

**Fact Sheet Describing Recombinant DNA and Elements Utilizing
Recombinant DNA Such as Plasmids and Viral Vectors, and the Application of
Recombinant DNA Techniques in Molecular Biology**

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June 3, 2002**

Introduction

Recombinant DNA (rDNA) has various definitions, ranging from very simple to strangely complex. The following are three examples of how recombinant DNA is defined:

1. A DNA molecule containing DNA originating from two or more sources.
2. DNA that has been artificially created. It is DNA from two or more sources that is incorporated into a single recombinant molecule.
3. According to the NIH guidelines, recombinant DNA are molecules constructed outside of living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell, or molecules that result from their replication.

Description of rDNA

Recombinant DNA, also known as *in vitro* recombination, is a technique involved in creating and purifying desired genes. Molecular cloning (i.e. gene cloning) involves creating recombinant DNA and introducing it into a host cell to be replicated. One of the basic strategies of molecular cloning is to move desired genes from a large, complex genome to a small, simple one. The process of *in vitro* recombination makes it possible to cut different strands of DNA, *in vitro* (outside the cell), with a restriction enzyme and join the DNA molecules together via complementary base pairing.

Techniques

Some of the molecular biology techniques utilized during recombinant DNA include:

1. **The study and/or modification of gene expression patterns**

Gene expression is the process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA (messenger RNA) and then translated into protein, and those that are transcribed into tRNA (transfer RNA) and rRNA (ribosomal RNA). Gene expression can be studied using microarray analysis, which is a method of visualizing the patterns of gene expression of thousands of genes using fluorescence or radioactive hybridization.

2. **Gene cloning**

Gene cloning utilizing recombinant DNA technology is the process of manipulating DNA to produce multiple copies of a single gene or segment of DNA.

3. **DNA sequencing**

DNA sequencing is a lab technique used to determine the sequence of nucleotide bases in a molecule of DNA.

4. **Creation of transgenic plants and animals**

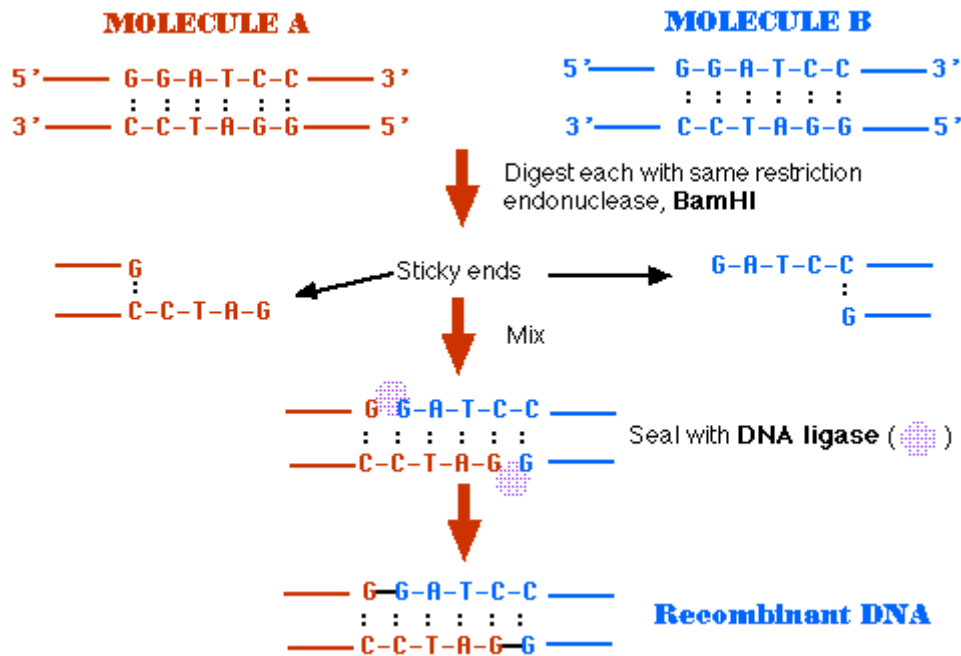
A transgenic plant or animal is one who has been genetically engineered, and usually contains genetic material from at least one unrelated organism, such as from a virus, other plant, or other animal.

Processes

The following is a summary of the process of making recombinant DNA (see Figure 1):

1. Treat the DNA taken from both sources with the same restriction endonuclease.
2. The restriction enzyme cuts both molecules at the same site.
3. The ends of the cut have an overhanging piece of single-stranded DNA called “sticky ends.”
4. These sticky ends are able to base pair with any DNA molecule that contains the complementary sticky end.
5. Complementary sticky ends can pair with each other when mixed.
6. DNA ligase is used to covalently link the two strands into a molecule of recombinant DNA.
7. In order to be useful, the recombinant DNA needs to be replicated many times (i.e. cloned). Cloning can be done *in vitro*, via the Polymerase Chain Reaction (PCR), or *in vivo* (inside the cell) using unicellular prokaryotes (e.g. *E. coli*), unicellular eukaryotes (e.g. yeast), or mammalian tissue culture cells.

Figure 1. A pictorial representation of the recombinant DNA process.



Examples

Some examples of the therapeutic products made by recombinant DNA techniques include:

1. **Blood proteins:** Erythropoietin; Factors VII, VIII, IX; Tissue plasminogen activator; Urokinase
2. **Human Hormones:** Epidermal growth factor; Follicle stimulating hormone; Insulin; Nerve growth factor; Relaxin; Somatotropin
3. **Immune modulators:** α -Interferon; β -interferon; Colony stimulating factor; Lysozyme; Tumor necrosis factor
4. **Vaccines:** Cytomegalovirus; Hepatitis B; Measles; Rabies

Cloning Vectors

A cloning vector is a DNA molecule that carries foreign DNA into a host cell (usually bacterial or yeast), where it replicates, producing many copies of itself along with the foreign DNA.

There are three features required for all cloning vectors:

1. Sequences that will permit the propagation of itself in bacteria or in yeast.
2. A cloning site to insert the foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes.
3. A method of selecting for bacteria or yeast containing a vector with foreign DNA; this is usually accomplished by selectable markers for drug resistance

General Steps of Cloning with Vectors

1. Prepare the vector and the DNA to be cloned by digestion with restriction enzymes to generate complementary ends.
2. Ligate (join) the foreign DNA into the vector with the enzyme DNA ligase.
3. Introduce the DNA into bacterial cells or yeast cells by transformation.
4. Select cells containing foreign DNA by screening for selectable markers (commonly drug resistance).

Types of Cloning Vectors

- Plasmid
- Phage
- Cosmid
- Bacterial Artificial Chromosomes (BAC)
- Yeast Artificial Chromosomes (YAC)

Plasmids

Plasmids are small, circular, extrachromosomal DNA molecules found in bacteria, which can replicate on their own, outside of a host cell. They have a cloning limit of 100 to 10,000 base pairs or 0.1-10 kilobases (kb). A plasmid vector is made from natural plasmids by removing unnecessary segments and adding essential sequences. Plasmids make excellent cloning vectors for various laboratory techniques, including recombinant DNA.

Transformation is the modification of the genotype of a cell (usually prokaryotic) by introducing DNA from another source. During transformation, genetic information is transferred via the uptake of free DNA. Often these sources of DNA come from plasmids that are deliberately introduced into a cell, transforming the cell with its genes. Transformation occurs naturally, and the resulting uptake of foreign DNA by the cell is not typically considered recombinant DNA.

If the plasmid being introduced to the cell has not been genetically altered, *in vitro*, then the plasmid is not considered to contain recombinant DNA.

Plasmids that have been genetically altered, such as by the insertion of an antibiotic resistance gene, do contain recombinant DNA because the original genetic composition of the plasmid has been artificially altered. These plasmids can be used to incorporate bacterial cells with the antibiotic resistance gene via transformation.

Phages

Phages are derivatives of bacteriophage lambda (λ phage), a virus which infects *E. coli*. They are linear DNA molecules, whose region can be replaced with foreign DNA without disrupting its life cycle. The major advantage of the λ phage vector is its high transformation efficiency, which is about 1000 times greater than that of the plasmid vector. They also have a larger cloning limit than plasmids, consisting of 8-25 kb.

Cosmids

Cosmids are extrachromosomal circular DNA molecules that combine features of plasmids and phages. They also have a high transformation efficiency, and their cloning limit of 35-50 kb is larger than that of plasmids or phages.

Bacterial Artificial Chromosomes (BAC)

BACs are based on bacterial mini-F plasmids, which are small pieces of episomal bacterial DNA that give the bacteria the ability to initiate conjugation with adjacent bacteria. They have a cloning limit of 75-300 kb.

Yeast Artificial Chromosomes (YAC)

- YACs are artificial chromosomes that replicate in yeast cells. They consist of:
- Telomeres, which are ends of chromosomes involved in the replication and stability of linear DNA.
- Origin of replication sequences necessary for the replication in yeast cells.

- A yeast centromere, which is a specialized chromosomal region where spindle fibers attach during mitosis.
- A selectable marker for identification in yeast cells.
- Ampicillin resistance gene for selective amplification.
- Recognition sites for restriction enzymes.

The procedure for making YAC vectors is as follows:

1. The target DNA is partially digested by a restriction endonuclease, and the YAC vector is cleaved by restriction enzymes.
2. The cleaved vector segments are ligated with a digested DNA fragment to form an artificial chromosome.
3. Yeast cells are transformed to make a large number of copies.
4. They are the largest of the cloning vectors, with a cloning limit of 100-1000 kb, however they have very low efficiency.

Yeast

Yeasts, eukaryotic unicellular fungi, contribute a great deal to the study of molecular genetics. They are popular organisms to clone and express DNA in because they are eukaryotes, and can therefore splice out introns, the non-coding sequences in the middle of many eukaryotic genes.

For the past two decades *Saccharomyces cerevisiae*, a species of yeast, has been an important model system for biological research because its entire genome has been base sequenced, and is used as a reference to human and other higher eukaryotic genes. This is because the basic cellular mechanics of replication, recombination, cell division and metabolism are generally conserved between yeast and larger eukaryotes, including mammals.

Also, yeast is easily genetically manipulated, which allows for convenient analysis and the functional dissecting of gene products from other eukaryotes.

Gene Therapy

Gene therapy can be defined as the transfer of genetic material into the cells of an individual resulting in a therapeutic benefit to the individual. It involves the intentional modification of genetic material with the aim of preventing, diagnosing, or curing a disease. These modifications include the correction of a genetic defect resulting from the absence or alteration of a protein, or the addition of genetic information to modify cellular characteristics.

Gene therapy allows the modification of specific genes without having to alter the disease phenotype using agents that either interact with the gene products (proteins), or are gene products themselves. The genetic modifications can be done *in vitro* or directly *in vivo* by using vectors that are capable of genetic transfer. Products used in gene therapy include viral vectors, genetically modified cells, and free or complex nucleic acids.

Currently the most efficient method for gene transfer is viral vectors. They cause infection of the target cell and transfer the therapeutic gene using their natural biological mechanisms. Viral vectors used in gene therapy must be unable to replicate and have no lytic (ability to rupture a cell) activity. The result of gene therapy is the permanent treatment of disease, hopefully with few or no side effects in the process.

Viral Vectors

A viral vector is a virus that carries a modified or foreign gene. They are commonly used in gene therapy where the viral vector delivers the desired gene to a target cell.

Some of the viruses used as vectors in gene therapy include:

- Retroviruses
- Adenoviruses
- Parvoviruses
- Herpesviruses
- Poxviruses

Retrovirus

Retroviruses are viruses belonging to the family *Retroviridae*. They are composed of a single RNA strand and use the enzyme reverse transcriptase to copy their genome into the DNA of the host cell's chromosomes. They are relatively genetically simple, and have the ability to infect a wide variety of cell types with high efficiency.

Retroviruses are the cause of many infections and cancers in vertebrates, including human T-cell leukemia and HIV. They also cause a variety of hematopoietic and neurological conditions, including paralysis, wasting, ataxia, arthritis, dementia, and neuropathy. Although vertebrate infection is mainly focused on, retroviruses have also been identified in virtually all organisms including invertebrates.

The genome of all retroviruses consists of two molecules of RNA, which are single stranded, (+) sense, and have 5' cap and 3' poly-(A) (which is equivalent to mRNA). They vary in size from approximately 8 to 11 kb. Retrovirus genomes have 4 unique features:

1. They are the only viruses that are truly diploid (*i.e.* contain a full set of genetic material which consists of paired chromosomes).
2. They are the only RNA viruses whose genome is produced by cellular transcriptional machinery, *i.e.* they do not require participation by a virus-encoded polymerase.
3. They are the only viruses whose genome requires tRNA for replication.
4. They are the only (+) sense RNA viruses whose genome does not serve directly as mRNA immediately after infection.

Avian and murine retroviruses are commonly used as cloning vectors in gene therapy.

Lentiviruses are a sub-group of retroviruses that are able to infect non-dividing cells or terminally differentiated cells, such as neurons. The main lentivirus vectors are derived from immunodeficiency viruses, including HIV.

Adenovirus

Adenovirus can be defined as a group of DNA containing viruses, which most commonly cause respiratory disease (ranging from one form of the common cold to pneumonia, croup, and bronchitis), and can also cause gastrointestinal illness, eye infections, cystitis, and rash in humans. Adenoviruses can also be genetically modified and used in gene therapy to treat cystic fibrosis, cancer, and potentially other diseases.

Some of the characteristic features of adenoviruses include:

- They are widespread in nature, infecting birds, and many mammals including humans. There are two genera: Aviadenovirus (avian) and Mastadenovirus (mammalian)
- They can undergo latent infection in lymphoid tissues, and become reactivated at a later time.
- Several types have oncogenic (cancer-causing) potential.

Adenoviral vectors can be used to express a wide variety of viral and cellular genes in mammalian cells. They can also be used to transfect cells for protein expression and *in vivo* characterization studies. *Transfection* is the uptake, incorporation, and expression of recombinant DNA by eukaryotic cells.

Adenoviral vectors are great to use for transfection because the adenovirus efficiently infects many different cell lines. The virus enters the cell but it does not replicate because the essential E1 (viral genes which are expressed early in the viral life cycle) are absent. This is termed *abortive infection*, and it can be used as a transfection system for introducing a functional gene into cells.

Adenoviral vectors are used in gene therapy when a high level of expression of the transferred gene is required for brief periods of time. The advantages of using adenovirus for the introduction of genes into cells include:

1. The process is quick, simple, and does not require any special equipment.
2. It is well tolerated, contrary to other methods. Post-infection viability is near 100% which means adenovirus can transfer genetic material into cells without toxic effects.
3. Adenovirus can infect most cell types.
4. Gene expression can be analyzed as early as a few hours after infection.
5. More than one protein can be expressed simultaneously.

Adenoviruses are medium-sized, nonenveloped, regular icosahedrons of 65-100 nanometers in diameter. The genome of Adenoviruses is a linear double-stranded DNA molecule containing 30,000-42,000 nucleotides.

Example

The following is an example of how adenovirus can be used in gene therapy to treat cystic fibrosis:

Adenoviruses are good vectors for use in human gene therapy because it can infect cells *in vivo*, rather than manipulating the cells *in vitro*, and returning the cells to the body. In order to become a vector, the genome of the adenovirus must first be altered by removing all the viral DNA, except for the minimum necessary for the virus to live and infect the cells. Genetically engineered viral vectors like these are harmless and typically can not live outside of the laboratory.

A copy of the Cystic Fibrosis gene is then inserted into the viral genome via *in vitro* recombination. *The viral genome is now considered recombinant.* Then purified proteins and enzymes are used to build a new virus, or “package” the DNA. To do this, the recombinant DNA is mixed with all of the protein components of the virus along with some viral enzymes that assemble the virus, and in the test tube a whole, intact virus is created with the new DNA inside. After infecting some cells in the laboratory, the new virus makes millions of copies of itself (carrying the Cystic Fibrosis gene), which can be purified and used for gene therapy.

Parvovirus

Parvoviruses are small DNA viruses that cause several diseases in mammals, such as canine parvovirus in dogs. Parvovirus B-19, which causes Fifth disease (erythema infectiosum) in humans, is the only form that is pathogenic to humans. In fact, many parvoviruses exhibit oncosuppressive properties (suppression of cancer-causing genes). Parvovirus-based vectors can be used to target the expression of therapeutic genes in tumors.

Herpesviruses

Herpesviruses include infectious human viruses including herpes simplex virus type-1 (HSV-1), which is common in the general population, but in rare cases can cause encephalitis. It is one of the most commonly used vector systems because it has a broad host cell range, the ability to transduce neurons, and a capacity to receive large inserts.

Poxviruses

Poxviruses are the largest and most complex viruses known. There are at least nine different poxviruses that are pathogenic to humans, the most common being vaccinia and variola virus (smallpox), which has since been eradicated. Poxviral vectors are successful in immunogenetic therapy protocols, due to their strong immunogenicity (ability to induce a high immune response). They cause the activation of immune responses against tumor antigens transported in dendritic cells.