UNIT-4

GENE THERAPY

There are more than 4000 known inherited disorders which lack effective therapy. There is one infant in every 100 that has genetic defect. Large numbers of genetic disorders are associated with liver (where numerous liver specific enzymes catalyze complex metabolic processes) and with haematopoietic organ (bone marrow).

Very few genetic disorders can be cured. Current methods of treatment of genetic diseases are mostly symptomatic treatments and in certain cases congenital abnormalities are corrected by surgery. Symptomatic treatments when analysed on the criteria of life increased, reproductive abilities and social acceptance show very low rate of success. It is completely successful only in 8 diseases (12% of the total), moderately successful in 26 genetic diseases (40% of the total) and useless in the rest. Apart from the low rate of success the other problems with symptomatic treatments currently practiced are : (a) high costs involved, (b) need of continuous treatment throughout the life, (c) dangers of transmission of AIDS virus and others during blood transfusion (d) high iron contents after repeated blood transfusions in thalasemic patients as side effect etc.

Some of the examples of current practices of symptomatic treatments for genetic diseases are

i) Replacement therapy for missing factor, e.g., coagulation factor for haemophilia.

ii) Long-term blood transfusion for thalassemia.

iii) Replacement with immunoglobulins for children with congenital hypogammaglobulinemia.

iv) Growth hormone therapy — for certain genetic dwarfism.

v) Insulin injections or oral for diabetes patients.

vi) Replacement of missing enzyme, e.g., in Gaucher's disease.

vii) Diet control — prevents accumulation of toxic metabolites, e.g., Phenylketonuria, Galactosaemia, fructuse intolerance,

viii) Bone marrow transplantation — to correct blood disorders.

Due to the problems mentioned which are associated with these treatments and low rates of successes alternatives are constantly being searched. The ability to introduce new genes into mammalian cells raises the possibility of

being able to correct genetic defects in humans by introducing a copy of normal functional gene into the appropriate cells.

There are four potential approaches to gene therapy:

(1) Addition of normal gene to replace the function of mutant (errant) gene.

(2) Replacement of mutant gene sequence with normal gene sequence.

(3) Establishment of alternative pathways to circumvent mutant functions.

(4) Altering regulation of normal or mutant genes.

In September 1990, at the National Institute of health in Bethesda, USA, Michaele Blease and W. French Andresow attempted gene therapy on human patient for the first time for SCID (Severe combined immunodeficiency) case. Human gene therapeutics, based on either *ex vivo gene* therapy or *in vivo* gene therapy or antisense therapy will enter into phase II and phase III trials in next 10 years. There are more than 15 companies which are currently working in this area of development.

- **Gene Replacement Therapy (Gene Augmentation) Vs. Corrective Gene Therapy**

There are two basic ways that gene therapy can be carried out. In both healthy counterpart of the defective gene is introduced into appropriate cells. In one the errant gene remains and healthy gene supplements to remove the deficiency. This is gene augmentation. As against this in corrective gene therapy errant defective gene is displaced by correct functional counterpart. There are many points of differences related to two ways. They can be documented as follows:

Gene Replacement Therapy (Gene Augmentation)	**Corrective Gene Therapy**
(1)Random insertion of healthy counterpart of defective gene somewhere in genome so that its product could be available.	(1) Directing insertion of healthy gene at specific site to displace defective gene is required.
(2) Suitable for recessive disorders and for single gene mutations.	(2) Possible for dominant disorders.
(3) No recombinational event required and nonspecific insertion will work so long as appropriate regulatory controls are provided for expression.	(3) Insertion at specific site would require some form of induced recombinational event.
(4) Approach is not useful for dominant nature disorders or where errant (defective) gene gives destructive or interfering substance.	(4)This approach would be ideal where errant gene produces destructive or interfering substance.
(5) This approach is feasible today and has effect similar to transplantation approach; only thing it being done still at root level of the defect.	(5) Extensive study is still required to direct gene at correct position in the genome.

Corrective Gene therapy approach is also possible if we try to bypass mutant gene by stimulating production of similar gene that was normally functional at different developmental stage. Corrective gene therapy may also be done by suppression of particular mutation by transfer RNA that was introduced into cell. For single gene disorders, e.g., premature stop codon use of suppressor tRNA which will insert amino acid into altered codons will work.

- **Somatic Therapy Vs. Germline Therapy**

Healthy genes can be introduced into germ cells (like sperms, eggs, early embryos) or into somatic cells (any other cells like blood cells, liver cells, skin cells, lung cells etc.) Introduction of healthy genes in germ cells is not encouraged due to both technical and ethical reasons. Somatic cell therapy is hot area of research today.

Somatic Therapy	Germline Therapy
(1) Genes are introduced into somatic cells.	(1) Genes are introduced into germ line cell and will get distributed in both germ cells and somatic cells.
(2) Changes are confined to the recipient.	(2) Changes will be passed to the future generations.
(3) No ethical issues attached.	(3) Ethical problems to be answered and precludes its use.
(4) Technical expertise for somatic cell manipulations *in vitro* introduction of gene of interest and replanting somatic cells in body to make them functional is developed.	(4) There are still many technical difficulties in introduction of genes into germ cells.
(5) Genes are tissue specific in most instances (in their expression) although not location specific in many. In many instances, it may not be possible to reconstitute normal level and tissue distribution.	(5) High frequency of insertional mutations are observed in this process and cause teratogenic consequences.
	(6) It is only abnormal embryo which is to be manipulated and to avoid causing harm to normal potential fetus early diagnosis and therapy (early stages of embryo development) is required.

- **Approaches to Targeting Somatic Gene Therapy**

There are two main approaches to target, the cells for gene manipulation. It may be done in *vitro (Ex vivo)* or *In vivo.*
(1) *Ex-vivo* approach: In gene augmentation.
(a) Target cells are removed from the patient. They will be isolated and cultivated *in vitro.*

(b) Recombinant (healthy) genes are introduced into these cells *in-vitro* when in culture.
(c) Then transferring these cells (transplanting them) back into the patient.

For this approach cells should be strong to withstand this handling in three stages, they should have long life (months, years or entire patients life) and also ease of removal of cells is primarily important.

Bone marrow cells, skin cells, Liver cells meet these criteria.

In Ex *vivo* gene therapy patient's own cells (autologous) are used for introduction of recombinant healthy gene *in vitro,* so that immunological reactions are not there when cells are transplanted back into patient. Getting autologous cells and genetic engineering of them for each patient becomes a separate exercise which is time consuming and costly. Hence research is underway to create "universal donor" cells that have most of the immunogenic antigens from cell surface so that they will raise no immunogenic response on range of patients.

Protocol used for gene transfer for *ex vivo* gene therapy has to be efficient and stable maintenance and persistent expression of healthy gene is important.

(2) *In vivo* approach: In second approach, recombinant genes might be introduced into cells *in vivo* by using vectors which are capable of targeting genes to specific sites in the body. Although this approach sounds very interesting, there is little progress in this direction.

Several different cellular targets are being explored for gene therapy. Due to considerable clinical experience in bone marrow culture and transplantation and possibility of manipulation of bone marrow cells they are the most obvious targets to correct many of the genetic defects caused by them and their progeny. Lymphocytes are another target cells for gene therapy. Skin cells can be used to make proteins of need, fibroblasts (a constituent of lower skin layer) is ideal for this purpose. Liver cells are another important target cells to correct dysfunctions of liver (e.g., hypercholesterolemia) and also for blood disease (e.g., haemophilia). Epidermal and epithelial cells are also considered as potential targets. Neurons cannot be removed for genetic alterations but fibroblasts could be used to produce proteins which diffuse into nerve cells.

There are different problems which are associated with different target cells.

- **Methods for Transport of Recombinant Genes to Target Cells**

Transport of recombinant genes to target cells is the key step in successful gene therapy. Method used for gene transport to target cells should be —

(1) highly efficient in bringing about transformation of large number of cells
(2) specific for various cell types and organs
(3) able to transform primary cells
4) able to introduce gene into cells without causing alterations in any genes

Methods used for gene transfer are:

(a) Microinjection
(b) Electroporation
(c) Calcium phosphate mediated transfection
(d) Use of retroviruses as vectors
(e) Use of other viruses as vectors
(f) Injection of liposomes

The details of the techniques involved in above-mentioned DNA transfer methods can be found in module no. 38. Here these methods will be discussed only with the reference to their importance in gene therapy.

a) Microinjection

This technique is useful for embryonic cells, primary cells and cells established m culture. 10-30 ng of DNA can be transferred into nucleus of target cell. The method is tedious only one cell at a time can be handled so laborious also, integration is random and aberrations in adjacent DNA are common. Frequency of stable integration is higher than any other method by several orders of magnitude.

b) Electroporation

This is becoming popular very fast and main advantage is large number of cells can be transformed in shorter time with more ease. 80-90% cells are killed but out of survivors 30 % are stably transformed. The method is suitable for primary cells such as hepatocytes epidermal cells, pancreatic B cells, pre-B cells and haematopoietic stem cells.

c) Calcium Phosphate Mediated Transfection

Copies of healthy genes are mixed with charged substances. Calcium phosphate, dextran, certain lipids may be used to make mixture with DNA which is then dumped onto recipient cells. Fraction of cells by endocytosis takes up the DNA. Frequency is very low — only one cell in 10^6 - 10^7 cells gets transacted. Mechanism of incorporation of transfected DNA is not clear. Low frequency, aberrations caused in DNA makes the method less applicable in gene therapy protocols. It is more used for gene transfer into cultured cells.

d) Use of Retroviruses as Vectors

This is the most popular method. Viruses which are non replicative and which do not cause pathological consequences are used. Thus mechanism of infection and transformation of cells by recombinant genes that are packed into viruses are the two properties which are taken advantage of in this method. Retroviruses are the choice as vectors because:

(i) Retroviruses are relatively harmless
(ii) They do not kill the cells they infect
(iii) They can carry more foreign genetic material than other viruses
(iv) Retroviruses can infect broad spectrum of host species and cell types
(v) Efficient integration with host

The retroviral vector requires that target cells must be dividing in order to be infected, whereas majority of the cells are quiescent when being treated for gene therapy which is a drawback.

e) Use of Other Viruses as Vectors

There is still not much progress in developing other viruses as vector. Main difficulties are getting the viruses:

(i) which are incapable of replication
(ii) which do not express viral genes
(iii) which do not harm host cell functions in their own interest
(iv) which can be manipulated to act as efficient vectors and pack more foreign genetic material.

Adenoviruses, Bovin Papilloma viruses, Vaccinia virus, Hepatitis virus and Simian virus 40 (SV 40) are amongst the others tried as vectors for gene therapy. Tissue specific viruses can be used while carrying out *in vivo* gene therapy so that viruses will take the healthy genes to defined region of the body. For example, herpes simplex virus infects nerve cells at their axonal terminal ends and is transported internally to the neuronal cell body, where it is maintained. For neurological disorders system involving herpes simplex virus can be used for delivery of remedial gene.

f) Injection of Liposomes

Intravenous injection of liposomes to deliver rat insulin gene to rat liver and spleen as model for diabetic treatment has been tried.

Whatever the method used, the goal of gene transfer is to achieve stable transformation of target cell by stable integration of the recombinant gene into chromosome of the host-cell. Other alternative could be, use of vector which will carry desired genes into host cell and will autonomously replicate into host cell as episomes without integration into host chromosome. This would be preferred and would be more attractive as it will cause no damage to host cell genes by insertional mutations or rearrangements. Also it may allow transient expression. SV40, Bovine Papilloma virus and Polyoma viruses based vectors which are thought in this regard express the viral genes which is not desired and also transfected gene may integrated in some cells which is a problem.

- **Cellular Targets for Somatic Gene Therapy**

Following cellular targets have been explored for gene therapy

(1) Bone marrow - totipotent stem cell
(2) Hepatic cells
(3) Fibroblasts
(4) Endothelium and pulmonary epithelium cells
(5) Endocrine cells

(6) Central nervous system
(7) Smooth muscle cells of blood vessel walls

(1) Bone marrow is ideal target for gene therapy because —

a) It is readily accessible for experimental manipulation.
b) Many diseases involving cells of hematopoietic lineage can be treated if totipotent stem cell is available for gene manipulation. Thalassemia, sickle cell disease, immunodeficiency states and diseases due to enzyme deficiencies in bone marrow derived cells can be tackled. Also diseases for which tissue specific function is not required can be also dealt with by bone marrow cells being used for gene therapy.
c) Totipotent stem cell gives rise to red blood cells, T cells, B cells, neutrophils, eosinophils, basophils, macrophages, kupfer cells, osteoclasts, platelets. Bone marrow contains stem cell at frequency of 10^{-4} to 10^{-5}. It is however difficult to isolate and culture totipotent stem cells.

(2) Hepatic cells

This is a difficult but important target for gene replacement therapy because many important metabolic and synthetic functions are normally associated with liver. Replacing genetically deficient hepatic cells with functional ones giving product will be useful for many liver related disorders. Liver cells manipulation is more difficult than that of bone marrow cells. Hepatocytes are relatively resistant to retroviral infections while carrying out gene manipulations. There is also less experience in field of hepatocellular transplantations. However, recently some successes are achieved on retroviral route for introduction of recombinant gene in hepatic cells. Both *ex vivo* and *in vivo* gene therapies are being researched for disorders concerned with liver cells. For Familial hypercholesterolemia and genetic diseases concerned with deficiencies involving serum proteins will be tackled by targeting hepatocytes for manipulations.

(3) Fibroblasts

These are interesting targets for gene therapy because it can be easily harvested, grown in culture and transformed with recombinant viruses. Few

genetic diseases directly involving the fibroblast and diseases which require secretion of protein, a hormone or provision of essential nutrients or clearance of soluble metabolites may be tackled using fibroblasts as cellular targets of gene therapy.

(4) Endothelium and pulmonary epithelium cells

Cells of many organs such as blood vessels, intestine, pancreatic ducts, bile ducts are important for gene therapy because they are readily accessible for infection by retroviruses *in vivo* and are also capable of substantial proliferation. Pulmonary epithelial cells can be used for gene therapy of cystic fibrosis.

(5) Endocrine cells

These will be targets for therapy of various endocrine and metabolic deficiency states. Endocrine cells are not infectable by recombinant retrovirus because they lack ability to divide. Other vectors are being tried for successful transformation of such cells. Pancreatic B cells for diabetes and adrenocortical cells for some hormone deficiencies may be used for gene therapy research.

(6) **Central nervous system**

There are several diseases in which brain is an affected organ. Introduction of genes into neurons in central nervous system is currently being studied. There are several major hurdles in considering gene therapy for central nervous system. These are complex architecture of brain, terminally differentiated neurons with little potential to divide. There is a report of construction of recombinant Herpes simplex virus carrying hypoxanthine phosphoribosyl transferase gene and it being used to infect rat neuronal cells and showing successful human enzyme activity.

(7) Smooth muscle cells of blood vessel walls

These are considered for *ex vivo* gene therapy because they are in contact with the circulatory system. Required protein may be released into blood stream by smooth muscle cell of blood vessel wall carrying a cloned gene.

Table: Diseases fro somatic gene therapy

Sr. No.	Disease	Symptoms	Current Therapy
1	Severe combined immunodeficiency	Loss of T and B cells.	Bone marrow transplantation, Adenosine deaminase replacement
2	Gaucher's disease	Accumulation of glucocerebroside in macrophages causing liver, spleen bone damage.	Symptomatic treatment. Removal of spleen antibiotics, repair bone damage, enzyme replacement.
3	Familial hypercholesterolemia	Elevated blood serum cholesterol, coronary artery disease	Diet, drugs, liver transplantation
4	Hemophilia A	Altered plasma protein that causes defective blood clotting, chronic internal bleeding into joints, and excessive bleeding after wounds	Concentrate of factor VIII by transfusion.
5	Phenylketonuria	Excess phenylalanine in blood stream of newborns causing mental retardation	Restriction of dietary phenylalanine
6	Emphysema	Deficiency of serum protein protease inhibitor, damage to the lungs, cirrhosis of liver	Replacement therapy, lowering environmental risks
7	Cystic fibrosis	Multisystem disease, pancreatic insufficiency, intestinal blockage, blocked airways of lungs	Antibiotics, physical clearing of lungs

8	Duchenne muscular dystrophy	Progressive muscle wasting confined to wheelchair	Good nutrition, aid in respiration. Only supportive treatment.
9	Sickle cell disease	Chronic anemia. Multisystem disease damage to spleen, liver, heart, kidney, brain	Blood transfusion, drugs.
10	Thalassemia	--	Blood transfusion

➢ Limitations and Requirements in Gene Therapy

1. Knowledge of exact cause of disease at DNA level.
2. Region bearing disorder must be well defined.
3. Only single gene defects can be tackled today.
4. Normal counterpart of defective gene is required for gene therapy.
5. Homologous recombination will have to occur for replacement of defective gene b, correct one.
6. Addition of functional gene and its random integration with genome is possible pan today.
7. Potentially curable diseases are single gene defects with recessive nature.
8. Affected tissue must be accessible for gene therapy.
9. Lasting presence of gene manipulated cells after re-transplantation is important. Self renewable cells are preferred targets so diseases involving such cells could be cured.
10. Corrected cells should have some selective advantage *in-vivo* over affected cells.
11. Spread of retrovirus throughout body and unwanted transmission of virus is the negative aspect possible.
12. The induction of oncogenesis as a result of provirus integration is another risk.

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- **Applications of genetic engineering**

1) **In Agriculture (Golden rice and Bt Cotton)**
2) **In Human and Animal health (Interferon and HBV vaccine)**
3) **In Industries (Strain improvement and Insulin)**
4) **In environment (Superbug and Bioremediation using GEMs)**

1) **In Agriculture (Golden rice and Bt Cotton)**

a) Golden rice

Golden rice is a variety of rice (*Oryza sativa*) produced through genetic engineering to biosynthesize beta-carotene, a precursor of vitamin A, in the edible parts of rice.[1] It is intended to produce a fortified food to be grown and consumed in areas with a shortage of dietary vitamin A.

Golden rice was created by transforming rice with two beta-carotene biosynthesis genes:

1. *psy* (phytoene synthase) from daffodil (*Narcissus pseudonarcissus*)
2. *crtI* (carotene desaturase) from the soil bacterium *Erwinia uredovora*

Golden rice is the result of an effort to develop rice varieties that produce provitamin-A (beta-carotene) as a means of alleviating vitamin A (retinol) deficiencies in the diets of poor and disadvantaged people in developing countries. Because traditional rice varieties do not produce provitamin-A, transgenic technologies were required.

Through the work of two European scientists, Dr. Ingo Potrykus of the Swiss Federal Institute of Technology in Zurich and Dr. Peter Beyer of the University of Freiburg in Germany, rice plants were developed containing two daffodil genes and one bacterial gene that carry out the four steps required for the production of beta-carotene in rice endosperm. Endosperm is the nutritive tissue surrounding the embryo of a seed and makes up the majority of the rice grain that we eat. The resulting plants appear normal except that after milling

(to remove the brown bran), their grain is a golden yellow color due to the presence of provitamin-A

How does golden rice produce provitamin-A?

Provitamin-A is not produced by traditional rice varieties. However, geranylgeranyl diphosphate (GGDP), a compound naturally present in immature rice endosperm, with the help of several enzymes not normally found in rice can be used to produce provitamin-A.

Two genes from daffodil and one from the bacterium Erwinia uredovora were inserted in the rice genome. These three genes produce the enzymes necessary to convert GGDP to provitamin-A. The inserted genes are controlled by specific promoters such that the enzymes and the provitamin-A are only produced in the rice endosperm.

b) Bt cotton

Bt cotton is a genetically modified organism (GMO) cotton variety, which produces an insecticide to bollworm. It is produced by Monsanto.

Strains of the bacterium *Bacillus thuringiensis* produce over 200 different Bt toxins, each harmful to different insects. Most notably, Bt toxins are insecticidal to the larvae of moths and butterflies, beetles, cotton bollworms and ghtu flies but are harmless to other forms of life. The gene coding for Bt toxin has been inserted into cotton as a transgene, causing it to produce this natural insecticide in its tissues. In many regions, the main pests in commercial cotton are lepidopteran larvae, which are killed by the Bt protein in the genetically modified cotton they eat. This eliminates the need to use large amounts of broad-spectrum insecticides to kill lepidopteran pests (some of which have developed pyrethroid resistance). This spares natural insect predators in the farm ecology and further contributes to noninsecticide pest management.

Bt cotton is ineffective against many cotton pests such as plant bugs, stink bugs, and aphids; depending on circumstances it may be desirable to use insecticides in prevention.

Mechanism

Bt cotton was created through the addition of genes encoding toxin crystals in the Cry group of endotoxin. When insects attack and eat the cotton plant the Cry toxins are dissolved due to the high pH level of the insect's stomach. The dissolved and activated Cry molecules bond to cadherin-like proteins on cells comprising the brush border molecules. The epithelium of the brush border membranes separates the body cavity from the gut whilst allowing access for nutrients. The Cry toxin molecules attach themselves to specific locations on the cadherin-like proteins present on the epithelial cells of the midge and ion channels are formed which allow the flow of potassium.[3] Regulation of potassium concentration is essential and, if left unchecked, causes death of cells. Due to the formation of Cry ion channels sufficient regulation of potassium ions is lost and results in the death of epithelial cells. The death of such cells creates gaps in the brush border membrane.

2) **In Human and Animal health**

a) Interferon
b) HBV vaccine

a) Interferon (Human Interferon Genes) (HIG)

Interferon is defined as "a protein which exerts virus non-specific antiviral activity, at least in homologous cells through cellular metabolic procedure involving the synthesis of both RNA and protein." Thus, interferon is secreted by human cells just to resist the immediate invasion by virus and multiplication of abnormal cells.

Interferon is used to cure many viral diseases such as common cold and hepatitis. It is species specific. In man there are 3 classes of interferon:

i) Alpha interferon (IFN-α) or leukocyte interferon (leukocytes of blood)

ii) Beta interferon (IFN-β) or fibroblast interferon (fibroblast of connective tissue).

iii) Gamma interferon (IFN-γ) or immune interferon (by lymphocytes of blood) and lymphoblastoid interferon by transformed leukocytes.

In 1980, IFN-α and IFN-β were successfully produced from genetically engineered *E. coli* cells (by isolation of mRNA from leukocytes and fibroblasts, production of cDNA, its integration into pBR322 and incorporation and cloning into *E. coli* cells). Production was estimated to be about 1,000 to 100,000

molecules of IFN-β per cell. The Swedish firm, Biogene, produced IFN-α and IFN-β through recombinant DNA techniques.

Later on hybrid plasmid containing cDNA of IFN-β genes was built up which needed a promoter site on plasmid to express in *E. coli* cells. Similarly, hybrid plasmids were also prepared that contained IFN- genes with *trap* promoter between the leader and ribosome binding sites, so that expression of interferon could be done. Expression of both the interferon could be optimised by varying the spacing sequence between *trap* Shine- Dalgarno sequence and the initiator condon.

b) HBV (Hepatitis B Vaccine)

Vaccines are chemical substances prepared from the proteins (antigen) of other animals which confer immunity to a particular virus. Some of the vaccines synthesized biologically through genetic engineering are briefly described as below:

***(a)* Vaccines for Hepatitis B Virus**

Hepatitis B virus (HBV) is wide spread in man and produces several chronic liver disorders such as Fulminant chronic hepatitis, cirrhosis and primary liver cancer. HBV DNA is a double stranded circular molecule of about 3Kb size and has a large single stranded gap which must be required with an endogenous polymerase before digestion with restriction enzyme for DNA cloning. Three types of viral proteins are recognised to be antigenic:

i) viral surface antigen (HBsAg),
ii) viral core antigen (HBcAg), and
iii) the e-antigen (HBeAg).

Although the whole viral genome has been cloned and sequenced, yet there is limited information about amino acid sequence of surface and core antigens. Recently, HBV DNA has been successfully cloned in *E. coli* and mammalian cells, and synthesis of HBsAg and HBcAg particles has been done in the cells. Burrell *et al.* (1979) inserted HBcAg genes in PBR322 near p-glactosidase gene. Production of these genes is needed in order to get production of vaccines

on a large scale. In yeast or mammalian systems, these antigens are synthesized more efficiently than in prokaryotes.

(i) Recombinant vaccine for Hepatitis B virus. After infection, HBV fails to grow and even in cultured cells it does not grow. This property has been explained to be due. to inhibition of its molecular expression and development of vaccines. Recombinant vaccine for HBV was produced by cloning HBsAg gene of the virus in yeast cells. The yeast system has its complex membrane and ability of secreting glycosylate protein. This have made it possible to build an autonomously replicating plasmid containing HBsAg gene near the yeast alcohol dehydrogenase (ADH) I promoter (Fig. 7.5). The HBsAg gene contains 6 bp long sequences proceeding the AUG that synthesises N-terminal methionine. This is joined to ADH promoter cloned in the yeast vector PMA-56. The recombinant plasmid is inserted into yeast cells. The transformed yeast cells are multiplied in trytophan-free medium. The transformed cells are selected. The cloned yeast cells are culture for expression of HBsAg gene. This inserted gene sequence expresses and produces particles similar to the 22 um particle of HBV as these particles are produced in serum of HBV patients. The expresed HBsAg particles have similarity in structure and immunogenicity with those isolated from HBV-infected cells of patients. Its high immunogenicity has made it possible to market the recombinant product as vaccine against HBV infection.
***(ii)* Indigenous Hepatitis-B vaccine.** India's first genetically engineered vaccine *(Guni)* against HBV developed by a Hyderabad based laboratory (Shantha Biotechnics Pvt. Ltd.) was launched on August 18, 1997. India is the fourth country (after the U.S.A., France and Belgium) to develop this highly advanced vaccine. The indigeneous yeast-desired HBV vaccine is one third the cost of the imported vaccine. This new vaccine had undergone human clinical trials at Nizam's Institute of Medical Sciences, Hyderabad and K.E.M. Hospital, Mumbai. The clinical trials clearly proved that the seroprotection is about 98%. It was found more effective than the imported vaccine. The Drug Controller General of India has permitted it for commercial manufacture.

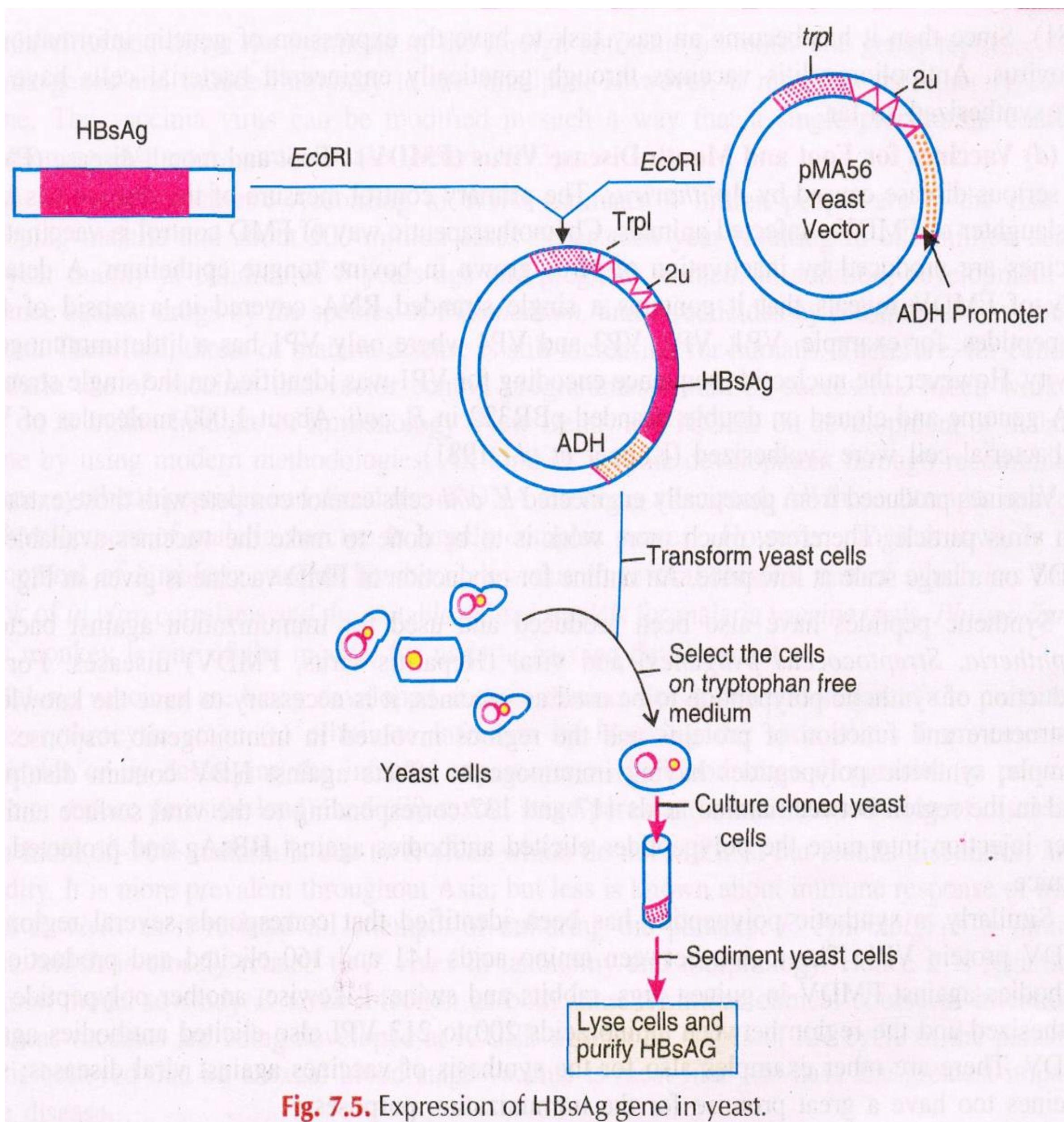


Fig. 7.5. Expression of HBsAg gene in yeast.

1) **In Industries**
a) **Strain improvement**
b) **Insulin**

a) Strain improvement

New alternatives have arisen through the transfer of nucleic acids between different organisms, which are part of the rapidly developing field of **combinatorial biology**. This involves the transfer of genes for the synthesis of a specific product from one organism into another, giving the recipient varied capabilities such as an increased capacity to carry out hydrocarbon degradation.

The genes for antibiotic production can be transferred to a microorganism that produces another antibiotic, or even to a non-antibiotic-producing microorganism. For example, the genes for synthesis of bialophos (an antibiotic herbicide) were transferred from *Streptomyces hygroscopicus* to *S. lividans.* Other examples are the expression in *E. coli,* of the enzyme creatininase from *Pseudomonas putida* and the production of pediocin, a bacteriocin, in yeast used in wine fermentation for the purpose of controlling bacterial contaminants.

DNA expression in different organisms can improve production efficiency and minimize the purification steps required before the product is ready for use. For example, recombinant baculoviruses can be replicated in insect larvae to achieve rapid large scale production of a desired virus or protein.

Transgenic plants may be used to manufacture large quantities of a variety of metabolic products. A most imaginative way of incorporating new DNA into a plant is to simply shoot it in using DNA coated microprojectiles and a gene gun.

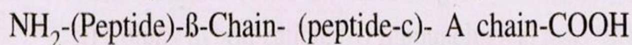
A wide range of genetic information also can be inserted into microorganisms using vectors and recombinant DNA techniques. Vectors include artificial chromosomes such as those for yeasts (YACs), bacteria (BACs), P1 bacteriophage-derived chromosomes (PACs), and mammalian artificial chromosomes (MACs). YACs are especially valuable because large DNA sequences (over 100 kb) can be maintained in the YAC as a separate chromosome in yeast cells.

A good example of vector use is provided by the virus that causes foot and mouth disease of cattle and other livestock. Genetic information for a foot-and-mouth disease virus antigen can be incorporated into *E. coli,* followed by the expression of this genetic information and synthesis of the gene product for use in vaccine production.

b) Insulin

(a) Insulin: This peptide hormone *i.e.* insulin is secreted by the Islets of Langerhans of pancreas which catabolizes glucose in blood. Insulin is a boon for the diabetics whose normal function for sugar metabolism generally fails.

Insulin consists of two polypeptide chains, chain A (21 amino acid long) and B (30 amino acid long). Its precursor is proinsulin which also contains two polypeptide chains, A and B, and is connected with a third peptide chain-C (35 amino acid long). However, the recent discoveries reveal that precursor of insulin is pre- pro-insulin which is about 109 amino acids long. The pre-pro- insulin is synthesized in beta cells of pancreas, the structure of which is given below :



NH_2-(Peptide)-ß-Chain- (peptide-c)- A chain-COOH

In the beginning, efforts were made to isolate mRNA for pre- pro- insulin from rats Islets of Langerhans of pancreas and to synthesize cDNA. Thereafter, it was inserted into a plasmid. The recombinant plasmids were transferred into the *E. coli* cells which secreted pro-insulin.

Itakura *et al.* (1977) chemically synthesized DNA sequence for two chains, A and B, of insulin and separately inserted into two pBR322 plasmids by the side of ß-galactosidase gene. The recombinant plasmids were separately transferred into *E coli* cells which secreted fused ß-galactosidase - A chain and ß- galactosidase - B chain separately. These chains were isolated by detaching from ß-glactosidase in pure form in a amount of about 10 mg/24 g of healthy and transformed cells (Sasson, 1984). Production of recombinant insulin is shown in Fig. 7.2.

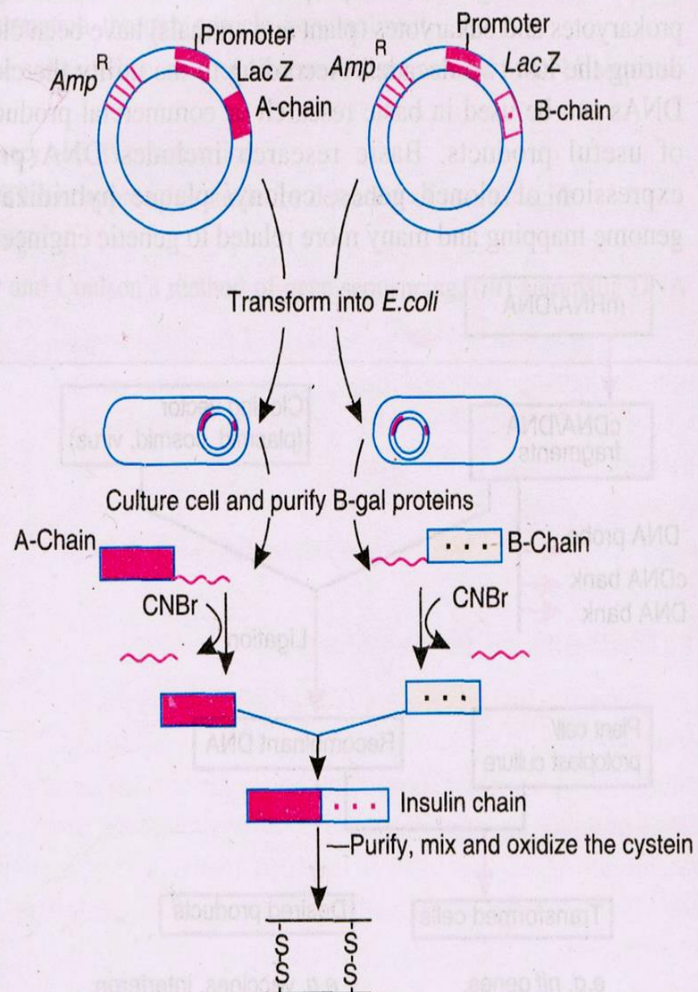


Fig. 7.2. Production of recombinant insulin in *E.coli.*

Detachment of proinsulin could be possible when an extra methionine codon was added at the N-terminus of each gene for A and B chains. The two chains (A and B) were joined *in vitro* to reconstitute the native insulin by sulphonating the two peptides with sodium disulphonate and sodium sulphite. Gilbert and Villakomaroff isolated mRNA for insulin from β cells of rat's pancreas and inserted into pBR322 plasmid in the middle of a gene normally coding the penicillinase, and

incorporated it into *E. coll* rells. *E. coli* cells produced a hybrid protein (penicillinase + proinsulin) from which the proinsulin was separated by using trypsin. It is estimated that clones of *E. coli* are capable of producing about one million molecules of insulin per bacterial cell. Human insulin (humulin) is the first therapeutic product produced by means of recombinant technology by Eli Lilly & Co. in 1980. Shreya Life Sciences, Pune (India) Co., has started producing the second generation rDNA-based insulin without using DNBr with the name 'Recosulin'.

4) In environment

a) Superbug b) Bioremediation using (GEMs)

a) Superbug

For detoxification and degradation of toxic chemicals, enzymes are encoded by specific genes present on plasmids. Chakraborty and co-workers (1979) succeeded in isolating the microbial culture which could utilize a number of organic chemicals, toxic in nature, such as salicylate, 2,4-D, 3 chlorobenzenes, ethylene, biphenyls, 1,2,4-trimethylbenzene, 2, 4, 5-trichlorophenoxyacetic acid, *etc.* (Chatterjee *et al.*, 1981; Kellogg *et al.*, 1981).

Genes responsible for degradation of environmental pollutants, for example, toluene, chlorobenzene acids, and other halogenated pesticides and toxic wastes have been identified. For every compound, one separate plasmid is required. It is not like that one plasmid can degrade all the toxic compounds of different groups. The plasmids are grouped into four categories:

(*i*) OCT plasmid which degrades, octane, hexane and decane,

(*ii*) XYL plasmid which degrades xylene and toluenes,

(*iii*) CAM plasmid that decompose camphor, and

(*iv*) NAH plasmid which degrades naphthalene.

Dr Anand Mohan Chakrabarty (an Indian born American scientist) produced a new product of genetic engineering called as superbug (oil eating bug) by introducing plasmids from different strains into a single cell of *P. putida.* This superbug is such that can degrade all the four types of substrates for which four separate plasmids were required (Fig. 7.12).

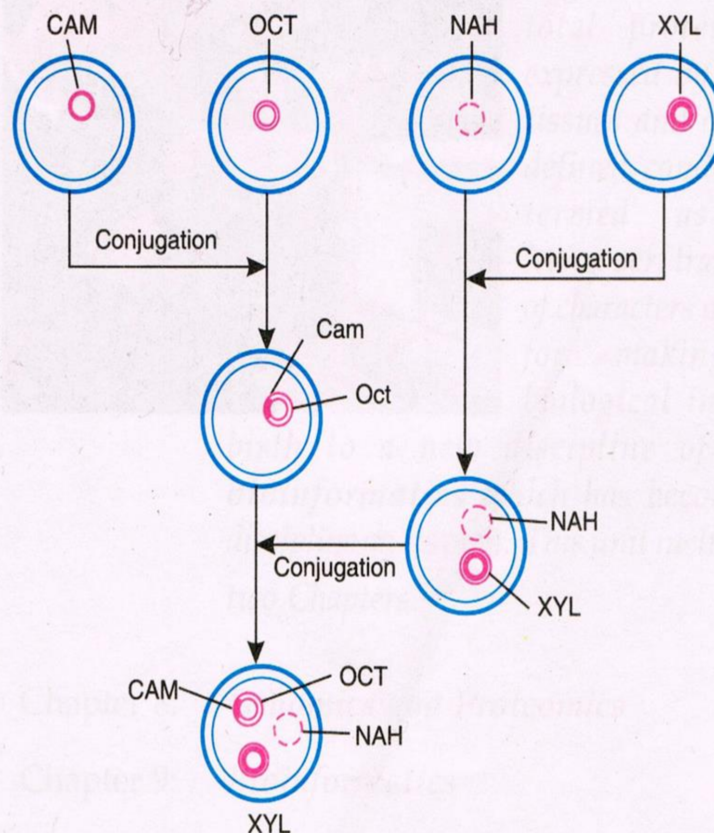


Fig. 7.12. Creation of a superbug (Diagrammatic).

The plasmids of *P. putida* degrading various chemical compounds are TOL (for toluene and xylene), RA500 (for 3, 5-xylene) pAC 25 (for 3-one chlorobenxoate), pKF439 (for salicylatetoluene). Plamid WWO of *P. putida* is one member of a set of plasmids now termed as TOL plasmid. WWO is propagated in *E. coli* (Chatterjee *et al.,* 1981; Kellogg *et al,* 1981).

b) Bioremediation using (GEMs)

Bioremediation is a waste management technique that involves the use of organisms to remove or neutralize pollutants from a contaminated site. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation. Recent advancements have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population's ability to break down contaminants. Microorganisms used to perform the function of bioremediation are known as **bioremediators**. However, not all contaminants are easily treated by bioremediation using microorganisms. For example, heavy metals such as cadmium and lead are not readily absorbed or captured by microorganisms.

Genetic Engineering has now become integrated with bioremediation since many microbes can be artificially designed which can consume the toxic waste and pollutants that are not usually taken in by normal microbes. This is done by first genetically altering the sequences of the desired microbe and enhancing its ability to digest the toxic particles of the pollutant or by genetically engineering a new microbe which has extraordinary ability to take in, consume and digest the pollutants. Thus, micro-organisms are designed specifically for bioremediation. A recent advancement in this section is the genetically modified bacterium Deionococcus radiodurans that is the most radio-resistant organism ever known. Deionococcus radiodurans can consume high amounts of radio-active ionic mercury and toluene from radioactive waste.

Genetically engineered microorganisms (GEMs) have shown potential for bioremediation applications in soil, groundwater, and activated sludge environments, exhibiting enhanced degradative capabilities encompassing a wide range of chemical contaminants.

The most direct application of GMOs in bioremediation is the development of a GMO that can be added to the contaminated site and will

degrade the xenobiotic in situ. GMOs are used for bioremediation of hydrocarbons, industrial waste, heavy metals, xenobiotics, pesticides.

The use of genetic engineering to create organisms specifically designed for bioremediation has great potential. The bacterium *Deinococcus radiodurans* (the most radioresistant organism known) has been modified to consume and digest toluene and ionic mercury from highly radioactive nuclear waste. Releasing genetically augmented organisms into the environment may be problematic as tracking them can be difficult; bioluminescence genes from other species may be inserted to make this easier.

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- **Ethical Issues of genetic engineering**

The use of biotechnology creates profound ethical questions.

1. Its use in reproduction and genetic screening brings unique questions of discrimination, exploitation of women,
2. Should we become architects of life itself? Should biotechnology be allowed to play god? Crossing the species boundaries in genetic exchanges which has resulted into inserting animal genes into human or human genes into animals and inserting plant genes into microorganisms and other species is not correct,
3. Patenting genetically engineered animal is equating it to status of manufactured product. Will living things have no more intrinsic value than automobiles or garments or any other commodity?
4. Will diagnostic procedures undermine individual privacy?
5. The failure to label genetically engineered foods means that persons who follow religious dietary restrictions will be unable to ensure compliance with their beliefs. The genes from prohibited foods could be engineered into other foods. Thus genetic material whose consumption violates religious restrictions may be present in vegetable, fruit, etc. Consumer will not know which processed foods are genetically engineered. Vegetarians may be forced inadvertently to take food containing genetic material from insects, fish, pigs or other animal. Religious and ethical beliefs will be greatly disturbed.
6. Would mothers be willing to buy infant formula with bioengineered ingredients extracted from udders of transgenic cow? (Human lactoferrin and human lysozyme are expressed in cow's milk.)

7. 70% of the public feel that, introducing animal genes into plants is 'unacceptable.' 90% of the public feel that human genes should not be introduced into animals and 98% people want genetically engineered (bovine growth hormone) bGH-milk labelled **so** that they can avoid it.
8. Should humans be genetically engineered? Gene therapy should be restricted to the alleviation of genetic diseases in individual patients and should not be used to change or enhance normal human traits. Genetic modification of reproductive cells or the germ cells which give rise to them should not at present be attempted.
9. An attempt to patent human DNA sequences which is our common heritage is an ugly side of research on Human genome.
10. Are we blurring the lines between species by creating transgenic combinations?
11. What are the known health risks associated with transgenics?
12. What are the long-term effects on the environment when transgenics are released in the field?
13. Are we inflicting pain and suffering on sentient creatures when we create certain types of chimeras?
14. Will transgenic interventions in humans create physical or behavioral traits that may or may not be readily distinguished from what is usually perceived to be "human"?
15. If the blending of nonhuman animal and human DNA results, intentionally or not, in chimeric entities possessing degrees of intelligence or sentience never before seen in nonhuman animals, should these entities be given rights and special protections?
16. Some individuals have argued that crossing species boundaries is unnatural, immoral, and in violation of God's laws.
17. There is a small but significant risk of the transmission of usually fatal zoonotic diseases, such as bovine spongiform encephalopathy (also known as "mad cow disease"), porcine endogenous retroviruses (PERVs), and Nipah encephalitis. The introduction of these diseases to the human population could have devastating consequences.
18. The risks and benefits of the experimental use of animals need to be discussed as well. Similarly, by combining animal DNA and human DNA with plant DNA, do we run the risk of creating new diseases for which there is no treatment? The long-term risks to the environment are unknown.

19. Various bioethicists, environmentalists, and animal rights activists have argued that it is wrong to create “monsters” or animals that would suffer as a result of genetic alternation (for example, a pig with no legs), and that such experimentation should be banned.