RECOMBINANT DNA AND GENE CLONING

Abstract

Recombinant DNA, a concept for enhancing desired qualities in organisms, has significantly contributed to human progress by manipulating the expression of target genes. However, in recent years, this field has made significant contributions to human progress through many technologies. This technique allows for the safe, affordable, and adequate production of critical proteins for health concerns and nutritional needs. The discovery of restriction endonucleases in the 1970s revolutionized molecular biology analysis by enabling more efficient DNA analysis and the creation of recombinant DNA fragments. Gene cloning, a process that enabled discoveries in gene structure, function, and regulation, has evolved over time. Although microarray analysis and PCR offer short cuts, gene cloning methods remain essential in many cases. This technique has broad applications and the ability to improve vital areas of life, such as health, food resources, and resistance to diverse unfavorable environmental impacts. Genetically engineered plants, particularly in agriculture, have boosted resistance to hazardous agents, increased product production, and demonstrated higher adaptation for better survival. Furthermore, recombinant medications are currently being utilised with confidence through many gene editing techniques (e.g.CRISPR).

Keywords: Recombinant DNA; Gene cloning; Cloning vectors; CRISPR.

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I. INTRODUCTION

Classical molecular biology studies provided a fundamental understanding of gene nature and expression, but it was unclear how to extend these principles to eukaryotic cells, as their genomes are thousands of times more complex than those of E. coli. The prospect of studying these genomes at the molecular level was daunting in the early 1970s [1].Recombinant DNA is increasingly important in the 21st century due to its potential impact on genetic diseases and agricultural areas. As genetic diseases become more prevalent, it will play a crucial role in various sectors. Recombinant DNA technology revolutionized molecular biology by enabling detailed studies of eukaryotic gene structure and function, overcoming obstacles in molecular biology advancements[2].Recombinant DNA, or simply rDNA, refers to any DNA fragment created by the in vitro ("in test tubes") synthesis of genetic material from several species. Recombinant DNA, also known as in vitro recombination, is a technique for creating and purifying desired gene[3]. Molecular cloning involves introducing recombinant DNA into a host cell for replication, moving desired genes from a complex genome to a simpler one. A patent or recombinant DNA, was filed by with S. N. Cohen and H. W. Boyer in 1974. Paul Berg in 1980 was awarded with Nobel Prize in chemistry for his work on Recombinant DNA[4]. The steps involved in making Recombinant DNA arethe use of same restriction endonuclease is used to process the DNA extracted from both sources. Both molecules are cleaved by the restriction enzyme at the same location. The cut's ends feature a single strand of DNA that hangs over the edge. This is referred to as "sticky ends." Any DNA molecule that has the corresponding sticky end can base pair with these sticky ends. When combined, complementary sticky ends can complement one another. The two strands are covalently joined together to form a molecule of recombinant DNA using DNA ligase. The recombinant DNA must be repeatedly duplicated (i.e., cloned) in order to be helpful [Fig.1] [5]. Cloning can be carried out in vivo (inside the cell) using unicellular prokaryotes or in vitro (using the Polymerase Chain Reaction, or PCR)[6].

II. TECHNIQUES FOR MAKING RECOMBINANT DNA

Recombinant DNA can be made using three techniques: Transformation, Phage introduction, Non-bacterial transformation[7].

1. **Transformation:** The selection of a DNA fragment to be inserted into a vector is the first stage in the transformation process. The second step is using a restriction enzyme to cut that segment of DNA, followed by DNA Ligase to ligate the DNA insert into the vector. The insert has a selectable marker that makes it possible to recognize recombinant molecules. It is common practice to utilize an antibiotic marker so that when a host cell is exposed to a certain antibiotic, the host with the vector will survive since it is resistant. Transformation is the process of inserting the vector into a host cell. E. coli is one illustration of a probable host cell. It is necessary to prepare the host cells specifically to accept the foreign DNA.

2. **Non- Bacterial Transformation:** Microinjection and biolistics are non-bacterial processes that use DNA directly into the nucleus of the transformed cell, while microinjection involves direct injection of DNA into the nucleus. Biolistics involves high-velocity micro projectiles coated with DNA, similar to transformation.

3. **Phage Introduction:** Phage introduction is a transfection process using phages instead of bacteria, using lambda or MI3 phages to produce phage plaques containing recombinants. These plaques can be identified using selection methods based on differences between recombinants and non-recombinants.

III. RECOMBINANT DNA AND GENE CLONING

DNA cloning involves creating identical copies of a specific DNA fragment by inserting it into a plasmid using enzymes(Restriction endonuclease and ligase), resulting in recombinant DNA assembled from multiple sources[8]. The recombinant plasmid is introduced into bacteria, which grow and replicate the plasmid, passing it on to offspring, creating multiple copies for experiments or new plasmids. Gene cloning involves: Identifying and isolating the desired gene, inserting it into a suitable vector, introducing it into a host organism, selecting a transformed cell and expressing the introduced gene[9].

1. **Restriction Endonuclease:** Viruses are small packages of DNA or RNA in a lipoprotein capsule, lacking cell machinery for protein production or replication. They reproduce by invading living organisms, seizing resources and replicating into new viruses[10]. Bacteria lack immune systems, making them vulnerable. They use restriction endonucleases for analyzing DNA chains, research, and genetic engineering. Over 3000 known restriction endonucleases, with over 600 currently commercially available, play crucial roles in various applications[11](Table 2).Molecular cloning involves inserting DNA fragments into a plasmid vector, creating multiple copies for easier study and manipulation. This process replicates in a bacterium, creating hundreds of identical copies per cell. Restriction enzyme cloning uses restriction enzymes to cut vectors and inserts at specific locations, forming recombinant DNA. After restriction with same enzyme in both vector and insert both are joined together by ligase enzyme. DNA ligase forms covalent phosphodiester bonds between DNA fragments, similar to Okazaki fragments, using the same restriction enzyme or compatible "sticky ends"[12].

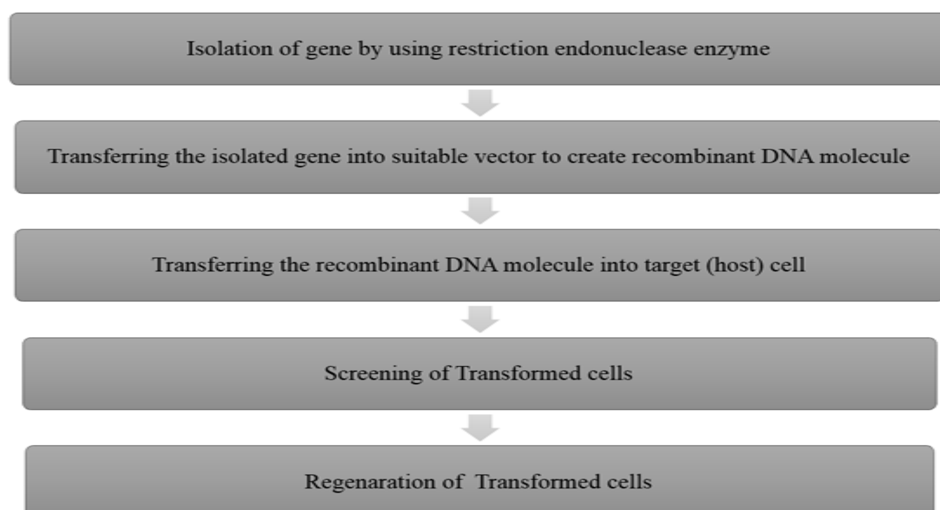

Figure 1: Steps involved in making Recombinant DNA

Table 1: Types of Restriction Endonuclease

	Type I	Type II	Type III	Type IV
Nuclease structure	Multimer; heterotrimer	Homodimer	Homodimer	
Recognition site pattern	Two sites, in any orientation	Small (4-8 bp);usually palindromic	Two sites, in head to head orientation; non palindromic	Weak specificity
Cleavage site	Variable distance from recognition site; non-specific cleavage	Cleavage within or outside the recognition site	Cleavage of one strand (nicking activity) 24-25 bp from recognition site	Methylated only
Cofactor	ATP,$Mg2^{+}$,SAM	$Mg2^{+}$	ATP,$Mg2^{+}$,SAM	ATP,GTP

Table 2: List of some of the Restriction Endonuclease and their Restriction Site

S. NO.	Restriction Enzyme	Cut site
1.	Alu1	AG↓CT
2.	BamH1	G↓GATCC
3.	Bfa1	C↓TAG
4.	Dpn11	↓GATC
5.	EcoR1	G↓AATTC
6.	Hae111	GG↓CC
7.	Hind111	A↓AGCTT
8.	Not1	GC↓GGCCGC
9.	Nru1	TCG↓CGA
10.	Rsa1	GT↓AC
11.	Sac1	GAGCT↓C
12.	Sma1	CCC↓GGG
13.	Tru91	T↓TAA
14.	Pst1	CTGCA↓G

2. **Cloning Vectors:** Recombinant DNA formation requires a cloning vector, which replicates within living cells. Vectors, derived from plasmids or viruses, contain genetic signals, insert foreign DNA, identify cells, and express it. The choice depends on the host organism, DNA size, and expression method.

   - **Plasmids:** Plasmid vectors are small circular DNA molecules that can replicate independently in bacteria without being associated with chromosomal DNA. They require an origin of replication and carry genes that confer antibiotic resistance, allowing bacteria to be selected. Plasmid vectors typically have 2 to 4 kb of DNA, compared to phage vectors' 30 to 45 kb, allowing easier manipulation of cloned DNA sequences.

- **Bacterial Artificial Chromosomes:** These resemble plasmid vectors from E. coli. It is derived from an innate F' plasmid. These are employed to research genetic diseases. Large DNA sequences may be accommodated without any harm.
- **Yeast Artificial Chromosome (YAC)** - The YAC is 11400 bp in size, and the cloning vector is 100-1000 kb. The marker is comparable to yeast's. It is synthetic and contains a yeast centromere obtained from Saccharomyces cerevisiae.
- **Bacteriophage** - It has a size of 48502 bp, of which one-third is not required. It can only recombinant around 4-5 kbp of donor DNA. Lambda genome is one such example
- **Cosmid** - The cosmid has a size of 7900 bp and a cloning limit of 30-50 kb. Super COS1 is an example of one with properties comparable to both phage and plasmid.
- **Human Artificial Chromosome** - It is an artificial chromosome that is used to transfer human genes and has no cloning restrictions since it may contain a human gene.

3. **Cloning Eukaryotic Genes**: Eukaryotic genes frequently have several, huge introns, making molecular cloning either impossible, unattractive, or both. Less than 10 kilo-base pairs (kbp) is the practical size limit for plasmid vectors, and more over roughly 10 kb makes PCR challenging.The mRNA is a condensed version of a eukaryotic gene that contains all the information necessary for protein coding, but lacks introns. An oligo-dT primer complementary to the polyA tail and the enzyme reverse transcriptase can be employed to create a complementary DNA (cDNA) molecule. By ligating adapters that include restriction endonuclease cleavage sites or PCR primer sequences, the cDNA may subsequently be cloned onto a plasmid or amplified by PCR[13].

4. **Expression of Foreign Genes in Bacteria**: Gene expression involves converting a gene's coded information into cell structures, such as mRNA, tRNA, and rRNA. Microarray analysis visualizes gene expression patterns while gene cloning involves manipulating DNA to produce multiple copies of a single gene. DNA sequencing determines nucleotide base sequences in a molecule.Plasmid vectors for cloning and expression in bacteria require an origin of DNA replication, restriction endonuclease sites, a selectable marker gene, a way to distinguish cells with the original plasmid from recombinant plasmids, and a promoter to drive transcription and translation of the inserted foreign gene[14]. The plasmid ends tend to re-ligate without a foreign DNA insert when plasmid and foreign DNA segments are joined, leaving the original, "empty" plasmid without a foreign DNA insert. Plasmid vectors have cloning sites within antibiotic resistance genes or the lacZ gene, which can disrupt the gene. E. coli bacteria with empty plasmids contain functioning beta-galactosidase, undamaged lacZ genes, and can cleave the colorless dye X-gal.These colonies remain white, discarded for further testing. Cloning into the 5′ end of the lacZ gene allows E. coli cells to express a protein encoded by the inserted DNA, allowing the lac promoter to regulate transcription and express protein coding sequences as a fusion protein[15].

5. **Expression of Foreign Genes in Eukaryotes:** The right eukaryotic promoters, downstream polyadenylation, and transcription termination signals must be present in vectors for the production of foreign genes in eukaryotic cells. These promoters must be located upstream of the cloning site. Bacterial plasmids harbouring foreign genes can be turned into single-celled organisms like yeast and cultivated cells. The plasmid DNA

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enters the nucleus and inserts itself into several chromosomes across the host cell. Delivering genes into the cells of multicellular creatures presents unique difficulties and necessitates the use of unique vectors and delivery techniques[9].

6. **Recombinant DNA-Containing Organisms' Characteristics:** Recombinant DNA organisms have typical phenotypes, with no changes in appearance, gestation, or metabolism. PCR testing are used to confirm recombinant sequences. If rDNA sequences result in an expressed gene, RNA and/or protein products can be identified using RT-PCR or western hybridization techniques[16]. However, unless the recombinant gene has been changed to produce spontaneous variation, large phenotypic changes are uncommon. Furthermore, recombinant DNA can produce undesirable consequences, such as insertional inactivation, which inserts rDNA into a host cell's gene, or unfavourable activation of previously unvoiced host cell genes. These things can occur when a recombinant DNA fragment with an active protagonist is found close to a previously quiet host cell gene or when a host cell gene that inhibits gene expression experiences insertional mutagenesis[17].

7. **DNA Sequencing:** Molecular cloning allows for the isolation of individual DNA segments for thorough characterization, including nucleotide sequence determination. This has revealed the structure of protein products and features of DNA sequences that govern gene expression. The coding sequences of newly isolated genes are often linked to those of previously characterized genes, and the functions of newly isolated genes are often surmised based on sequence similarities. Current DNA sequencing technologies are fast and precise, making cloning and sequencing easier than determining the amino acid sequence of a protein. Sequencing a cloned gene is the simplest technique to determine protein sequence[18]. The most prevalent technique of DNA sequencing is based on the presence of chain-terminating dideoxynucleotides in DNA polymerase operations, which causes premature termination of DNA synthesis[19]. DNA synthesis begins with a radio isotopically labelled primer and four distinct reactions, each containing one dideoxynucleotide. The incorporation of a dideoxynucleotide halts further DNA synthesis, producing a string of labelled DNA molecules. These fragments are electrophoretically separated based on size, and autoradiography is used to identify them. The size of each fragment's terminal dideoxynucleotide determines its size, ensuring consistent DNA sequence and order in the gel.

8. **Expression of Cloned Genes:** Molecular cloning allows for the determination of gene nucleotide sequences and amino acid sequences of protein products, providing new approaches to obtaining large amounts of proteins for structural and functional characterization[20]. Many proteins are present at low levels in eukaryotic cells and cannot be purified significantly by conventional biochemical techniques. However, engineering vectors that lead to high levels of gene expression in bacteria or eukaryotic cells can solve this problem. To express a eukaryotic gene in E. coli, the cDNA of interest is cloned into a plasmid or phage vector, which contains sequences driving transcription and translation of the inserted gene in bacterial cells. In many cases, inserted genes can be produced at levels high enough so that the protein that they encode makes up as much as 10% of the total amount of bacterial protein. Purifying the protein in quantities suitable for detailed biochemical or structural studies is a straightforward matter.High-level expression of cloned genes in eukaryotic cells is often beneficial for posttranslational

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modifications of proteins, such as adding carbohydrates or lipids. This can be achieved by inserting the cloned gene into a vector, such as baculovirus vectors, or using appropriate vectors in mammalian cells. Using yeast genetics methods to identify proteins that interact with other cloned proteins or specific DNA sequences is particularly useful for protein expression in eukaryotic cells[9].

IV. APPLICATION OF RECOMBINANT DNA IN CURRENT RESEARCH AREAS

1. **Gene Therapy:** Recombinant DNA technology is a rapidly expanding subject, with researchers all over the world creating novel methodologies, gadgets, and altered products for use in a variety of industries, including agriculture, health, and the environment. In compared to conventional human insulin, Lispro (Humalog) is a very effective and quick acting recombinant insulin [21]. Gene therapy faces challenges in delivering recombinant DNA into host cells. Viruses are promising techniques for delivering functional genes into newborns. Replacing viral replication genes with therapeutic human genes eliminates virus replication and co-opts the viral infection mechanism. However, only a small percentage of cells are infected and repaired, and the benefits of viral gene therapy are short-lived. A promising solution is geneticallymodifying stem cells, which can divide and replenish the body's cells for the patient's life. These stem cells can be returned to the patient's body, potentially providing and replenishing genetically modified blood cells and tissues.The first successful study of gene therapy for the treatment of a genetic condition offered a surer path towards healing the most lethal hereditary disorders [22, 23]. Adenosine deaminase deficiency (ADA-SCID), a primary immunodeficiency, can be successfully treated with this approach. Initial attempts at this technique failed due to a number of issues, including the need to keep patients on PEGylated ADA (PEG-ADA) during gene therapy and the need to target gene transfer to T-lymphocytes.

2. **Genome Editing – Crispr:** A genetically modified organism (GMO) is any creature that has been changed to carry additional genetic material, either from another species or synthesized in the lab. The goal of developing GMOs is generally to change one or more features of the modified organism, most commonly so that it expresses a new gene.CRISPR-Cas9 technology, developed by Jennifer Doudna, is a molecular technology that allows researchers to delete, add, or replace specific bits of DNA in a cell. CRISPR-Cas9 is a protein enzyme that binds short RNAs from the CRISPR gene library and recognizes corresponding DNA sequences that match the RNAs. This technology allows researchers to delete, add, or replace specific bits of DNA in a cell[24]. Cas9 targets the DNA site to be cut using a short guide RNA (sgRNA), allowing scientists to create asgRNA to target a specific DNA sequence in an organism. After cutting the DNA, the cell's DNA repair system trims the broken ends and ligates them together in non-homologous end-joining, which often creates a small deletion. If a homologous DNA sequence matches the sequences around the cut ends, the cell's DNA repair system can use the matching DNA as a template to repair the broken DNA. By providing Cas9 protein, sgRNA, and a homologous template DNA with a desired change, scientists have successfully made precise changes in the genomes of various cells and organisms, including cultured human cells. However, ethical discussions surrounding gene editing in humans are contentious, with most researchers believing the technology is not yet precise or accurate enough to attempt edits in human embryos in vivo.

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3. **Recombinant DNA in Agriculture:** Recombinant DNA technology, also known as genetic engineering, alters an organism's genetic makeup and characteristics by inserting DNA from another organism. Initially, the FlavrSavr tomato introduced GM food in 1994, offering longer shelf life and enhanced flavor[25]. Since then, GMOs have gained popularity due to their higher yields and reduced herbicide resistance. GMO crops like soybeans, corn, cotton, potatoes, and wheat are resistant to herbicides due to the introduction of an herbicide-resistant bacterial gene into plant DNA. This increases yield and reduces the need for frequent spraying compared to traditional crops [26]. BT is a safe and effective insecticide used in farming by inserting a toxin-producing gene from bacteria into corn and cotton DNA. This makes the crops resistant to insects and protects them from disease. Genetically-engineered plants, like papayas grown in Hawaii, are also resistant to certain viruses[27]. Research is still being done to create crops that can withstand high temperatures, have greater nutritional value, and perhaps generate human vaccinations or pharmaceuticals.

V. FUTURE PERSPECTIVE

Recombinant DNA methods were initially developed to address concerns about potential undesirable properties in organisms containing recombinant DNA[28]. Today, recombinant DNA molecules and proteins are generally not considered dangerous.Even though many of us are unaware of them, these applications have a hugely positive influence on our daily lives in numerous aspects.Current life sciences research trends make rDNA and gene cloning valuable for developing new therapeutics, including enhanced insulin, Epoetin alfa, and human growth hormone. This technology has numerous potential applications in hereditary diseases, oncology, and infectious diseases. Future market demand will be fueled by widespread uses of the recombinant DNA technology in the creation of genetically modified crops, veterinary products, gene therapy, and the manufacturing of biopesticides and biofuels.

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