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A REVIEW ON RECOMBINANT DNA TECHNOLOGY

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ABSTRACT

Recombinant DNA is artificially created from two or more DNA incorporated into a single molecule. Genetic engineering, recombinant DNA technology, genetic modification/manipulation and gene splicing are terms that are applied to the direct manipulation of an organism's gene. Genetics is the science of genes, heredity, and the variation of organisms. In modern research, genetics provides important tools in the investigation of the function of a particular gene. The development of these new technologies have resulted into production of large amount of biochemically defined proteins of medical significance and created an enormous potential for pharmaceutical industries. The biochemically derived therapeutics is large extracellular proteins for use in either chronic replacement therapies or for the treatment of life threatening indications.

Keywords: Recombinant DNA, Therapeutics, Plasmids.

INTRODUCTION

Recombinant DNA technology puts genetics in the main stream of medicine. Genetic tests will predict common diseases as well as many rare ones. The number of geneticists is inadequate to face this new current and other health care providers do not have sufficient background. The new technology can be used to locate and ultimately identify the genes for any disease for which alleles at one or a few loci account for most of the pathogenic effect. One of the first applications is genetic testing. DNA probes can be used to diagnose people with early symptoms and predict risks of disease in oneself or one's unborn offspring. Expression of the gene is not prerequisite for DNA-based tests. A small amount of DNA from any tissue at virtually any stage of development is all that is needed.

Eventually, the discovery of genetic defects will advance out knowledge of diseases and could lead to effective interventions. In meantime, testing will proceed for untreatable disorders. A recent survey of biotechnology companies identified that they are developing or planning to develop DNA based genetic tests and two others with related interests (Holtzman, in press). The use of predictive tests when no definitive treatment exists raises controversial issues. They include abortion of affected fetuses and denial of insurance and employment. Before discussing these issues, some of the limitations of DNA based tests and the obstacles to their safe and effective use need exposition. Policy implications are discussed throughout [1].

PLASMIDS

Plasmids are small, circular, double-stranded, extra chromosomal DNAs present in bacterial cells. They are inherited sharply without influence of chromosomal DNA. They replicate independently due to the presence of an origin of replication. The plasmids are 1 Kbp-200 Kbp in size and have limited number of genes. Most bacteria contain more than one copy of each plasmid. The number of copies of a plasmid present in a cell is called "copy number". The copy number varies from 1 to 50. However, it can be further increased by treating the bacterial culture with chloramphenicol (an inhibitor of protein synthesis).

The genes for antibiotics resistance, nitrogen fixation, nodulation, environmental stresses, etc. occur in plasmid DNAs. The antibiotics-resistance in plasmids can be used as genetic marker to identify the strains containing the plasmids. Some plasmids code for some secondary metabolites. Some plasmids under certain conditions integrate into the chromosomal DNA of the bacteria. Such plasmids are called "episomes". The integrated plasmid replicates along with the chromosomal DNA. Eg: F-plasmid. The eukaryotes except yeasts do not have plasmids. The yeast Saccharomyces. Cerevisiae contains

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YEp (Yeast episomal plasmid or 2-micron plasmid), YIp(Yeast integrating plasmid) and ARS (Automatically replicating sequence) in the cells [2].

CLASSIFICATION OF PLASMIDS

On the basis of conjugative transfer, plasmids are classified into two categories. They are

1. **Conjugative plasmids**: These plasmids are transferred from bacterium to the other during conjugation. They contain ' tra genes' for conjugative transfer. Eg: F-plasmid.
2. **Non-conjugative plasmids**: These plasmids do not pass from one bacterium to another bacterium during conjugation. Eg: Col E1 plasmid.

On the basis of the functions, plasmids are classified into five types. They are

1. F-Plasmid: F-plasmid or fertility plasmid contains some genes expressing the maleness in bacteria. The genes are known as ' tra genes'. Eg: F-plasmid.

2. R-Plasmids: R plasmids (Resistance plasmids) contain some genes giving resistance to bacteria against antibiotics and heavy metals. Eg: Psc101 gives the organism tetracycline resistance.

3. COL Plasmids: Col plasmids code for the synthesis of bacterial toxin 'colicin' Colicin kills other closely related of the bacteria. Eg: Col E1, Col B, etc.

4. Degradative Plasmids: Degradative plasmids code for enzymes that degrade toxic substances such as toluene, xylene, salicylic acid, parathion, 2,4-d etc. TOL Plasmid of Pseudomonas putida involves in the breakdown of toluene.

5. Virulence Plasmids: Virulence plasmids provide pathogenicity to bacteria [3].

ARTIFICIAL PLASMIDS

pBR322 is an artificial plasmid.It is a gene cloning vector for E.coli. It was constructed from two plasmids pSC101 and Col EI and a transposon Tn3.

In the plasmid pBR322

i) 'p' indicates that it is a plasmid.

ii) 'BR' indicates the names of workers F.Bolivar and Rodriguez who created the plasmid.

iii) 322 is the specific number to distinguish the plasmid from others pBR322 is a circular, double stranded plasmid DNA. It costs of 4363 base pairs. The plasmid has 528 restriction sites for 66 restriction enzymes. Among these 20 restriction enzymes cut it at unique restriction sites [4].

The pBR322 has two selectable gene markers. They are

1. Tetracycline resistance gene(Tet^r)

2. Ampicillin resistance (Amp^r)

The Tet^r gene has unique sites for six restriction enzymes. If a gene is inserted into any of these restriction sites, the tetracycline resistance gene becomes inactive. The Amp^r gene has unique sites for three restriction enzymes. If a gene is inserted into any one of these unique sites, the ampicillin resistance gene becomes inactive. The sequences other than Tet^r gene and Amp^r gene have unique sites for 11 restriction enzymes. There is no insertional inactivation when a gene is inserted into any one of these sites [5].

Advantages of pBR322

1. pBR322 is a small plasmid consisting of 4363 base pairs.

2. The copy number of pBR322 is 15.It can be increased upto 300 by adding chloramphenicol to the bacterial culture.

3. Bacterial cells can uptake DNAs of 15 kbp size from the culture. But pBR322 is only 4.4kbp in size. So it can carry relatively large DNA segments of 5-10kbp.

4. pBR322 has two selectable gene markers for selection of recombinants by insertion; on activation method.

5. The regulation and expression of a gene inserted into the plasmid is good.

6. pBR322 is used as a base plsmid for the invitro construction of derived plasmid vectors such as pUC8,pUC9,pUC10 etc and cosmids.

Uses

pBR322 is used to introduce desired genes into E.coli cells.

Eg: Somatostatin gene of man is introduced into E.coli through pBR322.

pUC8

pUC8 is a cloning vector constructed from pbr322.It is 2676 bp in size. It has an ampicillin resistance sequence(Amp^r) and an origin of replication from pbr322 and lac Z gene of E.coli. The lac Z gene has a multiple cloning sequence (MCS). Foreign gene is inserted into the MCS and it inactivates the lac Z gene. The recombinants are screened by growing the cells in a medium containing X-gal. When a rDNA introduced into a Lac Z mutant E.coli, the cells fails to form β-galactosidase because of α-polypeptide. So the bacterial colony remains white. The other plasmids of pUC series include pUC7, pUC9, pUC12, pUC13, pUC18 and pUC19.All these plasmids are similar but they differ from each other in specific multiple cloning sequence.

Advantages

1. pUC8 is smaller than pBR322.So it can carry large DNA segments upto 12.4kbp size**.**
2. As it has MCS, DNA segments with different types of cut-ends can be inserted into the plasmid**.**
3. The screening of recombinants is easier than that of recombinant pBR322**.** E.coli having recombinant pUC8 is selected by growing it in a medium containing X-gal [6].

STEPS INVOLVED IN R-DNA

There are five steps involved in r-DNA. They are i. Preparation of desired gene, ii. Preparation of plasmid, iii. Insertion of desired gene into plasmid, iv. Introduction of r-DNA into bacteria, v. Selection of recombinants.

i. Preparation of Desired Gene

Insulin gene is taken as an example for desired gene which can also be obtained from genomic DNA, insulin mRNA or from free nucleotides by chemical synthesis. The genomic DNA contain insulin gene along with numerous genes. The cell DNA is isolated in following steps.

Cells treated with SDS (sodium dodecyl sulphate) to disrupt cell membrane
↓
Lysed cell treated with proteinase k to digest cellular proteins and histones bound to DNA
↓
Treated with phenol-chloroform mixture to precipitate proteins
↓
Proteins are centrifuged out. Supernatant solution is treated with ribonuclease to digest RNAs.
↓
Supernatant is treated with acetate-ethanol solution. White fibrous precipitate of DNA develops.
↓
Obtained precipitate is washed with TE buffer and with ESAM solution.
↓
DNA precipitate which is obtained is suspended in TE buffer and stored at -20 C.

The procedure that is explained above is most widely used. But the desired gene can also be obtained from mRNA and free nucleotides. Insulin gene is also made from mRNA. At first mRNAs are isolated from cells of Islets of Langerhans of human pancreas and then fractionated by electrophoresis method. Each fraction of mRNAs is then allowed to synthesis protein in invitro in a cell free translation system. Those mRNA fractions that produce insulin protein isolated from this system and treated with reverse transcriptase and deoxyribonucleotides. This enzyme synthesizes a DNA strand called cDNA. The cDNA is separated from DNA-RNA hybrid by alkali treatment and also with DNA polymerase and deoxyribonucleotides. Insulin gene is also synthesized from free nucleotides by using a gene machine. The gene machine synthesizes small oligonucloetides. These oligonucleotides are joined together by ligase chain reaction to form duplex DNA [7,8].

ii. Preparation of plasmid

Isolation of Plasmid

Bacterial Culture
↓ Chloramphenicol
Bacterial Culture having high copy number of Plasmid
↓ EDTA+ Lysozyme
Bacterial Spheroplasts
↓ SLS solution
Cell Lysate
↓Sodium acetate
Centrifugation
↓ Precipitate (proteins, chromosomal DNA and large RNAs
Supernatant
↓ Isopropanol
UltraCentrifugation
↓ Supernatant
Precipitate
↓ ESAM solution
UltraCentrifugation
↓ ESAM solution
Precipitate of plasmids
↓
Mixture of plasmids
↓
Ultracentrifugation in CsCl2 density gradience
↓
Plasmids from a specific layer
↓
Storage in TE buffer

Preparation of Plasmid

The Plasmid has been a popular vector for gene cloning in E.Coli. It is an artificial plasmid.
The isolation of plasmid pBR322 involves the following steps

Bacterial cell wall
↓ Lysozyme and EDTA
Cell lysed with SLS
↓ Cell wall debris and proteins
Centrifugation
↓
Supernatant
↓ Fractioned by cesium chloride density Gradient centrifugation
Plasmids
↓
Centrifuged out

iii. Insertion of desired gene into plasmid:

The cell DNA is cut with a restriction enzyme to get small fragments. Among these fragments one fragment contains the desired gene. As we don't know in which fragment the desired gene resides, the DNA fragments are separated from one another by electrophoresis. Each fraction is then cloned separately. The plasmid is cut with restriction enzyme that has unique site at its antibiotics resistance gene and that was used to cut the cell DNA.

Linearised plasmid DNA
↓ Mixed with fraction of cell DNA In 1:3 ratio
Cohesive ends are formed
↓
Plasmid DNA and cell DNA
↓
Complimentary base pairing
↓ DNA ligase
Link between two DNAs
↓
Recombinant DNA

In such a way rDNAs are constructed with DNA fragments of each and every fraction of cell DNA. Of these different rDNAs at least one type contains the desired gene. cDNA clones and chemically synthesized desired genes are blunt ended. Adaptors, linkers or homopolymer tails are used to insert such DNAs into plasmid [9,10].

iv. Introduction of rDNA into Bacteria

Recombinant pBR322 is introduced into bacterial cell by "bacterial transformation" process.

r DNA added to the bacterial culture
↓
Culture is treated with 50Mm calcium chloride
↓
$Cacl_2$ adheres the rDNA on surface of bacterial cell
↓
Modifies the bacterial cell wall to intake rDNA
↓
Bacterial culture heated gently at 42^0C
↓
Stimulates bacterial cells to intake the rDNAs
↓
Bacterial cells become recombinants

v. Selection of Recombinants

Recombinants are cells or organisms harboring recombinant DNAs. They are selected from non-recombinant cells by following methods.

- Lose of antibiotics resistance
- Colony hybridization
- Immunochemical method

Lose of Antibiotics Resistance

Bacterial transformation
↓
Bacterial culture placed in agar medium containing antibiotic
↓
Prepare a replica plate
↓
Keep the plate on agar medium containing antibiotics
↓
Colonies that fails to produce their replica in replica plate
↓
recombinants
↓
Obtained recombinants are cultured in suitable medium
↓ Subjected
Screening tests

Colony Hybridization

Recombinants are diluted
↓ placed
Agar medium
↓
Replica plate made on nitrocellulose filter
↓ filtered
Cells on filter disc are lysed
↓treating with chloroform & alkali
DNA is denatured
↓
Filter paper dipped in hybridization solution
↓
Solution contains radioactive probe DNA for desired gene
↓
Hybridization and washing
↓
Autoradiogram
↓
Colonies produce dark spots on site of filter paper
↓
Picked up and grown separately
↓
Recombinants containing desired gene are obtained

Immunochemical Method

It is another screening method used to select recombinants harbouring specific desired gene. This method is applicable when the target protein acts as antigen in mice to produce antibodies. Human insulin has no antigenic role in mice. So, this method is not suitable for this purpose [11,12].

APPLICATIONS

There are many potential applications of r-DNA in medicine, agriculture and industry and in many fields. Some of the applications are

Importance of Vaccines

Vaccine development is one of the first and most promising fields of application of biotechnology. At present, an enormous amount of research is going in this area in an effort to develop new, safer and more efficacious vaccines which give the highest protection to animal economically.

There are still a large number of animal diseases for which protective vaccines are yet to be available, especially against parasitic diseases. Some antigens have been virtually impossible to incorporate into vaccines because of the difficulty in growing the micro-organisms or isolating the antigens, or because of their complexity or an inability to adapt them to a viable production method. Research activities are now focused subunit, recombinant DNA, synthetic peptide, anti-idiotype, deletion mutant, re assortment and vaccinia vectored vaccines. It is expected that in the next few years an increasing number of genetically engineered vaccines will be marketed.

***Recombinant Vaccine for Hepatitis B Virus* (HBV):**

After infection, HBV fails to grow and even in cultured cells it does not grow. This property is due to inhibition of it molecular expression and development of vaccines. Recombinant vaccine for HBV was produced by cloning HBsAg gene of 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. The HBsAg gene contains 6 bp long sequence preceding the AUG that synthesizes N-terminal methionine. This is joined to ADH promoter coned in the yeast vector PMA-56.The recombinant plasmid is inserted into yeast cells.The transformed yeast cells are multiplied in tryptophan-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 22µm particle of HBV as these particles are produced in serum of HBV patients. The expressed HBaAg 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 [13].

***Indegeneous Hepatits-B Vaccine*:**

- HepatitisB surface antigen(HBsAg) is isolated from the virus hepatitsB.
- The vector pma56 Is selected for DNA recombination.
- The specific region of desired DNA (HBsAg) and the plasmid pma56 are cut by restriction digestion enzyme which is called EcoR1.
- Now, the desired DNA is inserted into vector pma56 thereby rDNA is constructed.
- The constructed rDNA is introduced into yeast cells for multiplication.
- After incubation period is completed the recombinants can be identified by colony hybridization method.
- The identified recombinants are taken for screening of the hepatitis surface antigen which is given for treatment of hepatitis infection.

***Vaccines for Rabies Virus*:**

Research has been made to synthesize vaccines including genetically engineered E.coli cells. However, attempt has been made to isolate mRNA encoding viral protein from rabies infected cells. First step is the genes coding for the production of rabies virus glycoprotein coat has been transferred to E.coli which leads to the production of anti rabies virus vaccine. The glycoprotein stimulates antibody production in animals. The genetically engineered virus synthesized ant rabies vaccine for animals. The recombinant vaccinia virus did not cause rabies in those animals that received rabies genome that encoded antigenic molecules and activated immune system against rabies infection. This new vaccine can be administered orally in animals and decrease the risk of human death due to bite of animals receiving rabies virus [14].

***Vaccines for Foot and Mouth Disease Virus* (FMDV):**

Foot and mouth disease (FMD) is a serious disease caused by Aphthovirus. The primary control measure of the disease has been the slaughter of FMDV – infected animals. Chemotherapeutic way of FMD control of vaccination Vaccines are produced by inactivation of virus grown in bovine tongue epithelium. A detailed study of FMDV reveals that it contains a single stranded RNA covered in a capsid of four polypeptides. For eg: VP_1, VP_2, VP_S and VP_4 where only VP_1 has immunogenic activity.

However, the nucleotide sequence encoding for VP_1 was identified on the single stranded RNA genome and cloned on double stranded PBR322 in E.coli. About 1,000 molecules of VP_1 per bacterial cell were synthesized. Vaccines produced from genetically engineered E.coli cells cannot compete with those extracted from virus particle. Therefore, much work is to be done to make the vaccines available for FMDV on large scale at low price.

Synthetic peptides have also been produced and used for immunization against bacterial and viral diseases. Bacterial diseases like diphtheria, streptococcus pyrogens and viral diseases like Hepatitis virus and FMDV etc. For the production of synthetic polypeptide to be used as vaccine it is necessary in immunogenic response. For example synthetic polypeptides having immunogenic effects against HBV contain disulphide bond in the region between amino acids 117 an 137 corresponding to the viral surface antigen. After injection into mice the polypeptides elicited antibodies against HBsAg and protected half of mice.

Similarly, a synthetic polypeptide has been identified that corresponds several regions of FMDV protein VP_1 .The region between amino acids 141 and 160 elicited and production of antibodies against FMDV in guinea pigs, rabbits and swine. Another polypeptide was synthesized and the region between amino acids 200 to 213 VP_1 also elicited antibodies against FMDV. There are other examples also for the synthesis of vaccines against viral diseases [15].

- ***DNA Sequencing:***

Recombinant DNAs are used in DNA sequencing in chain termination method. The large genomic DNA is cut into several pieces and each piece is inserted into RF of M13 phage. Single stranded DNA is isolated from the recombinant M13 phage and used as a template for DNA sequencing [16,17].

***Diagnosis of HIV infection*:**

The antibody test (ELISA or Western blot test) uses a recombinant HIV protein to test the presence of antibodies that has produced due to HIV infection. This test looks for presence of HIV genetic material using reverse transcriptase polymerase chain reaction (RT-PCR) [16].

- ***Gene Cloning*:**

Production of multiple copies of a desired DNA in vivo by constructing an rDNA and introducing it into a bacterium called gene cloning. In the recombinant the desired DNA is propagated along with the vector sequence of the rDNA. The vectors selected for gene cloning do not integrate with genomic DNA of the host cell. So, reisolation of the desired DNA from the bacteria is easy.

- ***Establishment of Gene Banks:***

To establish gene bank of an organism, its DNA is cut into many small fragments, each of which is inserted into vector DNA. The collection of bacteria having all DNA of an organism is called gene bank. Gene banks maintain the genes and provide required gene for gene manipulation works [18].

Fig 1. Restriction map of pBR322 showing unique restriction sites

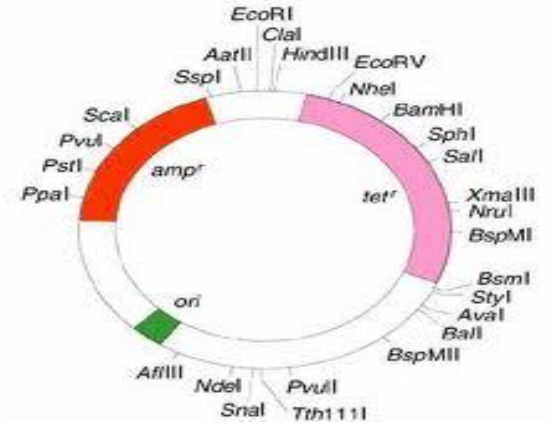


Fig 2. Preparation of Desired Gene

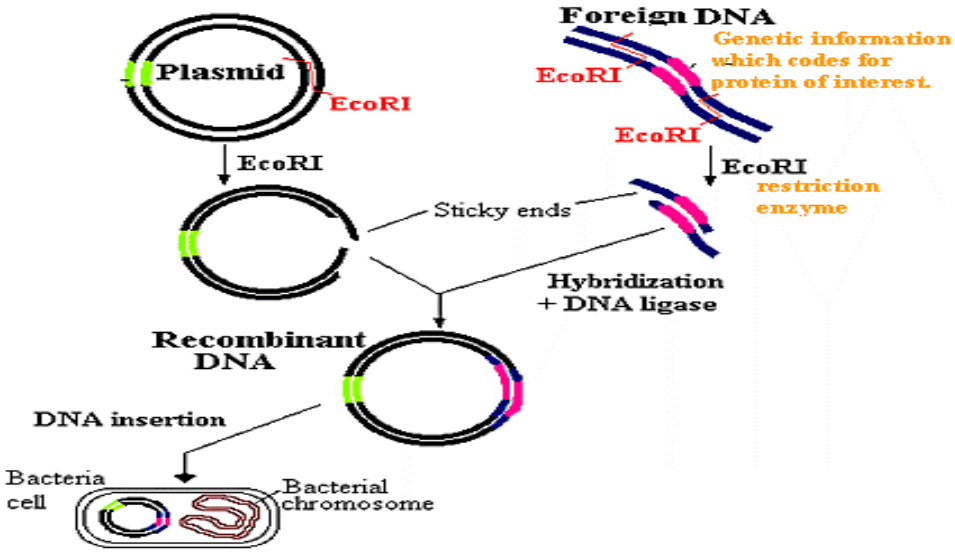
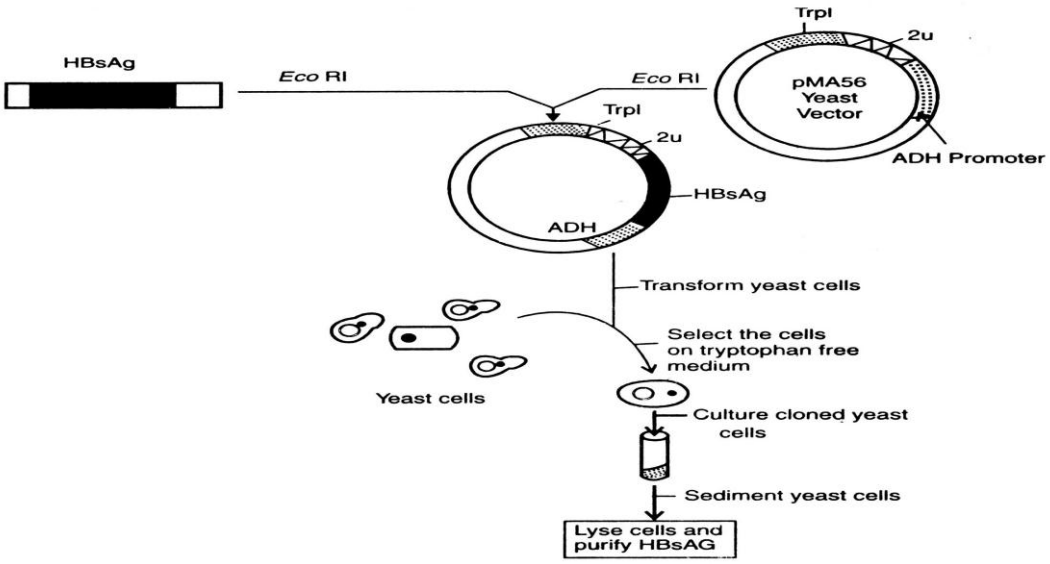


Fig 3. Indegeneous Hepatits-B Vaccine



CONCLUSION

Recombinant DNA is artificially created from two or more DNA incorporated into a single molecule. Genetic engineering, recombinant DNA technology, genetic modification/manipulation and gene splicing are terms that are applied to the direct manipulation of an organism's gene. The development of these new technologies have resulted into production of large amount of therapeutics is biochemically defined proteins of medical significance and created an enormous potential for pharmaceutical industries. The biochemically derived large extra cellular proteins for use in either chronic replacement therapies or for the treatment of life threatening indications. Recombinant DNA technology has also an important role in forensic science in identification of criminals, DNA profiling to study kinship analysis and in paternity testing.

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