on replication deficient retroviruses. The vectors are mainly derived from **Rous sarcoma virus**, **avian leukosis virus**, and **murine leukemia virus.**

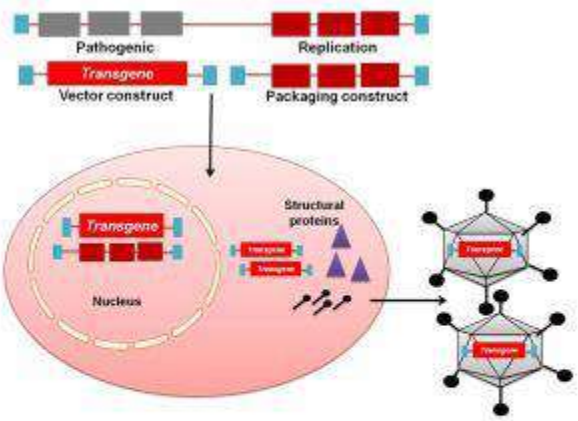
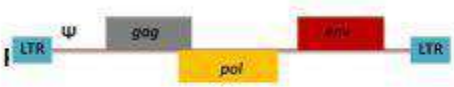
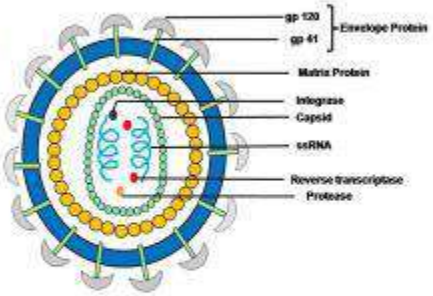


Figure 5: Basic strategy to design a viral vector:
Virus genome codes for pathogenic and replication fragments. The pathogenic segment may be replaced by transgene. The cells are transfected with transgene and packaging construct which assembles to form vector. Alternatively, the packaging constructs can also be stably expressed in stable cell line. The stable cell line containing packaging proteins is used to transfect the viral vector containing transgene under the control of tissue specific promoter.

Retrovirus

Retroviruses are a large family of enveloped RNA viruses found in all vertebrates. The enveloped virus particle contains two copies of the viral RNA genome, which are surrounded by a cone-shaped core. The viral RNA contains three essential genes, *gag* , *pol* , and *env* , and is flanked by long terminal repeats (LTRs).





- Gag protein is proteolytically processed into
  - MA (matrix)
  - CA (capsid)
  - NC (nucleocapsid)
- Pol protein encodes enzymes
  - PR (protease)
  - RT (Reverse Transcriptase which has both DNA polymerase and RNase H activities)
  - IN (Integrase)
- Env protein encodes
  - SU surface glycoprotein (gP120)
  - TM transmembrane protein (gP41)
  - They mediate virus entry.

- The transduction of **retroviral vector is limited** because of **its ability to have limited cellular tropism (**cells and tissues of a host that support growth of a particular virus).

- The cellular tropism of the retrovirus is broadened by the incorporation of envelope from related or unrelated viruses, making them **pseudotype virus**. Incorporation of vesicular stomatitis virus glycoproteins into the retrovirus virion or the envelope of murine leukemia virus allows the broad host range to the transducing vectors. Pseudotyping of retrovirus by the use of lyssavirus glycoprotein makes it transducible to the brain while incorporation of surface protein of ebola virus helps in transducing airway epithelium.
- The use of each coreceptor corresponds to viruses with different biological properties and pathogenicity. Viruses isolated at the beginning of infection use the **CCR5** co-receptor, which is the major coreceptor for macrophage-tropic strains (M-tropic).
- In full-blown AIDS cases, new viral species appear with high level of replication, cytopathic effects and they use the coreceptor **CXCR4**, which is the major receptor for T-cell strains (T-cell tropic).
- The retrovirus that binds to the receptor of mice cells are called **ECOTROPIC** virus while those binding to both human and mice is called as **AMPHOTROPIC** virus.

Replication cycle of retrovirus

- Virion attachment to a specific cell surface receptor
- Virion penetration into the cell
- Reverse transcription of ther genome.
- Transfer of viral DNA to the infected cell **nucleus**
- **Integration of viral DNA** randomly to the cellular DNA to form the provirus
- Viral RNA synthesis by cellular RNA polymerase II using the proviral template
- Transcripts processing to viral genome and mRNAs
- Virus protein synthesis
- Assembly and budding from cell surface

- Processing of capsid proteins
    - After binding to its receptor (CD4 receptor on T cells), the viral capsid containing the RNA genome enters the cell through membrane fusion. The viral RNA genome is subsequently converted into a double-stranded viral DNA by the viral enzyme reverse transcriptase. The viral DNA is heavily associated with viral proteins like nucleocapsid, reverse transcriptase, and integrase, and translocates to the nucleus where the viral enzyme integrase mediates integration of the viral DNA into the host cell genome to form PROVIRUS.
    - Alternatively, the vector can also be produced by the transfection of plasmid expressing the structural proteins. The latter method is less time consuming as it avoids the use of packaging cell line. Viruses are recovered from the supernatants of actively growing producer cells.

Lec.5 Gene Therapy

Mechanism of suicidal gene therapy

- **Retroviruses** or lentiviral vectors are designed to carry a therapeutic gene.
- Since the retroviruses only grow in dividing cells so they will specifically multiply in tumor cells.
- Retroviral vectors are designed to express the **thymidine kinase gene (tk)** from **herpes virus.**
- The tk gene sensitizes the tumor cells for a prodrug **ganciclovir**. Herepes virus tk is a normal substrate for the ganciclovir while the host tk is not affected by the drug. **Therefore host cells surrounding the tumors will not have any effect of ganciclovir.**
- Ganciclovir is phosphorylated by the herpes virus tk into monophosphate form; the ganciclovir is then converted into triphosphate form which **inhibits the DNA synthesis in tumor cells**. The tumors cells are specifically killed by this process as the host tk has little affinity with the ganciclovir.

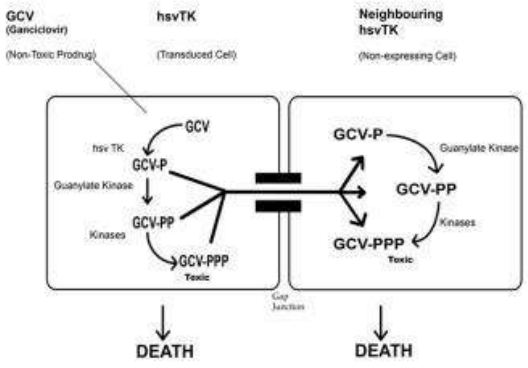


Figure 4: Schematic representation of suicidal gene therapy:
Herpes simplex virus contains an enzyme called **THYMIDINE KINASE** which is used in targeting cancer cells for suicidal gene therapy. The **Ganciclovir,** an antiviral drug is used for suicidal gene therapy. The mechanism behind the killing of a cancer cells is by **phosphorylation of Ganciclovir.**

Adenovirus

- Adenoviruses are isolated from wide varieties of animals and human beings. The list of adenovirus serotypes that affects human are more than 50.
- Adenoviruses are associated with the respiratory diseases, conjunctivitis and gastroenteritis in humans. Adenoviruses are also associated with the tumor formation in animals.
- Adenoviruses are non-enveloped and icosahedral particles usually around 90 nm in diameter Adenovirus has a double stranded DNA (ds-DNA) genome of approximately 35-36 kb in length. The ds-DNA genome contains transcription segments in an overlapping fashion. The genome contains more than 50 proteins that are formed by splicing, 11 of which are structural proteins.

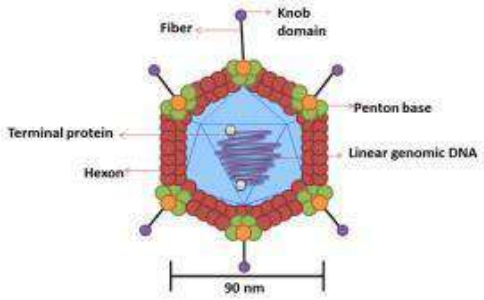


Figure 6: Schematic representation of adenovirus:

Mature virion contains penton base, which is surrounded by 5 different proteins and hexon proteins, which are surrounded by 6 different proteins. The penton base contains a fiber that interacts with the host cell receptors.

The fiber protein projects from the virion, and the carboxy-terminal (knob) forms a high-affinity complex with the receptor present on the host cell.

The virion consists of a shell called capsid which surrounds DNA containing core. The Coxsackie virus and adenovirus receptor (CAR) present in respiratory epithelium, nervous system, liver, lung, and intestinal lining acts as a receptor for the adenovirus. While entering the cell through CAR, the virions follow caveolae mediated endocytosis (clathrin independent).

-

Virus replication

- The virus replication and life cycle is divided into late and early stages. After entering the cell, the virion is **endocytosed** in lysosome which has acidic environment.
- The capsid of the virion gets disrupted by the acidic environment releasing the subvirion into the cytoplasm. Subvirion of the adenovirus interacts with the nuclear pore complex leading to its migration into the infected cell nucleus.
- As soon as it reaches the nucleus, early proteins are synthesized which are required for viral genome replication, transcription, and translation. These early proteins make their way out in the cytoplasm in order to facilitate the process. There are five early proteins which are E1A, E1B, E2, E3 and E4 Among these E1 and E4 are responsible for DNA replication, E2 for RNA polymerase while E3 is responsible for virus specific immune response.

- The late proteins are formed later during the infectious cycle and comprise mainly of capsid or structural proteins (L1–L5).
- Inverted terminal repeats (ITRs) are present on the 5' and 3' ends of the viral genome and are the packaging signals responsible for packaging of viral genome into the icosahedral capsid.
- Adenovirus also forms an **RNA intermediate** called **virus -associated (VA) RNA**. The VA RNA is not fully functional because it doesn't form any protein. The viral transcription segments are transcribed by the help of cellular RNA polymerase II, whereas the VA is transcribed by RNA **polymerase III.**
- Infection of adenovirus **induces a high immune response** in the host body. The initial immune response comprises of cytokines such as tumor necrosis factor and interleukins 1 and 6 followed by specific cytotoxic T lymphocyte. The high level of immune response against the adenovirus is a major hurdle in making adenoviral vector as a gene therapy tool.

Adenovirus as a vector

- Adenoviruses have been explored as a gene therapy vector for quite some time with varying degree of success. Generally adenovirus serotype 5 is widely used but serotypes 2, 4, 7, and non human adenovirus isolates were also explored for gene therapy vector.
- Adenovirus is made replication deficient after removing the coding regions of the viral genome. The first generation of adenoviral vectors is made by removing E1 region of the genome with a transgene (gene of interest). The E1 protein in adenovirus is essential to activate the expression of genome specific transcripts; absence of E1 makes the virus replication deficient in most of the cell lines.
- Alternatively, the E1 deficient virus can be grown in cells that contain the E1 proteins in *trans*, such as 293 cells. Removal of E1 from the genome reduces its size which is further used to put the transgene of around 4.5 kb at the same position.
- The cloning capacity of E1 deleted adenoviral vectors can be further increased by deleting the nonessential region of E3 protein. Minimum quantity of viral gene in the vector reduces the chances of recombination with the host cell DNA.
- The second generations of adenovirus vectors are generated by deleting E1 as well as part of E2 and/or E4 gene segments.
- The deletion of E2 along with E1 further increases the capacity of adenovirus vector to accommodate a larger transgene.
- E2 proteins are essential for viral genome replication and for successful rescue of adenoviral vector it has to be supplied in *trans* in the packaging cells.
- Many deletions in other genes on the adenovirus genome have been tried by scientists in order to increase the capacity to clone a transgene.
- The deleted regions of the adenovirus genome must be provided in *trans* in order to rescue recombinant virus particles carrying the gene of interest.
- In theory it is possible to make an adenovirus that lacks almost all of

its proteins except the ITRs. The vectors made in this way don't have any virus specific sequences and are termed as **GUTLESS vectors** (A helper dependent virus also termed a gutless virus is a synthetic viral vector dependent on the assistance of a helper virus in order to replicate).

Figure : Gutless vector

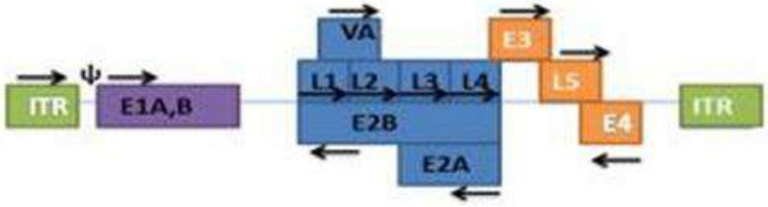


Figure 7: Adenoviral genome organization

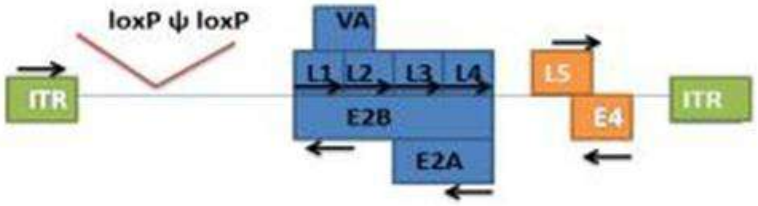
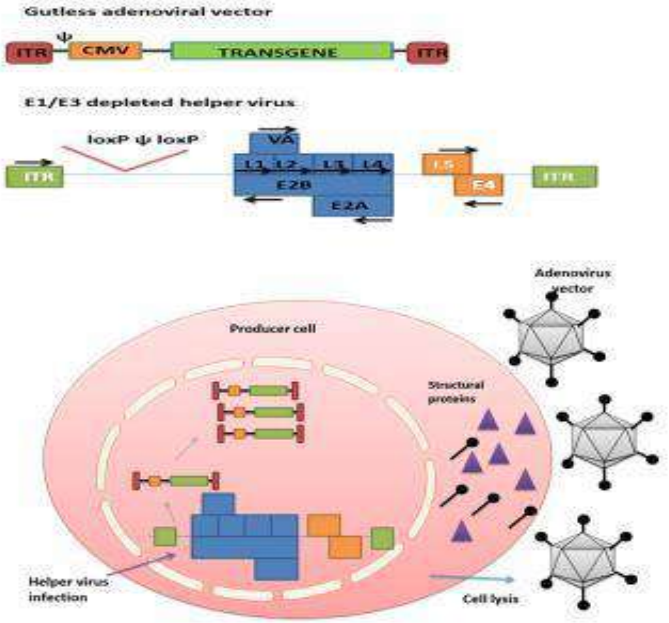


Figure 8: depleted helper virus


Gutless adenoviral vector
ITR
Ψ
CMV
TRANSGENE
ITR
E1/E3 depleted helper virus
loxP Ψ loxP
ITR
VA
L1
L2
L3
L4
E2B
E2A
L5
E4
ITR
Adenovirus vector
Producer cell
Structural proteins
Helper virus infection
Cell lysis

Lec.6 Gene Therapy

Adeno-associated virus

Adeno-associated virus as a gene therapy vector

- The first AAV gene therapy vector was generated by replacing internal *rep* and *cap* gene with the **transgene**. The recombinant virus was produced by the co-infection of **helper virus** and transfection of **plasmid** expressing *rep* and *cap* proteins in *trans* .

The method still generates the vector having ITR sequences (around 400bps) of the viral genome. The presence of ITR sequences may lead to production of wild type virus in the subsequent generation.

- The next generation of AAV contains **only part of ITR sequences** essential for the production and integration. Essentially the AAV vector system contains a promoter and a transgene flanked by the ITRs, the method prevents the production of replication competent AAV production. The adenovirus E1A, E1B, E2A, VA, and E4 proteins are essential for the production of a successful virus particle. To avoid the use of replication adenovirus particles, scientists have developed a system that requires only E2A, VA, and E4 proteins to produce viable AAV vector. The system requires transfection of E2A, VA, and E4 gene expressing plasmid in Human embryonic kidney 293 cells.

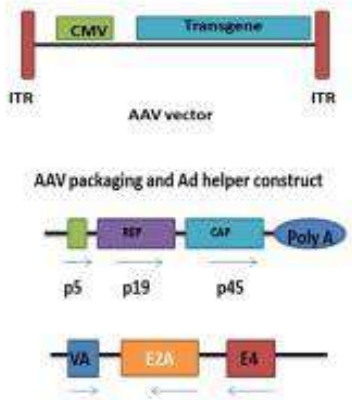
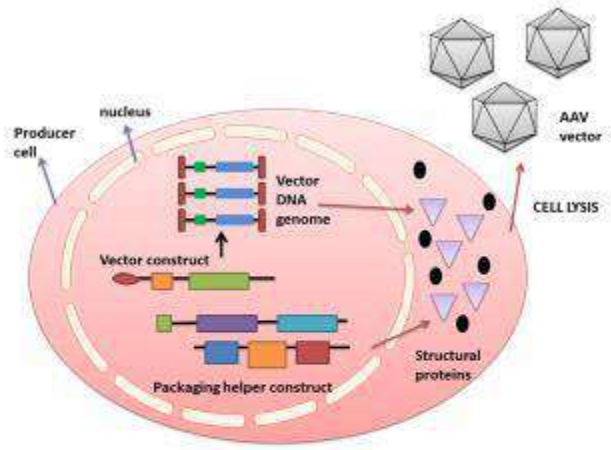


Figure 9: Adeno-associated virus vector development:
AAV vectors are made by replacing rep/cap genes by transgene and a **tissue specific promoter CMV**. A packaging construct containing endogenous promoters p5, p40 and p1 used which provides the regulation for the expression of *rep* and *cap* genes. Helper adenovi expresses E2, E4 and VA genes in producer cells. The **293 cells** are generally used as a producer cell line as it **provides the E1 proteins** for the generation of AAV particles. Prod cells thus produce non replicating AAV vector. ssDNA is packaged by the structural protein followed by its release after cell lysis.



Interesting points:

293 cells are known to express adenovirus E1A and E1B gene products.

Difference between adeno and adeno-associated virus

Character	Adenovirus	Adeno-associated virus
Integration in host	No	Yes
Expression of vector	Transient	Stable
Transfection efficiency	High	Low
Immune response	High	No
Genome	36kb ds DNA	4.7kb ssDNA
Envelope	No	No
Receptor	CAR	Heparan Sulphate
Cell line	293 HEK	293 HEK
Entry	Caveolae mediated	Clathrin mediated
Proteins	Five early and five late	Four for replicase and three for capsid
Capsid size	40 nm	25 nm

Tropism of adeno-associated virus

- AAV-2 is the most common serotype used to develop a gene therapy vector. The cap protein of AAV-1, -2 and -3 share a great homology

with each other and hence are known to use the heparan sulfate receptor.

- The capsid proteins of AAV-4 and -5 are different from other serotypes and probably involved in using different cellular receptors to infect the susceptible host.
- The AAV-1 is more tropic towards muscle and liver while AAV-5 is more tropic to retina (eye). AAV-3 is more tropic towards the hematopoietic stem cells.
- The tropism of AAV serotypes can by modulated by **shuffling** the capsid proteins.
- To be more efficient AAV genome should be of **dsDNA** instead of parental ssDNA, the expression of transgene increases to a large extent after the production of dsDNA from ssDNA by host cell machinery. The dsDNA form of the AAV vector can persist for a long time in a transduced cell by head to tails recombination with ITR sequences.
- The major hurdle in AAV mediated gene therapy is regarding the limitation of transgene capacity. Another hurdle is regarding the presence of neutralizing antibodies against the AAV serotypes in the human population because of natural AAV infection. In addition a single injection of AAV can mount a huge humoral immune response that inhibits the second injection of the same serotype. Using AAV serotypes of different capsid protein may overcome this hurdle. The AAV offers a very promising tool for the gene therapy vector and its efficacy and potential is increasing day by day. Currently many gene therapy trials are going on using AAV as a vector expressing different transgene.

Genome, transcriptome and proteome

- The AAV genome is built of single-stranded deoxyribonucleic acid (ssDNA), either positive- or negative-sensed, which is about 4.7 kilobase long. The genome comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading

frames (ORFs): rep and cap. The former is composed of four overlapping genes encoding Rep proteins required for the AAV life cycle, and the latter contains overlapping nucleotide sequences of capsid proteins: VP1, VP2 and VP3, which interact together to form a capsid of an icosahedral symmetry.

ITR sequences

- The Inverted Terminal Repeat (ITR) sequences comprise 145 bases each. They were named so because of their symmetry, which was shown to be required for efficient multiplication of the AAV genome. The feature of these sequences that gives them this property is their ability to form a hairpin, which contributes to so-called self-priming that allows primase-independent synthesis of the second DNA strand. The ITRs were also shown to be required for both integration of the AAV DNA into the host cell genome (19th chromosome in humans) and rescue from it as well as for efficient encapsidation of the AAV DNA combined with generation of a fully assembled, deoxyribonuclease-resistant AAV particles.
- With regard to gene therapy, ITRs seem to be the only sequences required in cis next to the therapeutic gene: structural (cap) and packaging (rep) proteins can be delivered in trans. With this assumption many methods were established for efficient production of recombinant AAV (rAAV) vectors containing a reporter or therapeutic gene. However, it was also published that the ITRs are not the only elements required in cis for the effective replication and encapsidation. A few research groups have identified a sequence designated cis-acting Rep-dependent element (**CARE**) inside the coding sequence of the rep gene. CARE was shown to augment the replication and encapsidation when present in cis.
- ***rep*** **gene and Rep proteins**
- On the "left side" of the genome there are two promoters called p5 and p19, from which two overlapping messenger ribonucleic acids (mRNAs) of different length can be produced. Each of these contains an intron which can be either spliced out or not. Given these possibilities, four various mRNAs, and consequently four various Rep proteins with overlapping sequence can be synthesized. Their names

depict their sizes in kilodaltons (kDa): Rep78, Rep68, Rep52 and Rep40. Rep78 and 68 can specifically bind the hairpin formed by the ITR in the self-priming act and cleave at a specific region, designated terminal resolution site, within the hairpin. They were also shown to be necessary for the AAVS1-specific integration of the AAV genome. All four Rep proteins were shown to bind ATP and to possess helicase activity. It was also shown that they upregulate the transcription from the p40 but downregulate both p5 and p19 promoter

cap gene and VP proteins

- The right side of a positive-sensed AAV genome encodes overlapping sequences of three capsid proteins, VP1, VP2 and VP3, which start from one promoter, designated p40. The molecular weights of these proteins are 87, 72 and 62 kiloDaltons, respectively. The AAV capsid is composed of a mixture of VP1, VP2, and VP3 totaling 60 monomers arranged in icosahedral symmetry in a ratio of 1:1:10, with an estimated size of 3.9 MegaDaltons.
- The *cap* gene produces an additional, non-structural protein called the Assembly-Activating Protein (AAP). This protein is produced from ORF2 and is essential for the capsid-assembly process.[39] The exact function of this protein in the assembly process.
- All three VPs are translated from one mRNA. After this mRNA is synthesized, it can be spliced in two different manners: either a longer or shorter intron can be excised resulting in the formation of two pools of mRNAs: a 2.3 kb- and a 2.6 kb-long mRNA pool. Usually, especially in the presence of adenovirus, the longer intron is preferred, so the 2.3-kb-long mRNA represents the so-called "major splice". In this form the first AUG codon, from which the synthesis of VP1 protein starts, is cut out, resulting in a reduced overall level of VP1 protein synthesis. The first AUG codon that remains in the major splice is the initiation codon for VP3 protein. However, upstream of that codon in the same open reading frame lies an ACG sequence (encoding threonine) which is surrounded by an optimal Kozak context. This contributes to a low level of synthesis of VP2 protein,

which is actually VP3 protein with additional N terminal residues, as is VP1.

- Since the bigger intron is preferred to be spliced out, and since in the major splice the ACG codon is a much weaker translation initiation signal, the ratio at which the AAV structural proteins are

  synthesized *in vivo* is about 1:1:20, which is the same as in the mature virus particle. The unique fragment at the N terminus of VP1 protein was shown to possess the phospholipase A2 (PLA2) activity, which is probably required for the releasing of AAV particles from

  late endosomes

Serotypes, receptors and native tropism

- Until the 1990s, virtually all AAV biology was studied using AAV serotype 2. However, AAV is highly prevalent in humans and other primates and several serotypes have been isolated from various tissue samples.
- Serotypes 2, 3, 5, and 6 were discovered in human cells, AAV serotypes 1, 4, and 7–11 in nonhuman primate samples. As of 2006 there have been 11 AAV serotypes described, the 11th in 2004.
- AAV capsid proteins contain 12 hypervariable surface regions, with most variability occurring in the threefold proximal peaks.
- All of the known serotypes can infect cells from multiple diverse tissue types. Tissue specificity is determined by the capsid serotype and pseudotyping of AAV vectors to alter their tropism range will likely be important to their use in therapy.
- **Serotype 2**
- Serotype 2 (AAV2) has been the most extensively examined so far. AAV2 presents natural tropism towards skeletal muscles ,

  neurons, vascular smooth muscle cells and hepatocytes.
- Three cell receptors have been described for AAV2: **heparan sulfate proteoglycan (HSPG)**, a**Vβ5** integrin and fibroblast growth

  factor receptor 1 (**FGFR-1**). The first functions as a primary receptor, while the latter two have a co-receptor activity and enable AAV to enter the cell by receptor-mediated endocytosis.

- HSPG functions as the primary receptor, though its abundance in the

extracellular matrix can scavenge AAV particles and impair the infection efficiency.

- Studies have shown that serotype 2 of the virus (AAV-2) apparently kills cancer cells without harming healthy ones.This could lead to a new anti-cancer agent.
- **Synthetic Serotypes**
- There have been many efforts to engineer and improve new AAV variants for both clinical and research purposes.
- Such modifications include new tropisms to target specific tissues, and modified surface residues to evade detection by the immune system. Beyond opting for particular strains of recombinant AAV(rAAV) to target particular cells, researchers have also explored AAV pseudotyping, the practice of creating hybrids of certain AAV strains to approach an even more refined target. The hybrid is created by taking a capsid from one strain and the genome from another strain. For example, research involving AAV2/5, a hybrid with the genome of AAV2 and the capsid of AAV5, was able to achieve more accuracy and range in brain cells than AAV2 would be able to achieve unhybridized.
- Researchers have continued to experiment with pseudotyping by creating strains with hybrid capsids. AAV-DJ has a hybrid capsid from eight different strains of AAV; as such, it can infect different cells throughout many areas of the body, a property which a single strain of AAV with a limited tropism would not have. Other efforts to engineer and improve new AAV variants have involved the ancestral reconstruction of virus variants to generate new vectors with enhanced
- properties for clinical applications and the study of AAV biology.

Lec.7 Gene Therapy

Non viral vectors

Introduction

- Therapeutic gene expression has emerged as a potent tool in modern medicine. The main goal of gene therapy is to treat the disease caused by loss of function/ mutation, by introducing specific gene and its regulatory elements. For stable expression at physiological levels the therapeutic gene must be maintained within the nucleus, replicated and passed on to subsequent generations.
- Viruses particularly retro viruses are preferred systems for gene delivery owing to

their in vivo transfection efficiency. But immunogenicity and cytoxicity of viral vectors have limited their clinical use.

- Moreover, the phenomenon of insertional mutagenesis associated with use of viruses is another cause of concern.
- Non viral vectors on the contrary are much safe, in terms of reduced pathogenicity and capacity of insertional mutagenesis as well as their low cost and ease of production.

Traditional non viral methods of transgenesis

With the advent of using exogenous genetic element for treating diseases various methods for delivering transgene have been developed.

These conventional methods can be broadly classified into physical and cationic polymer methods.

Physical methods of transgenesis

a. Hydrodynamic pressure techniques :

In this technique intravascular injection of plasmid DNA is given to drive DNA molecules out of the blood circulation and into the tissue.

Electroporation :

In this method controlled electric shocks are given to cells resulting in formation of large cytoplasmic pores, through which polynucleotide can move into the cell.

Such methods are mostly restricted to in vitro applications but development of new electrodes designed for in vivo applications has allowed its use in certain areas of the body, such as tumors, muscles and liver tissue in animal models .

c. Ballistic delivery :

In ballistic delivery, DNA-coated metal microparticles are allowed to penetrate cell membranes at high velocity. In vivo this technique is limited to cutaneous applications.

d. Microinjection :

In this technique DNA is injected into cells resulting in efficient transgene expression but is laborious and limited to ex vivo applications, such as for the delivery of artificial chromosomes.

Cationic polymer method of transgenesis

- a. Lipofection : This method involves the use of cationic lipids mixed with DNA in aqueous solution forming liposomes that encapsulate DNA. Such DNA preparations are taken in either by pinocytosis or phagocytosis, depending on the cell type. Use of liposomes in vivo is limited by its rapid plasma clearance and high toxicity.
- b. Cationic peptides : These are chain of basic amino acids, which compact DNA into spherical complexes, or chromatin components such as histones which compact DNA in a structured manner allowing it to enter cell by their interaction with sulfated membrane-bound proteoglycans.
- c. Polyethylenimine : Polyethylenimine is a linear or branched polymer with many protonable amino nitrogen atoms that allows efficient DNA condensation and cell entry. The polymer behaves as an effective ‘proton sponge' that causes the rupture of endosome by osmotic swelling and releases the polyethylenimine–DNA complexes into the cytoplasm, but it is toxic due to induction of apoptosis
- d. Receptor-mediated delivery : In this method polycations conjugated DNA bound to a cell-specific ligand is targeted to cells via receptor-mediated internalization.

Lipofection Cationic peptides

All these non viral vectors have proven well in vitro transfection but lacks in vivo transfection efficiency and only allow transient transgene expression.
This inefficiency of non viral vectors can be attributed to the following:

- Interaction of the non viral–DNA complex with blood plasma proteins, undesirable cells and extracellular matrix.
- Inability to escape from liposome or endosome enclosed moiety.
- sensitivity to cytoplasmic degradative enzymes.
- Inability to pass through the double membrane nuclear envelope and subsequent degradation during breakdown of nuclear membrane at mitosis.

DNA Vaccines

- The earliest form of protection against the diseases has been in the form of vaccines.
- Discovery of vaccines by Edward Jenner in the year 1796 witnessed a revolution in human medicine.
- A vaccine is a biological formulation that confers immunity to a specific disease. The role of the vaccine is to stimulate the immune system, recognize the attacking agent, eradicate it and keep it in memory so that if there is any repeated exposure of the same disease then the destruction of such agent would be easier.
- There are several types of vaccines like killed, attenuated, live, subunit, conjugated and Nucleic acid vaccines.
- Nucleic acid vaccine means the vaccination done using RNA or DNA vaccines.
- In general DNA vaccines may be defined as those vaccines that provide immunity by transfecting host cells with DNA that encodes an antigen.

- The DNA vaccine seems to be a promising candidate as vaccines because they are very cost effective but still these are not listed for human use. There are some DNA vaccines for veterinary use though.

- The advantages of DNA vaccines:

1) It is easy to form and construct the DNA vaccines as compared to attenuated viruses and subunit protein vaccines.
2) It is cheap and cost effective.
3) It is quite stable at room temperature as compared to attenuated viral vaccines.
4) The protection offered by DNA vaccines favors bias towards cellular immunity, which is trusted to be vital for successful vaccination against intracellular pathogens.

Trials with DNA vaccines:

- After several failures the first fairly successful DNA vaccination result in humans was attained with a malaria- specific DNA vaccine. HIV-infected patients

- Recently investigations based on DNA vaccines have shifted largely to tumor antigens and the reason behind it might be the excelling funding opportunities in this field.

How safe are DNA vaccines

- Usually DNA vaccines are granted safe for both patient and environment.
- However due to the use of genetic vaccines there is a potential threat that the genome of the plasmid may get integrated into host genome of somatic cells and may lead to the production of transformed cells or oncogenes.

RNA vaccines

- RNA vaccines have been known in the field of vaccine development is quite well known.

- RNA vaccines, including those based on mRNA and self-amplifying RNA replicons, have the tendency to surpass the restrictions of plasmid DNA and viral vectors.

- Possible difficulties related to the cost.

Lec.8 Gene Therapy

tRNA gene targeting

In gene-dense genomes, mobile elements are confronted with highly selective pressure to amplify without causing excessive damage to the host. The targeting of tRNA genes as potentially safe integration sites has been developed by retrotransposons in various organisms such as the social amoeba Dictyostelium discoideum and the yeast Saccharomyces cerevisiae. In D. discoideum, tRNA gene-targeting retrotransposons have expanded to approximately 3 % of the genome. Recently obtained genome sequences of species representing the evolutionary history of social amoebae enabled us to determine whether the targeting of tRNA genes is a generally successful strategy for mobile elements to colonize compact genomes.

Background

Mobile elements are obligate genomic parasites that amp- lify as selfish DNA and play important roles in driving the evolution of their hosts. Retrotransposons mobilize by reverse transcription of RNA intermediates and integra- tion of the resulting DNA copies at new locations of their host's genomes. Retrotransposons encode proteins that me- diate their mobility and they can be distinguished by their overall structures and retrotransposition mechanisms . The supergroup of retrotransposons bearing long terminal repeats (LTRs) is classified into vertebrate retroviruses (Retroviridae), hepadnaviruses, caulimoviruses, Ty1/copia (Pseudoviridae), Ty3/gypsy (Metaviridae), BEL, and DIRS (Dictyostelium intermediate repeat sequence) . Non- LTR retrotransposons are a diverse group of mobile elements that lack LTRs and can be further distin- gushied by structural features such as the presence of an encoded apurinic or apyrimidinic site DNA repair endonuclease or a type IIS restriction endonuclease instead of a retroviral integrase and the presence or absence of a ribonuclease H (RNH) domain as part of the reverse transcriptase (RT) .

Dictyostelids are soil-dwelling protists that belong to the supergroup of Amoebozoa. Unfavorable en- vironmental conditions, such as a lack of food, triggers social behaviors in single cells that aggregate and form fruiting bodies to spread some of the population as dor- mant spores into the environment . Dictyoste- lium discoideum, the model organism in studying the biology of social amoebae, has a 34-Mb haploid genome in which two thirds of the chromosomal DNA code for proteins and intergenic regions are mostly below 1 kb in length . The gene density of this genome limits the available space for transposable elements to expand without causing damage to the host. Therefore, it is re- markable that the genome of D. discoideum is inter- spersed with a variety of mobile elements that add up to nearly 10 % of nuclear DNA .

The D. discoideum DIRS-1 element has inverted terminal repeats instead of LTRs and a complex arrangement of open reading frames (ORFs) that include an RT/RNH and a tyrosine recombinase (YR) instead of a canonical integrase (IN). DIRS-1 has a strong preference to integrate into existing DIRS-1 cop- ies by a mechanism that probably involves YR-mediated homologous recombination. Therefore, DIRS-1 forms complex clusters located near chromosome ends and contributes ~50 % of centromeric DNA of D. discoi- deum chromosomes.

DGLT-A and Skipper are related Ty3/gypsy-type LTR retrotransposons with strikingly different integration preferences. Skipper contains two ORFs coding for enzymatic activities required for retrotransposition arranged in the order RT-RNH-IN .

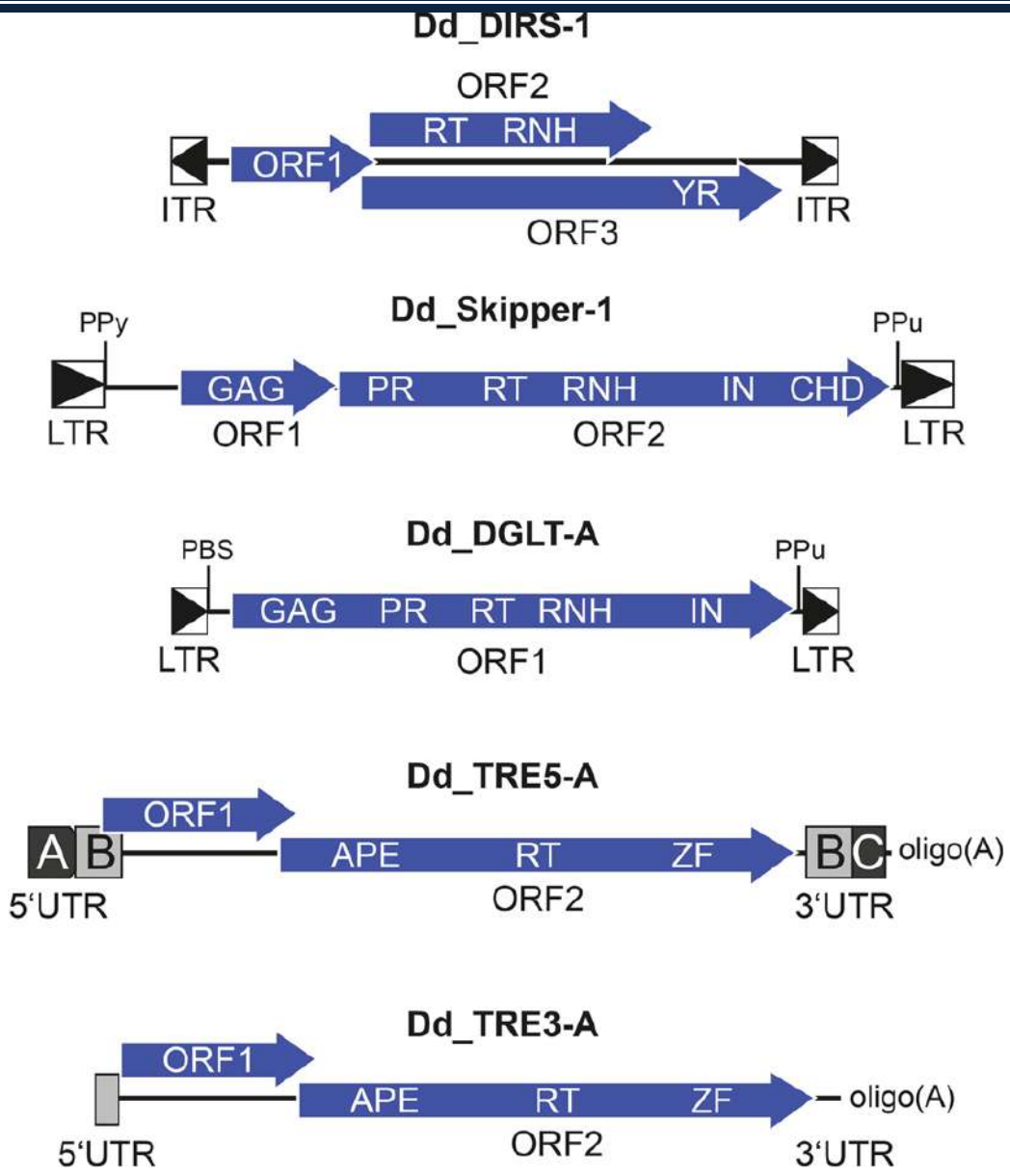


Fig. Overview of retrotransposons in the *D. discoideum* genome. DIRS-1 is the founding member of the class of tyrosine recombinase retrotransposons.

Lec.9 Gene Therapy

Transgenic animal models

Genetically modified animal models also called as transgenic animals represent a promising tool in biology to understand the host pathogen interactions and gene function in the purview of disease susceptibility and its progression. Apart from many animal models mice represent one of the best tools to understand many of the above important

roles in the discovery and development of new disease treatments.

9.1 Types of Transgenic Animals

Transgenic animals are genetically altered with specific characteristics which otherwise would not be present in that specific animal. In general, transgenic animals have either DNA added (to express an additional gene) or have their genome altered (to abolish or modify the expression of an existing gene). Rodents particularly mice comprise of over 95% of transgenic animals used in biomedical research. The mouse is the model organism of choice because of the following reasons:

- Complete mice genome sequence is available.
- Easy genetic manipulation of mice cells and embryos.
- Short gestation period and large litter size.
- Availability of major antibodies and other molecular biology tools.
- Possibility to perform physiologic and behavioral tests that can be directly linked to human disease.

Other transgenic species include cattle, pig, sheep and rats. Their use in pharmaceutical research has so far been limited due to technical constraints. Recent advances in molecular biology techniques may allow us to use transgenic rat for the development of many human therapeutics where the rat is a better model than the mouse.

.2 Uses of transgenic animals:

Transgenic animals are useful in the discovery of new therapy for important human diseases.

Transgenic animals are fundamentally similar to their counter wild type variety except for some genomic heterogeneity.

Transgenic animals are used in the gene therapy experiment to understand the importance of malfunctioned or mutated gene.

Transgenic animals are also used to check the efficacy and safety of the drugs or vaccines used in the clinical trials.

Transgenic mice can be generated to express human targeted gene that can be further used to design new therapy. Transgenic mice can also obviate the use of animals such as monkeys for testing drugs for many human diseases, eg. hypercholesterolemia and HIV.

Some terminology:

The number of offsprings produced at one birth by an animal is called Litter size.

Normal pregnancy period is called as gestation period.

Presence of high levels of cholesterol in the blood is called as hypercholesterolemia.

LDL receptor (LDLR) present in liver helps to eliminate the excess cholesterol from the body. The deficiency of LDLR in human body leads to increase in blood cholesterol level, condition called as HYPERCHOLESTEREMIA.

n hypercholesteremia chances of getting heart attack is higher because of the deposition of cholesterol in the lining of blood vessels. Blood plasma is purified for any unwanted substance in order to prolong the life of a patient (eg low density lipoprotein [LDL] from blood) by a method called PLASMAPHERESIS.

The historical example of a transgenic animal goes to successful generation of a mice where the mouse gene for metallothionein-I was fused to the human growth hormone (GH) and introduced into mice. The resultant transgenic mice showed altered growth characteristics and served as a valuable resource for human disease condition where production of excess growth hormone modulates many physiological processes.

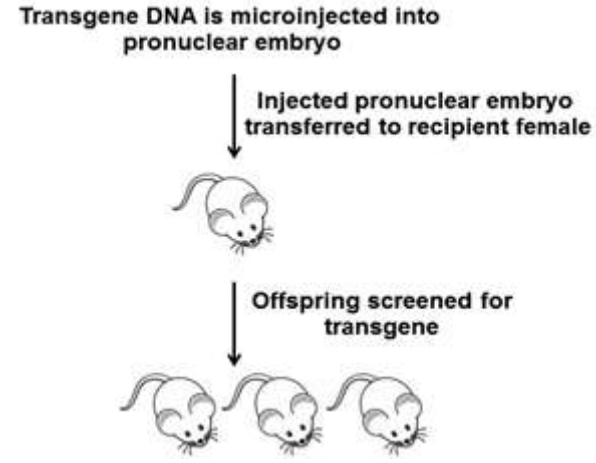
9.3 Generation of transgenic animal

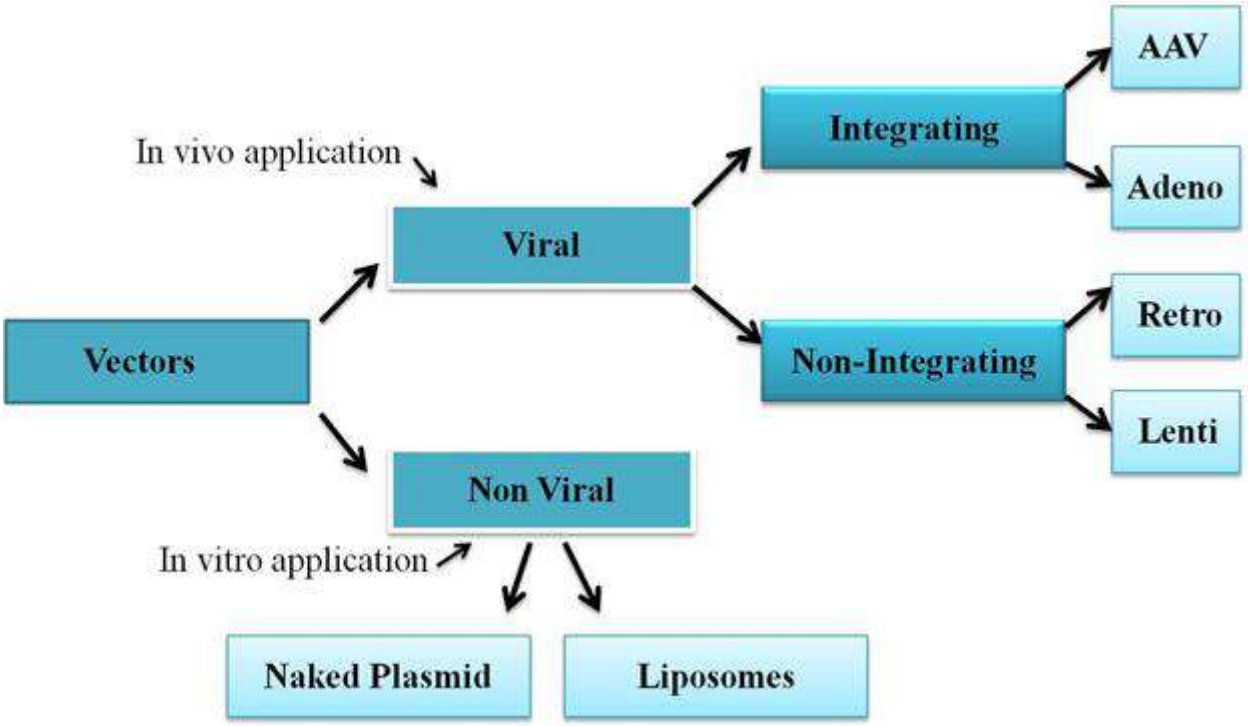
Transgenic animals can be generated by following methods:

- Retroviral infection of pre- or pro-implantation embryos
- DNA injection of embryos at pronuclear stage.
- Microinjection of genetically modified embryonic stem cells into blastocysts.

The transgenic animals are created mostly by a well- known technique where fertilized embryos were microinjected by plasmid DNA containing a fused protein *in vitro* . A micro needle is used to inject the targeted plasmid DNA into the embryo that leads to integration of foreign DNA into the host genome. The pronuclear embryo containing the foreign DNA is then implanted into the recipient animal (Figure 9.1).

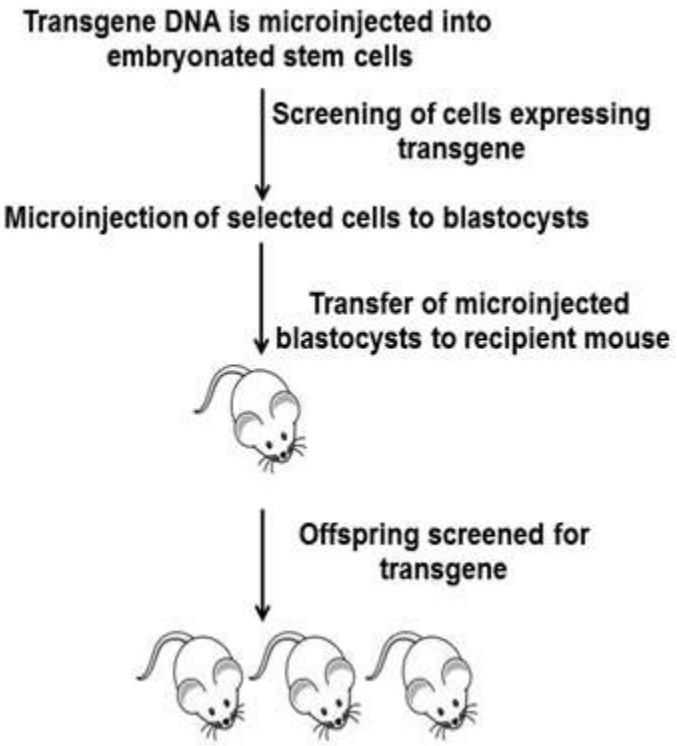
Figure 9.1 Standard transgenic approach:





In another approach, the pluripotent stem cells derived from embryonic blastocysts are elctroporated by foreign DNA containing the gene of interest. The microinjected stem cells are reintroduced into the blastocysts which are then transferred into the uterus of a pseudopregnant recipient animal (Figure 9.2).

Figure 9.2 Alternate approach:



Both the methods are successful only after multiple generations of breeding and selection of the most stable transgenic line. Plasmid DNA containing gene of interest can be coupled with a tissue specific promoter to make it as an inducible system. Cytomegalovirus promoter is used widely as a promoter for the inducible system. Alternatively the recombination of gene of interest can also be achieved by *Cre-lox* pathway.

10.1 *Cre-lox* system:

This system allows the genetic manipulation of target cells to control its gene expression, delete specific DNA sequences, or modify the genomic content. The *Cre* recombinase is a site-specific integrase isolated from bacteriophage P1. It catalyzes the recombination of DNA between specific*loxP* sites in DNA. Generally, this system is created after generating two strains, one expressing *Cre*recombinase and the other having *loxP* site flanked with the gene of interest. Both the strains are crossed in order to allow independent recombination and their outcome is determined by the location and orientation of *loxP* site containing gene of interest. If the *loxP* sites are oriented in opposite directions, *Cre* recombinase mediates the inversion of the gene of interest (Figure 10.1). However, *Cre* recombinase mediates a translocation event if the *loxP* sites are located on different chromosomes (Figure 10.2) and deletion event if the *loxP* sites are oriented in the same direction on a chromosome segment (Figure 10.3).

Some terminology:

Pluripotent embryonic stem cells are undifferentiated early embryonic cells derived from the inner cell mass of mouse blastocysts.

Nuclear localization sequences (NLS) are important for directing a protein to nucleus. Generally NLS contains PK3RKV amino acid residues in the protein. The proteins are directed to the nucleus with the help of nuclear pore complex and with the combined effect of RAN-GTPase and IMPORTIN molecules.

Figure 10.1 *Cre-lox* inversion:

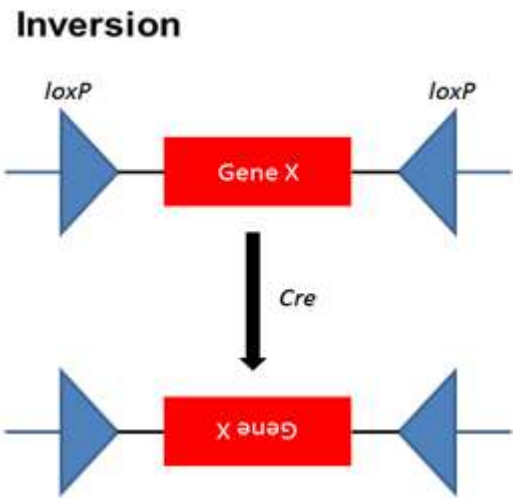


Figure 10.2 *Cre-lox* translocation:

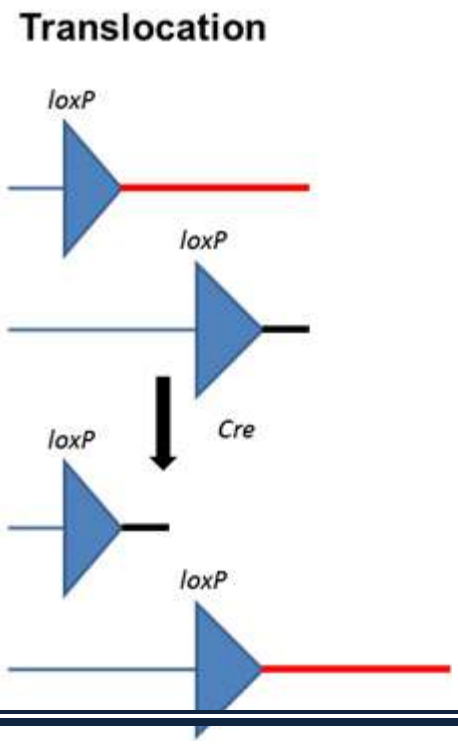
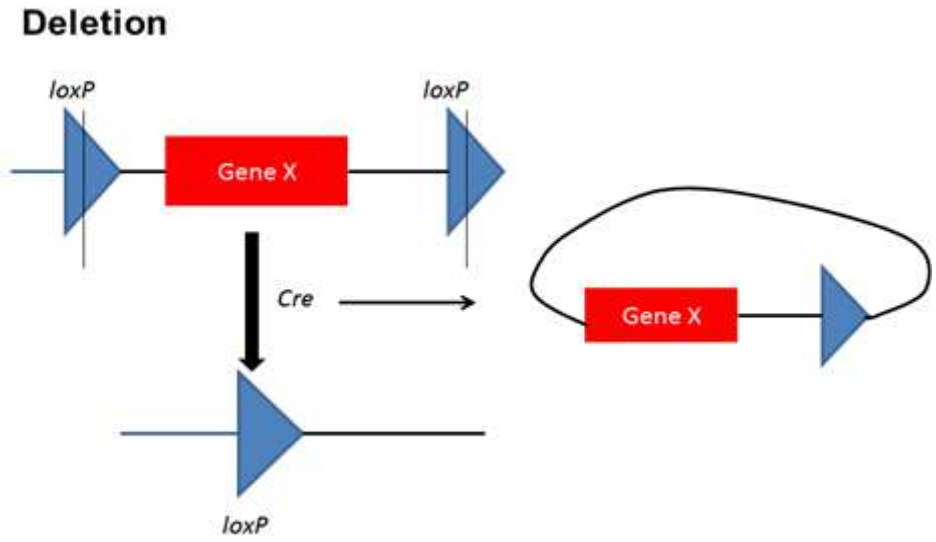


Figure 10.3 *Cre-lox* deletion:



10.2 Humanized mouse:

A mouse carrying functional human genes or cells is called humanized. Humanized mice are made by introducing the functional copy of a human gene into a recipient mouse in the form of expression plasmid. The mice containing the functional copy of a human gene are bred with a knockout mouse. Alternatively the humanized mouse can also be made by directly injecting the human transgene into pronuclear embryos from mice that are knockout for the corresponding murine gene. In addition, the human gene can also be introduced using a routine homologous recombination in embryonic stem cells with the corresponding mouse gene.

10.3 Knockout mice:

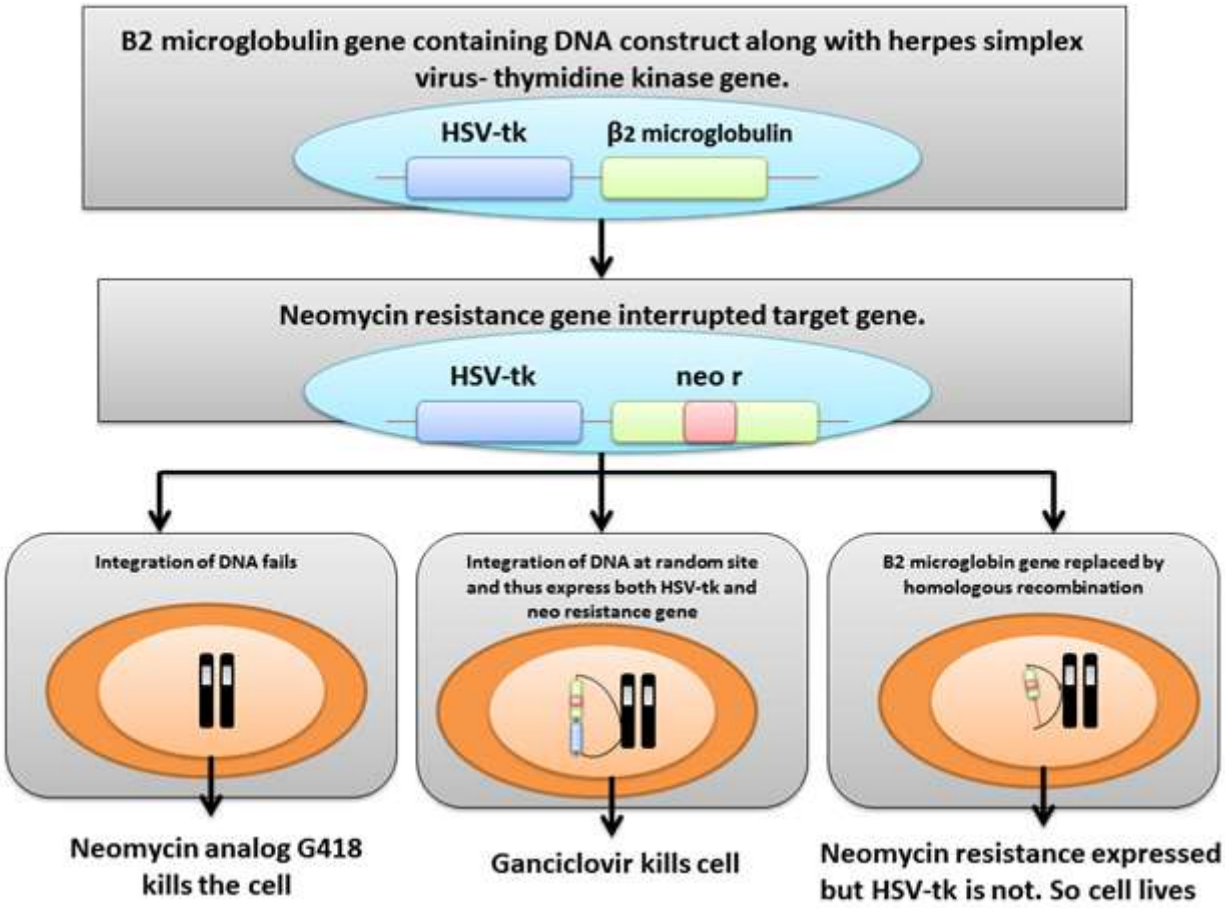
A mouse in which an endogenous gene has been modulated in such a way that its normal expression is altered is called knockout mice. The knockout mice are important in understanding the complex biological pathways, human embryonic developmental pathways, and gene therapy. The knockout models are made for a very specific gene of interest. Alteration of the gene takes place at a precise position using an embryonic pluripotent stem cell. Recombinant DNA vector containing the gene of interest is interrupted with an antibiotic resistance gene such as G418 (Figure 10.4). To ensure targeted integration has occurred, the flanking DNA contains the thymidine kinase gene (tk) from herpes simplex virus. There are three possibilities following the integration of the recombinant DNA:

1. Cells or embryo will die if they fail to integrate when grown in presence of neomycin and in absence of resistance gene.

2. The cells or embryo will die under Ganciclovir if the integration occurs at random site, since the expression of tk will kill it.

3. Cells and embryo will survive if the integration is site specific since the knocked out cells will survive both in G418 and Ganciclovir.

Figure 10.4 knockout mice generation:



Lec.10 Gene Therapy

Artificial Human Chromosome Is New Tool for Gene Therapy

BIOLOGISTS have accumulated wonderfully detailed insights into the genetic causes of human disease but in many cases still lack the tools to make use of the knowledge. A treatment of great promise, gene therapy, has long been stalled by the lack of suitable vehicles to get genes into human cells and maintain them in working order.

But that obstacle could dissolve if a strange new biological invention works as well as its makers hope.

The invention is an artificial human chromosome.

Synthesizing just the essential components of a human chromosome, researchers found that human cells would assemble the inserted pieces into a working chromosome, which could serve as a vehicle for therapeutic genes, they report in today's issue of the journal Nature Genetics.

Gene therapy holds such promise in combating inherited diseases, heart disease and cancer that the Federal Government has been pouring $200 million a year into research, and the pharmaceutical companies' research efforts are said to be even larger.

But so far, almost all attempts to introduce curative genes into patients have fizzled. A report prepared for the National Institutes of Health in December 1995 concluded, "While the expectations and the promise of gene therapy are great, clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol." Little has changed since, said a co-author of the report, Dr. Arno G. Motulsky of the University of Washington in Seattle.

A major reason is that the stripped-down viruses used in many experiments to move human genes into cells do not provide a stable platform for the genes to operate. The viruses are good at sneaking into cells, as nature designed them to do, but some are not large enough to carry a full human gene and all its genetic control switches, while others provoke attack by the immune system.

So most introduced genes have lacked stable expression, the biological term for a gene producing its protein. The artificial human chromosome could sidestep many of these problems because it promises to provide a stable, natural platform for human genes to function and to be passed to daughter cells when the parent cell divides.

Commenting on the development, Dr. James M. Wilson, director of the Institute for Human Gene Therapy at the University of Pennsylvania, said, "Stability of expression is a big deal, and if we can in fact create a genetic element that functions like a chromosome, it is a really important advance."

Dr. W. French Anderson, director of the gene therapy laboratories at the University of Southern California School of Medicine, described the advance as "fascinating science" that would be "very helpful for the development of gene therapy vectors." He predicted, however, that improved viral vectors were likely to be in use before artificial chromosomes could pass manufacturing and regulatory hurdles.

The artificial chromosome is the fruit of more than a decade's work by Dr. Huntington F. Willard, a human geneticist at the Case Western Reserve University School of Medicine, together with colleagues at the university and

at Athersys, a Cleveland biotechnology company.

The project is based on the insight that if the minimum essential components of a human chromosome were to be introduced into a cell, the cell's natural repair system might be able to stitch them together into a a working whole.

The Cleveland team synthesized the ends of human chromosomes, short runs of DNA known as telomeres, and a long stretch of DNA designed to correspond to the centromere, a vital structure toward the center. Human centromeres, the staging posts for the apparatus that spins dividing chromosomes apart during cell division, are still somewhat mysterious. Synthetic versions have not been made until now, although several groups have been trying.

The synthetic telomeres and centromeres and a long test region of human DNA were all inserted separately into test-tube cultures of human cells with the help of a fat-based chemical that helps DNA squeeze through cell membranes.

As the researchers had hoped, not only did the cells' repair systems knit together the fragments of DNA, but the assembled DNA molecule also became clothed in chromatin, the special proteins characteristic of chromosomes.

The human cells treated the artificial chromosomes like one of their own, dutifully furnishing a copy to each daughter cell throughout the 240 cell divisions of the six-month experiment.

Dr. Willard and his colleagues, in their report, called the development an important step "toward building a prefabricated artificial chromosome capable of introducing and stably maintaining therapeutic genes in human cells." His colleagues were Drs. John J. Harrington, Gil Van Bokkelen, Robert W. Mays and Karen Gustashaw.

The Cleveland biologists believe that human genes built into the artificial chromosomes would operate normally, but they have not yet tested the idea. The DNA used in these preliminary experiments carried a marker gene, which was expressed, but no known human genes. "We have every reason to expect human genes would be expressed just fine," Dr. Willard said.

Dr. Van Bokkelen, president and chief executive of Athersys, the company that hopes to exploit the invention, said the immediate plan was to improve the artificial chromosomes as vectors for moving genes into cells. After that, the technology might be used to treat blood cell disorders like sickle-cell disease, hemophilia and immune deficiencies. These diseases have long been a target of gene therapy because blood-forming cells can be removed, treated and injected back into the body.

In the longer term, Dr. Van Bokkelen said, the new technique could be applied

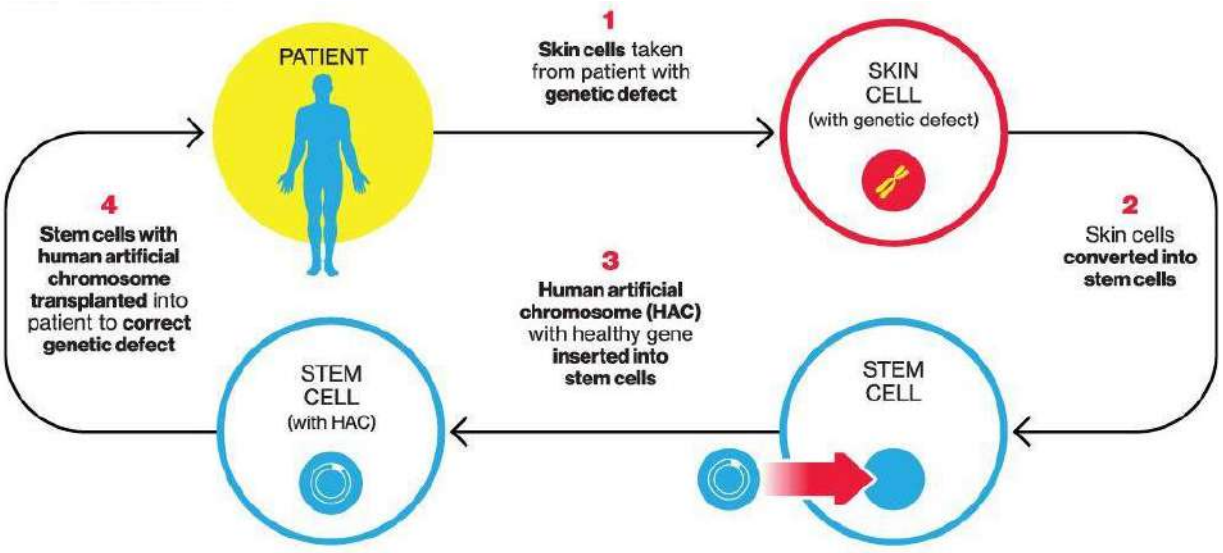
to developmental diseases like muscular dystrophy and cystic fibrosis and to forms of hereditary cancer.

Among the advantages of the artificial chromosomes, he noted, are their mitotic stability, which means that they are reliably reproduced and passed on every time the cell divides. There appears to be no immediate limit to the number of genes and regulatory elements that could be loaded onto them, he added.

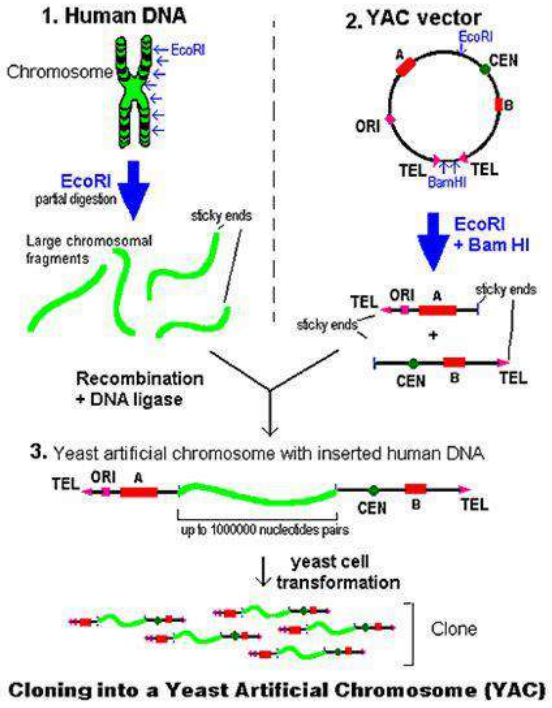
Some cancers develop when a cell's tumor suppressor genes get knocked out of action by a mutation. The ample carrying capacity of the artificial chromosome makes it possible to think of packing it with spare copies of tumor suppressors and other disease-preventing genes, along with switches to turn them on should the natural copies fail.

"One of the great appeals of using artificial chromosomes is to introduce all the subtleties of the genetic controls that exist at the chromosomal level.

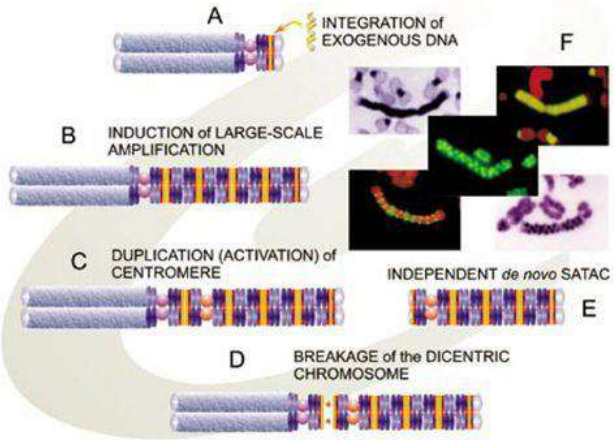
Artificial chromosomes were first created in yeast, a model organism much studied by biologists, in the early 1980's, but efforts to do the same for humans have long been frustrated. Building a working centromere was the missing piece.



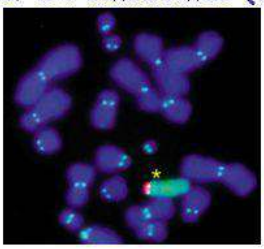
Artificial Chromosomes



Cloning into a Yeast Artificial Chromosome (YAC)



Mammalian satellite DNA-based artificial chromosomes (SATACs)



Lec.11 **Gene Therapy**

CARCINOGENIC AGENTS

Chemical carcinogens Physical agents Microbiological agents

DIRECT ACTING CARCINOGENS

Direct-acting agents require no metabolic conversion to become carcinogenic. have highly reactive eletrophile groups that directly damage DNA, leading to mutations and eventually cancer.

Examples are : cancer chemotherapeutic drugs (e.g., alkylating agents)

Used for e.g., leukemia, lymphoma, Hodgkin lymphoma, and ovarian carcinoma, non-neoplastic disorders, such as rheumatoid arthritis or Wegener granulomatosis.

May evoke later a second form of cancer, usually leukemia

INDIRECT ACTING CARCINOGENS

The designation indirect-acting agent refers to chemicals that require metabolic activation & conversion to an ultimate carcinogen before they become active

Indirect-acting agents are not active until converted to an ultimate carcinogen by

endogenous metabolic pathways e.g.. endogenous enzymes like cytochrome p-450 oxygenase.

Examples :

Benzopyrene, polycyclichydrocarbons, aromatic amines and azo dyes , aflatoxin b1, insecticides, fungicides, nitrites used as food preservatives

PHYSICAL CARCINOGENS

IONIZING RADIATION

Ionizing radiation includes: x-rays, gamma rays, as well as particulate

radiation; alpha, beta, protons, neutrons and primary cosmic radiation. all

forms are carcinogenic with special sensitivity in:

Bone marrow: acute leukemia occurs before other radiation-induced neoplasia (seven year latent period in atomic bomb survivors).

Thyroid: carcinoma occurs in 9 % of those exposed during infancy or childhood.

Lung: increased frequency of lung cancer in miners exposed to radon gas (an

alpha particle emitter).

The oncogenic properties of ionizing radiation are related to its mutagenic effects; it causes chromosome breakage, translocations, and, less frequently, point mutations.

Double-stranded DNA breaks seem to be the most

important form of DNA damage caused by radiation.

there is also some evidence that non-lethal doses of radiation

may induce genomic instability.

DNA TUMOR VIRUSES IN HUMAN CANCER

Papilloma viruses

Cause natural cancers in animals

Cause benign warts

Epitheliotropic - most human tumors are malignancies of

epidermodysplasia verruciformis

malignant wart

squamous cell carcinoma

Adenoviruses

highly oncogenic in animals Only part of virus integrated Always the same part

Early functions

E1a region: 2 T antigens E1b region: 1 T antigen E1a and E1b = oncogenes

Herpes viruses

Considerable evidence for role in human cancer

Some very tumorigenic in animals

Viral DNA found in small proportion of tumor cells

Hepatitis B Virus Strong correlation between HBV and hepatocellular carcinoma

China: 500,000 - 1 million new cases of hepatocellular carcinoma per year Relative risk of getting HCC

DNA Viruses	Associated Human Cancer	Areas of High Incidence	Other Suspected Risk Factors
Epstein-Barr virus (EBV)	Burkitt's lymphoma	Tropical Africa	Malaria
	Nasopharyngeal carcinoma	Southern China South East Asia Eskimos in Alaska and Greenland	Salted fish in infancy Histocompatibility genotype
Hepatitis B virus (HBV)	Hepatocellular carcinoma (hepatoma)	South East Asia Tropical Africa	Aflatoxin from fungal contamination of food Alcoholism
Human papilloma virus (HPV) (subtypes 16 and 18)	Carcinoma of the uterine cervix	Worldwide	Smoking