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Genetic Engineering and Cloning: Focus on Animal Biotechnology

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Additional information is available at the end of the chapter

1. Introduction

1.1. What is genetic engineering?

Over the last 35 years the term genetic engineering has been commonly used not only in science but also in others parts of society. Nowadays this name is often associated by the media forensic techniques to solve crimes, paternity, medical diagnosis and, gene mapping and sequencing. The popularization of genetic engineering is consequence of its wide use in laboratories around the world and, developing of modern and efficient techniques. The genetic engineering, often used with trivia, involves sophisticated techniques of gene manipulation, cloning and modification. Many authors consider this term as synonymous as genetic modification, where a synthetic gene or foreign DNA is inserted into an organism of interest. Organism that receives this recombinant DNA is considered as genetically modified (GMO). Its production are summarized in simplified form in five steps: 1) Isolation of interested gene, 2) Construction, gene of interested is joined with promoters (location and control the level of expression), terminator (indicates end of the gene) and expression marker (identify the gene expression), 3) transformation (when the recombinant DNA is inserted into the host organism), 4) Selection (selection of those organisms that express the markers), 5) Insertion verification of recombinant DNA and its expression [1].

1.2. How to apply genetic engineering in our everyday

One of the main firstlings of genetic engineering is that genetic information is organized in the form of genes formed by DNA, which across some biotechnologies can be manipulated to be

applied in various fields of science. Currently, genetic engineering is widely used at various branches of medicine to produce vaccines, monoclonal antibodies, animals that can be used as models for diseases or to be used as organ donors (such as pigs). Another function of genetic engineering is gene therapy which aims to restore correct gene expression in cells that have a defective form. In the industry, genetic engineering has been extensively used for the production bioreactor able to express proteins and enzymes with high functional activity. Already in agriculture, genetic engineering is being very controversial because it tends to produce genetically modified foods resistant to pests, diseases and herbicides.

1.3. Concept is already old

However, all the knowledge obtained in the present day was only possible by discoveries of Gregor Mendel, considered the father of genetics. The results obtained in 1865 by the Austrian monk generated genetics studies related to heritability and variation. The term formerly called "element" by Mendel was later termed "genes" by Wilhelm Johanssen in 1909. Sutton and Boveri (1902) have proposed that these genes were grouped in the form of chromosomes, which in turn constitute the genetic material of eukaryotes. In 1953, James Watson and Francis Crick unraveled the structure of DNA as double helix, creating a period of intense scientific activity that culminated in 1966 with the establishment of the complete genetic code.

Major new discoveries were made in 1967 when DNA ligase was isolated that has the ability to join DNA fragments. The first restriction endonuclease enzyme was isolated in 1970 and it functions as a scissors cutting a specific DNA sequence. These discoveries allowed the development of the first recombinant DNA molecule, which was first described in 1972. In 1973 restriction enzymes (scissors) and DNA ligase (adhesive) were used to join a DNA fragment in plasmid pSC101, which is a circular extrachromosomal bacterial DNA. Thus, E. coli was transformed with the recombinant plasmid and it was replicated, generating multiple copies of the same recombinant DNA. The experiments conducted in 1972 and 1973 were crucial to the establishment of new genetics and genetic engineering.

1.4. Genome: Structure, organization and function

Genome is considered long chains of nucleic acid that contains the information necessary to form an organism [2], consisting of small subunits called nucleic bases that are inheritable. Thus, the genome contains a complete set of features that are inheritable. The genome can be divided functionally into sets of base sequences, called genes. Each gene is responsible for coding a protein, and alternative forms called alleles. A linear chain gene is named chromosome and each gene assumes a specific place, locus. Therefore, the modern view of genetics genome is a complete set of chromosomes for each individual. According to the central dogma (Figure 1), each gene sequence encodes another sequence of nitrogenous bases of single stranded RNA. The RNA sequence, complementary to a genomic DNA, will encode amino acids that form the protein. As previously mentioned, each gene relates with expression of one protein and for that each codon (the sequence of 3 nitrogenous bases of DNA) represent only one amino acid, but each amino acid can be represented by more than one codon.
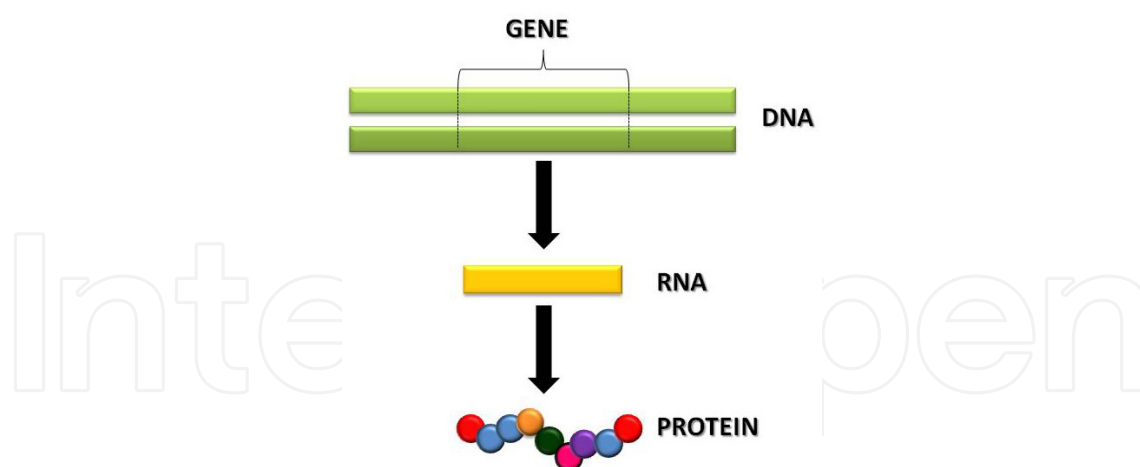
Figure 1. Central Dogma, gene codes a RNA sequence that is complementary of DNA and it encodes a protein.

1.5. DNA and RNA structure

The DNA is considered as genetic material of bacteria, viruses and eukaryotic cells having a basic structure the nucleotide, which is formed by a nitrogenous base (purine ring or pyrimidine), sugar and phosphate. In 1953, Watson and Crick proposed that DNA is a double polynucleotide chain organized as a double helix. In this model, the double helix was linked by hydrogen bounding between nitrogenous bases. The base is linked to the 1-position by a pentose glycosidic bond from N7 of pyrimidines or N9 of purine. The nuclear acid is named by the type of sugar. DNA has 2`-deoxyribose, whereas RNA has ribose. The sugar in RNA has an OH group in a 2` position of pentose ring. A nucleic acid is a long chain of nucleotides and the sugar can be linked in 3´or 5´ position to the phosphate group and the backbone of chain consist in a repeated sequence of sugar (pentose) and phosphate residues. One pentose ring is connected at 5`position to a forward pentose that is linked by the 3` position via phosphate residues; in this way, the sugar-phosphate backbone is 5´-3` phosphodiester linkages (Figure 2).

Nucleic acid contains 4 types of base, 2 purines (adenine (**A**) and guanine (**G**), which are present in DNA and RNA) and two pyrimidines (cytosine (**C**) and thymine (**T**) for DNA and for RNA uracil (**U**) instead of thymine). Therefore, DNA contains **A**, **G**, **C**, **T** and RNA contains **A**, **G**, **C** and **U**. Other important discover were that the **G** bounded specifically with **C**, and **T/U** with **A**; these named base pairing (complementary), and that the chains had apposite directions (antiparallel).

2. Genetic engineering: Timeline

The chronological order of main events of genetic engineering and cloning are described above.

1866 - Gregor Mendel proposed the law of independent, of segregation and basic principles of heredity; principles that created a new science called genetic.
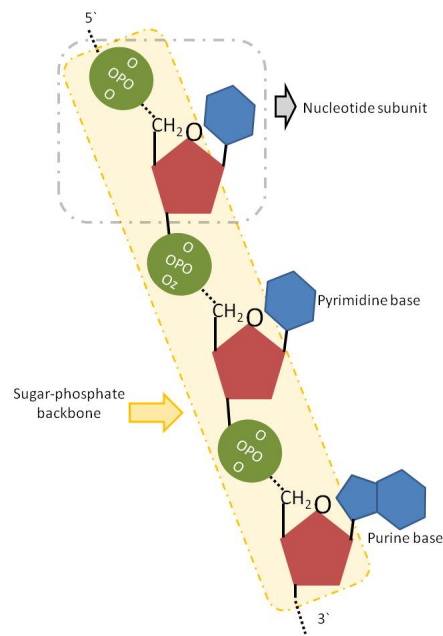
Figure 2. Polynucleotide chain, 5´-3´sugar phosphate linkages (backbone) and structure of nucleotide subunit - Adapted from Lewin, B (2004)[2]

1900 - Mendel´s principles were rediscovered by Hugo de Vries, Carl Correns and Eric von Tschermak

1908 - Chromosome Theory of Heredity was proposed by Thomas Hunt Morgan

1944 - Was established that DNA contains the heredity material.

1946 - First electronic digital computer was created

1952 - The first cloned animal (Northern Leopard Frog)

1953 - Watson and Crick described DNA structure and proposed the double helix model.

1955 - Protein sequencing method was established by Frederick Sanger and insulin was sequenced

1965 - Atlas of protein sequences was created

1966 - Genetic code was cracked

1970 - Algorithm for DNA sequence was created

1972 - Establishment of DNA recombinant technology by Stanley Cohen, Herbert Boyer and Paul Berg

1973 - The first recombinant DNA organism was created

1976 - The first genetic engineering company is founded.

1977 - DNA sequencing method was established

1980 - Was done the first molecular mapping of a human chromosome

1982 - GeneBank started to be public

1983 - Mullis developed PCR (Polymerase chain reaction)

1984 - "Genetic Fingerprinting" techniques was developed and human genome sequencing started

1986 - National Center for Biotechnology was developed in USA and automatic machine for DNA sequencing was created

1990 - Dolly, the first cloned animal, was born and blast program was created

1995 - First complete bacterial genome was sequenced

1997 - E. coli complete genome sequence was published

1999 - Complete sequence of human chromosome 22 was published

2000 - Drosophila genome was sequenced and first holy genome from plant was published

2002 - Mouse genome sequence was published

2003 - Human genome sequence was published

2004 - Chimpanzee genome sequence was published

3. Cutting and pasting the DNA

3.1. Discovering restriction endonuclease and a Nobel Price in 1978.

Molecular biology and genetic were innovated in middle of 70th decade the discover of restriction endonuclease by W Arber, D Nathans e H Smith that wan the Nobel price in 1978. When phage λ attacks an E. coli strain B a specific restriction endonuclease (EcoB) cuts just the DNA from phage λ and infections is blocked. E.coli methylates its own DNA by action of DNA methylase to protect this DNA from itself enzyme. Restriction endonuclease recognizes short sequences of duplex DNA as cleavage target and the enzyme cuts this point of DNA every time this target sequence occurs. When the DNA molecule is cleaved by restriction endonuclease DNA fragments are produced. Analyzing restriction fragments is possible to generate a map of the original DNA molecule (restriction map, a linear sequence of DNA separated in defined fragment size) [1, 2]

3.2. Types of restriction endonuclease enzyme: Nature, structure, application, recognition site of action and nomenclature

Restriction endonuclease are classified in types I, II and III by sequence specificity, nature of restriction and structural differences (table 1). Types I and III have a restrict use in molecular biology and genetic engineering but the type II is largest used because it cleaves the DNA a specific recognition sequence, separate methylation, no additional energy requirement is necessar, high precision and do not match actions. Type II restriction endonuclease are

classified by the size of recognition sequence such as tetracutter, hexacutter or octacutter (4, 6 and 8 base paired respectively) [3].; and generally that sequences are palindromic (nitrogenous bases sequence read the same backwards and forwards). Restriction enzymes also could be classified as neoschizomers (recognize the same sequence) and isoschizomers (recognize and cleave in the same location).

	Type I	Type II	Type III
Enzyme structure	Complex of three subunits with independent recognition endonuclease and methylase function	Separate monomeric enzymes for endonuclease and methylase action, both recognize the same target sequence	Separated dimeric enzymes for endonuclease and methylase with one common subunit
Requirement for activation	ATP and Mg2+ S-Adenosyl methionine	Mg^{2+}	ATP and Mg2+ S-Adenosyl methionine Enhance activity
Recognition site	Double-stranded DNA	Generally palindromic sequence of Double-stranded DNA	Single-stranded DNA
Nature of restriction	Cleaves the DNA at a random sequence at one Kb away of recognition site	Cleaves the DNA at a specific sequence near or at the recognition site	Cleaves the DNA about 25pb downstream of the recognition site at a random sequence

Table 1. Properties of restriction endonucleases – Adapted from Satya, P[3] (2007)

The nomenclature of restriction endonuclease is derivate from the species that it was isolated (Ex. ECORI, from *Escherichia coli Ry13*); First two letters from enzyme name identify the species and the third identify the different strains from the same organism (Table 2). The number classifies the different enzymes from the same organism and strains in chronological order of discover (Ex. Hind III, is the third RE isolated from *Haemophilus influenza*). Restriction endo-nuclease cut the DNA in two different ways: blunt end (two DNA strands are cleaved at the same position) or sticky end (the enzyme cut each DNA strand at different position, generally two until four nucleotides apart). So in the sticky, DNA fragments have short single-stranded overhangs at each end.) [1-4]

3.3. Linking of DNA fragments: DNA ligase

Restriction endonuclease type II cuts the double-stranded DNA in specific target sequence but this enzyme do not joined back again the DNA fragments, this is essential to create a new hybrid DNA. Joining two DNA fragments by 5`→3` phosphodiester bond is an energy dependent process (ATP or NAD, depending the kind of enzyme that is being used). DNA ligase is a specific enzyme that is responsible to join DNA fragments spending and two blunt

Enzyme	Recognition sequence	Type of ends	End sequences
*Alu*I	5'-AGCT-3'	Blunt	5'-AG ↕ CT-3'
	3'-TCGA-5'		3'-TC ↕ GA-5'
*Sau*3AI	5'-GATC-3'	Sticky, 5' overhang	5'- ↕ GATC-3'
	3'-CTAG-5'		3'-CTAG ↕ -5'
*Hin*fI	5'-GANTC-3'	Sticky, 5' overhang	5'-**G** ↕ ANTC-3'
	3'-CTNAG-5'		3'-CTNA ↕ **G**-5'
*Bam*HI	5'-GGATCC-3'	Sticky, 5' overhang	5'-**G** ↕ GATCC-3'
	3'-CCTAGG-5'		3'-CCTAG ↕ **G**-5'
*Bsr*BI	5'-CCGCTC-3'	Blunt	5'- ↕ NNNCCGCTC-3'
	3'-GGCGAG-5'		3'- ↕ NNNGGCGAG-5'
*Eco*RI	5'-GAATTC-3'	Sticky, 5' overhang	5'-**G** ↕ AATTC-3'
	3'-CTTAAG-5'		3'-CTTAA ↕ **G**-5'
*Pst*I	5'-CTGCAG-3'	Sticky, 3' overhang	5'-CTGCA ↕ **G**-3'
	3'-GACGTC-5'		3'-**G** ↕ ACGTC-5'
*Not*I	5'-GCGGCCGC-3'	Sticky, 5' overhang	5'-GC ↕ GGCCGC-3'
	3'-CGCCGGCG-5'		3'-CGCCGG ↕ CG-5'
*gg*I	5'-GCCNNNNNGGC-3'	Sticky, 3' overhang	5'-GCCNNNN ↕ NGGC-3'
	3'-CGGNNNNNCCG-5'		3'-CGGN ↕ NNNNCCG-5'

*N = any nucleotide.

–

*Note that most, but not all, recognition sequences have inverted symmetry: when read in the 5'→3' direction, the sequence is the same in both strands.

Table 2. Same restriction endonuclease used in genetic engineering - Adapted from Brown, TA (2002)[4]

ends can be joined easily spending two ATPs molecules and this blunt end is very popular in genetic engineering. However, the efficiency of this process is very low because the DNA ligase just joins adjacent DNA fragments and it cannot bring the DNA end nearby. Action of enzyme to catalyze the reaction is a random process that depends of vicinity of DNA fragments in solution. Joining DNA fragments with blunt ends is generally used to short oligonucleotides because concentration of free ends and enzyme are high, increasing the efficiency of process. Presence of sticky ends increase process efficiency because complementary ends come together by a random diffusion event in the solution and transient base pair might form between the two complementary strand. This ligation is not very stable but may persist for enough time to join DNA fragments by DNA ligase catalysis and synthesis of phosphodiester bonds [4].

The greater efficiency of sticky-end ligation stimulated the creation of new methods, such linkers or adaptors. They are short double-strand molecules that cover the blunt-end and insert a recognition sequence for a restriction endonuclease to create a sticky-end. The linkers need to be digest by a restriction endonuclease to have a stick-end but the adaptor is a final sequence, digestion is not necessary and fragments can be direct joined by DNA ligase.

4. DNA cloning

In modern molecular biology the ability to manipulate DNA molecules by restriction endonuclease and DNA ligase is named by DNA cloning and, a recombinant DNA can be constructed. However, a single copy of recombinant DNA is not enough. Replication machinery of one organism generally is used to increase the number of copies. The DNA is inserted in the organism for a propagation or transfer. Generally, the vector has autonomic replication system that is independent of the cell cycle, increasing the number of copies. Majority systems of DNA cloning use bacterial as a host and common plasmid vector is classified in low copy number (<10) or high copy number (>20). To select recombinant cell some parameters need to be present: have restriction sites in which de exogenous DNA is inserted (just one site for each restriction endonuclease) and vector needs to have a marker gene multicloning sites (one site for several restriction endonuclease) makes the vector more useful [3, 4].

5. Isolation, sequencing and synthesis DNA

The transgenic animal technology involves in first place, the isolation or artificially synthesis of a gene, which will be molecular manipulated and used for transformation leading to the transgenic production. The need of knowledge involving this target gene can be overcome by its sequencing, conducting to the understanding of its structure. The main of this subject is briefly described the mechanisms involved in isolation, sequencing and synthesis of a gene.

5.1. Isolation of genes

The first gene isolation was reported in 1969. Two specialized transducing phages, bacteriophages λ and $\Phi80$, which carry the *lac* operon of *Escherichia coli* was inserted in reverse orientation into their DNA, being used as a source of complementary sequences to prepare pure *lac* operon duplex [5]. This method besides being resourceful work did not have general applicability.

Now a day, several methods are in progress for isolation of a gene. A most traditional method used largely in research is the construction of a genomic or complementary DNA (cDNA) library. A genomic library represents the total DNA of a cell including the coding and non-coding sequences cloned on a vector and a cDNA library is a combination of cloned fragments from the mRNA inserts into a collection of host cells, creating in both cases, a portion of organism transcriptome.

To produce a genomic library after the extraction of genomic DNA, these molecules are digested into fragments of reasonable size by restrictions endonucleases and then inserted into a cloning vector generating a population of chimeric vector molecules.

On the other hand, to create a cDNA library is necessary first, to produce a cDNA, which can be obtained from a mature mRNA isolate from a tissue or cells actively synthetizing proteins. The extraction of mRNA is easy due to poly-A tail present in eukaryotic mRNAs. Than the extracted mRNA is used for copying it into cDNA using the reverse transcriptase enzyme, method that create a single strand cDNA, which is converted in a double strand cDNA with DNA polymerase, coiling and nucleases. This cDNA are cloned into a bacterial plasmid, which is transformed into bacterial competent cells, amplified and selected.

Once a genomic or a cDNA library is available, they can be used for the identification and isolation of a gene sequence.

There are many commercial kits to create a genomic or cDNA library. Normally, the genomic library is created with lambda or cosmid vectors while a cDNA library is produced with plasmid vectors (*more information see item 5*). These kits usually try to improve the classical laborious techniques, enabling rapid construct of the libraries and ensuring generating of full-length clones.

The isolation of a gene using a genomic or cDNA library can be done by colony hybridization. In this technique the fragments containing a gene or parts of it can be identified by the use of DNA probes, which can be tagged or labeled with a molecular marker of either radioactive or fluorescent molecules. The commonly used markers are phosphorus 32 and digoxigenin, a non-radioactive, antibody-based marker.

The DNA of bacteria carrying the chimeric vectors is fixed on the filter, which is hybridized with the labeled probe carrying a sequence related to the gene to be isolated. The colonies carrying moderate to high similarity to the desired sequence are detected by visualizing the hybridized probe via autoradiography or other imaging techniques. In this way, the original chimeric vectors carrying the target gene sequence can be recovered from original colonies and used for advance researches.

If the library available were in the form of phage particles, instead of colonies are plaques that can be hybridized in the same way described above for colonies. This method of identification and isolation of genes are called plaques hybridization.

5.2. Isolation of genes related to a protein

To identify a gene related to a protein the inverse pathway (from protein to DNA) should be simulated. For start is necessary to have the protein product in a pure form. To purify a protein several methods typically used are in a series of steps. Each step of protein purification usually results in some degree of product loss, so, an ideal strategy is one in which the highest level of purification is reached in the fewer steps. The properties of the protein product like size, charge and solubility; determines the selection of which steps to use. These steps can be precipitation and differential solubilization; ultracentrifugation or chromatographic methods.

Thus, having the protein product is possible to produce antibodies probes for this protein by immunizing animals. This production require reliance upon animals immune system to levy responses that result in biosynthesis of antibodies against the inject molecule. Antigens must be prepared and delivered in a form and manner that maximizes production of a specific immune response by the animal.

These antibodies probes can be used to precipitation of polysomes engaged in synthesizing the target protein leading to the achievement of the mRNA coded for this protein. This method combined with immunoadsorbent techniques brings the possibility of application at less abundant proteins expression [6]. Then the mRNA are isolated and purified from the polysome fraction, being after used for synthesizing cDNA for a cDNA library preparation, described above.

Thereby, to identify the specific cDNA clone for the target protein immunological and electrophoretic analysis methods are used, screening a complete or partial genomic library [10].

5.3. DNA Sequencing

The basic concept of DNA sequencing is the mechanism involved in determining the order of nucleotides bases (adenine, guanine, cytosine and thymine) in a strand of DNA. F. Sanger and coworkers reported the first DNA sequencing, which was genome of DNA ΦX174 virus. Thus, at that moment, two methods of DNA sequencing were developed: one proposed by A. Maxam and W. Gilbert, known as chemical method of DNA sequencing, and the other developed by F. Sanger, S. Nicklen and A. R. Coulson known as chain termination method.

The chemical method of DNA sequencing consists in determines the nucleotide sequence of a terminally labeled DNA molecule by breaking it at adenosine, guanine, cytosine and thymine with chemical agents. Partial cleavage at each base produces a nested set of radioactive fragments extending from the labeled end to each of the positions of the base. The autoradiograph of a gel produced from four different chemical cleavages, shows a pattern of bands from which the sequences are read directly [7].

The chain termination method depends on DNA replication and termination of replication at specific sequences. For that, Sanger's technique is based on an enzymatic synthesis from a single-stranded DNA template with chain termination on DNA polymerase, using dideoxynucleotides (ddNTPs). The principle of this method relies on the dideoxynucleotide lacking a 3'OH group, which is required for extension of the sugar phosphate backbone. Thus, DNA polymerases cannot extend the template copy chain beyond the incorporated ddNTP [3, 8].

Both methods rely on four-lane high-resolution polyacrylamide gel electrophoresis to separate the labeled fragment and allow the base sequence to be read in a staggered ladder-like fashion. Sanger sequencing was technically easier and faster, becoming the main basis of DNA sequencing, being modified and automated to aid large scale sequence procedure [3, 8, 9].

5.3.1. Automatic sequencing

An automatic sequencing is an improvement of Sanger sequencing, through the use of different fluorescent dyes incorporated into DNA extension products primers or terminator. The use of

different fluorophores in the four based (A, C, G and T) specific extension reactions means that all reactions can be loaded in a single lane. For each base one color are used, emitting a different wavelength when excited. Throughout electrophoresis, the fluorescence signs are detected and recorded [10, 11].

The classic electrophoresis methods used in automated sequencing are slab gel sequencing system or capillary sequence gel system, both described below.

5.3.2. Slab gel sequencing systems

The slab gel sequencing system consists of using ultrathin slab gels, about 75μm, and comprises running of at least 96 lanes per gel. By this instrument, fluorescent-labeled fragments were loaded to the top of vertical gel and electric filed was applied, as the negatively charged DNA fragments migrated through the gel they were sized and fractionated by the polyacrylamide gel. The fragments were automatically excited with a scanning argon laser and detected by a camera [12].

The loading of sequencing gels samples can be done manually or automatically. The automation consists in the use of a plexiglass block with wells in same distance from each other as the comb teeth cut in a porous membrane used as a comb for drawing samples by capillary action. The loading of samples automation achieve up to 480 samples per gel [9].

5.3.3. Capillary sequence gel systems

Alternatively, the capillary sequence gel system instead of continuous polyacrylamide gel slabs, DNA is sent through a set of 96 capillary tubes filled with polymerized gel [3, 9].

In this system fused silica capillaries of 50-100 μm in diameter and 30-80 cm in length, heat resistant, are filled with a separation matrix consisting of a gel and electrode buffer. Solution phase DNA molecule are injected into the capillary either by pressure or electrokinetic injection and separated inside the capillary according to their size under high voltage conditions. The molecules are detected using UV light absorption or laser induced fluorescent detection at the end of the capillary [3, 12].

5.3.4. Direct sequencing by PCR

PCR has relieved much of the experimental toil of molecular biology improving the procedure's sensibility and facilitating the rapid cloning and sequencing of large numbers of samples [13]. The amplification of target DNA by PCR followed by direct sequencing of amplified DNA has emerged as a powerful strategy for rapid molecular genetics analysis bypassing the time consuming cloning steps and generating accurate DNA sequence information from small quantities of precious biological samples [14].

The direct PCR sequencing involves two steps 1- generation of sequencing templates through PCR and 2- sequencing of PCR products using thermolabile or thermostable DNA polymerases [15].

Some enzymes as *Taq* polymerase are thermostable and can be used in automated sequencing reactions such as cycle sequencing. Others, such as Klenow polymerase and reverse transcriptase are thermal instable, being able to both direct sequencing by PCR products and cloned template, although cannot be used in cycle sequencing. Another enzyme, Sequenase, has also been used effectively in both radioactive and fluorescence cycle sequencing [8].

One sequencing strategy of form any PCR-amplified DNA template are the sequenase approach. First, the PCR-amplified DNA is denatured to single strands, annealing the sequencing primer to complementary sequence on one of the template strands. Then, the annealed primer is extended by DNA polymerase by 20-80 nucleotides, incorporating multiple radioactive labels into the newly synthesized DNA, under non-optimal reactions conditions, retaining the enzyme functionality low, for the synthesis of only short stretches DNA. After, the labeled DNA chains are extended and terminated by incorporation of ddNMPs [14].

On the other hand, cycle sequencing strategies can be used for PCR-amplified DNA. These methods generate high-intensity sequence ladders due to the advantage of automated cycling capability of thermal cyclers. First, the PCR-amplified DNA is denatured to single strands, and then it is annealed of a 32p-labeled sequencing primer. After, it is extended and chain-terminated by a thermostable DNA polymerase and denatured in the next sequencing cycle. This step releases the template strand for another round of priming reactions while accumulates chain-terminated products in each cycle. These steps are repeated 20-40 cycles to amplify the chain-terminated products in a linear fashion [14].

5.3.5. DNA sequencing by microarray

A DNA microarray technology brings the possibility of large scale sequence analyses by generating miniaturized arrays of densely packed oligonucleotide probes [9, 16].

The word microarray has been derived from the Greek word *mikro* (small) and the French word *arrayer* (arranged). This technology can be described as an ordered array of microscopic elements on a planar surface that allows the specific binding of genes or gene products [17, 18].

The DNA sequencing by microarray uses a set of oligonucleotide probes to examine for complementary sequences on a target strand of DNA. Briefly, after cleavage DNA segments are hybridized to the definitely arranged probes on a gene chip, the detection is made with a light driven. Then, to reconstruct the target DNA sequence, the hybridization pattern is used. To analyze the data and determinate the DNA sequence specific software are used [3, 16].

The array elements react specifically with labeled mixtures, producing signals that reveal the identity and concentration of each labeled species in solution. These attributes provide miniature biological assays that allow the exploration of any organism on a genomic scale [17].

The array technology has been widely used in functional genomics experiments designed to study the functions and interactions of genes within the context of the overall genome distinct plant and animal species. To sequence a DNA fragment by microarray a series of laboratory procedures are involved, from RNA extraction, reverse transcription and tagging fluorescent hybridization to the end, which invariably introduce different levels of additional variation

data. On the other hand, experiments with microarrays are still considerably expensive and laborious and, as a consequence, are generally conducted with relatively small sample sizes. Thus, the conducting tests on microarrays require careful experimental design and statistical analysis of the data [19].

5.3.6. DNA sequencing by MALDI TOF mass spectrometry

The Matrix assisted laser desorption/ionization is very rapid and combined with time-of-flight (MALDI-TOF) became an efficient and less time consuming (range of several microseconds) in acquire DNA sequence information by sensitive discrimination of their molecular masses.

The technique consists in embedded the samples to be analyzed in a crystalline structure of small organic compounds (matrix) and deposited on a conductive sample support. Then, the samples are irradiated with an ultraviolet (UV) laser with a wavelength of 266 or 337nm. The energy of the laser causes structural decomposition of the irradiated crystal and generates a particle cloud from which ions are extracted by an electric field. Following acceleration through the electric field, the ions drift through a field-free path and finally reach the detector. The results come from the calculation of ion masses by measuring their TOF, which is longer for larger molecules than for smaller ones. Due to single-charged, nonfragmented ions are mostly produced, parent ion masses can be determined from the resulting spectrum without the need for complex data processing. The masses are accessible as numerical data for direct processing and subsequent analysis [20].

The development of MALDI-TOF for an efficient DNA analyses happens due to needed of high throughput, parallel processing, simplified handling and low-cost techniques. The method uses an initial PCR amplification, which, PCR is carried out with a DNA polymerase that accepts ribonucleoside triphosphates (NTP) substrates. One of the four deoxynucleotides is replaced by an NTP. Fragments are generated by simple alkali backbone cleavage at the ribo-bases of the PCR products, generating oligonucleotide fragments each terminating with the ribonucleotide of the cycled primer extension reaction. Analysis is carried out by MALDI-TOF mass spectrometry. Differences between the unknown sample and a reference sequence are determined by changes in the results pattern [21, 22].

Nowadays, with the advent of genome sequencing projects been accomplished, sequences of DNA can be obtained and compared through electronically databases, than physically from clone libraries (described above). The available databases include locus information, organism species, the whole gene sequence, the reference authors and the status of the sequencing. The most used resource is the GenBank [23] provided for the *National Center for Biotechnology Information* (NCBI).

5.4. Synthesis

The gene synthesis methods had their main development during 1980s and 1990s. DNA gene synthesis is the process of writing the DNA. As DNA carries the genetic information of an organism, it could be viewed like a kind of information resource, enabling its reading (sequencing, described above) and writing (synthesis).

The oligonucleotides synthesis can be done rapidly and in high yields with different kinds of methods. The gene synthesis, together with the knowledge of full genomes, molecular cloning, and protein expression profiles, improved the biotechnology field, making possible to explore the whole functionality of an entire complex organism.

5.4.1. Gene synthesis machine

The gene synthesis machine is fully automated instrument, which synthesizes predetermined polynucleotide sequence. The principle involved is based on a combination of organic chemistry and molecular biological techniques.

Automatic gene machines, synthesize specific DNA sequences by programming the apparatus for the desired sequence. Briefly, the chosen sequence is entered in a keyboard and a microprocessor automatically opens the valve of nucleotide, chemical and solvent reservoir, controlling the whole process [15].

Containers of the four nucleotides (A, T, C and G) and reservoirs for reagent and solvent supports are connected with the synthesizer column. This column is packed with small silica beads, which provides support for assembly of DNA molecules. The desired sequence is synthesized on the silica beads which are later removed chemically [23].

Commercial services for gene synthesis are available from numerous companies worldwide. This gene synthesis method provides the possibility of creates entire genes without the need of a DNA template.

5.4.2. Gene synthesis from mRNA

The reports of a ribonuclease-sensitive endogenous DNA polymerase activity in particles of RNA tumor viruses by H.M. Temin and D. Baltimore enable the synthesis of complementary DNA (cDNA) using mRNA as template [9, 15, 24].

This enzyme, known as reverse transcriptase, are largely used in biotechnology research, and combined with the polymerase chain reaction create a methodology for DNA synthesis and amplification of the product.

To use the mRNA as a template first is necessary purify this molecule of the cell, or tissue. This can be done using oligo-dT cellulose spin columns, oligo-dT/magnetic beads and coated plates. The principle involved at isolation of mRNA relies on base pairing between the polyA residues at the 3' end of most mRNA, and the oligo (dT) residues coupled to the surface of cellulose spin columns or, magnetic beads or, a pre-coated 96 oligo-dT plate.

Independently of efficiency the three kinds of mRNA isolation are available commercially, facilitating the lab work.

Since the mRNA is available, the cDNA can be produced. To produce the cDNA the reaction should be done using mRNA template and a mix of, primers, reverse transcriptase, solution of four dNTPs and buffers. Depending on the experiment, ligo (dT)12-18, random hexanucleotides, or gene-specific antisense oligonucleotides can be used as primers for synthesis of first-strand cDNA [25].

The correctly native gene synthesis by this method depend on the fidelity of copying mRNA and also on the stability of DNA thus synthetized. Moreover, since mRNA of a gene does not have the complete transcript of the gene *in vivo* (intronic regions are dismissed) the synthesized gene will be smaller than the gene in vivo, but contain just the coding sequences, what could be a great advantage for research [9].

5.4.3. Synthesis by PCR

The gene synthesis by PCR, as described first for W. P. C. Stemmer and coworkers were reported having four steps. First the olygos are synthetized, and then the gene is assembled, amplified and cloned. Since single-stranded ends of complementary DNA fragments are filled in during the gene assembly process, cycling with DNA polymerase results in the formation of increasingly larger DNA fragments until the full-length gene is obtained [26].

The classical method involves the use of oligonucleotides of 40nt long that overlap each other by 20nt. The oligonucleotides are designed to cover the complete sequence of both strands, and the full-length molecule is generated progressively in a single reaction by overlap extension PCR, followed by amplification in a separate tube by PCR with two outer primers [27].

Variations of the classical approach were done, such as ligation of phosphorylated overlapping nucleotides, modified form of ligase chain reaction combinations with asymmetrical PCR and thermodynamically balanced inside out.

Nevertheless, most of them are based on phosphorylation of oligos at the 5' ends', annealing of overlapping ends, filling the gaps by enzymatic extension at 3' ends and join nicks with DNA ligase. Then the full length double stranded DNA can be cloned on a plasmid/phage vector and multiplied in *E. coli* or, amplified by PCR, separated on electrophoresis, purified from gel and cloned [9].

The most commonly synthesized genes range in size from 600 to 1,200 bp although, much longer that genes made by connecting previously assembled fragments of fewer than 1,000 bp. In this size range it is necessary to test several candidate clones confirming the sequence of the cloned synthetic gene by automated sequencing methods [23].

6. Cloning vectors

The molecular cloning brings the possibility to isolate, analyze, synthetize and clone individual genes or segments of DNA, creating a recombinant DNA. After isolated and purified the DNA target sequence must be mounted on an appropriate carrier molecule, the cloning vector.

A cloning vector is a small piece of DNA into which a foreign DNA is inserted for transfer or propagation in an organism, with the ability to self-replicate. The purpose of a vector is to allow efficient high-level expression of cloned genes or still, the need to increase the number of copies of a recombinant DNA [28].

6.1. Need to increase the number of copies of recombinant DNA

Besides having a DNA molecule already recombined, single copies are not sufficient to construct a recombinant DNA. The *in vitro* manipulation like, purification and transfer to a target cell, of a single copy is not possible. Thereby the recombinant construct should be propagated to increase the copy number. A convenient way to copy such fragments is to use the replication machinery of an organism, inserting the donor DNA in a cloning vector [29].

The essence of molecular cloning is to use restriction nucleases to cut DNA molecules in a starting DNA population (the target DNA) into pieces of manageable size, then attach them to a replicon (any sequence capable of independent DNA replication) and transfer the resulting hybrid molecules (recombinant DNA) into a suitable host cell which is then allowed to proliferate by cell division. Because the replicon can replicate inside the cell (often to high copy numbers) so does the attached target DNA, resulting in a form of cell-based DNA amplification [11].

6.2. Cloning vectors

In principle, any molecule of DNA that can replicate itself inside a cell system could work as a cloning vector, but many factors as, small sizes, mobility between cells, easy production and detection mechanism should be considered [28].

The type of host cells used in a particular application will depend mainly on the purpose of the cloning procedure. Host cells exploited are modified bacterial, fungal cells (*e.g.* Yeast), or still virus, being the bacterial system (*e.g. E. coli*) the most used due to their capacity for rapid cell division and for attend the major vectors requirements.

The vector may have an origin of replication that originates from either a natural extrachromosomal replicon or, in some cases, a chromosomal replicon [11]. Besides the structure the vectors should contain a sequence that make possible to select the recombinant cells, like a marker gene and in third place they should contain restriction sites into which the DNA can be inserted [29].

The types of cloning vectors are plasmids, phages, cosmids, phagemids, artificial chromosomes, viral vector and transposons. Each of them will be briefly describe in this section.

6.2.1. Plasmideal vectors

Plasmids are small circular double-stranded DNA molecules, which exist in the cell as extrachromosomal units. In a cell, they have the ability for self-replicating, and copy numbers maintenance. Due to their capacity of copy numbers they can be classified as: single copy plasmids or multicopy plasmids.

The single copy plasmids are maintained as one plasmid DNA per cell, instead the multicopy plasmids that are maintained as 10-20 copies per cell. Another kind of plasmids consists in ones that are under relaxed replication control, allowing their accumulation in numbers up to 1000 copies per cell, being the used ones as cloning vectors [15].

The plasmids vectors are designed to work in bacteria cells. An important property in these vectors is the detection of the same in the host cells. Usually, the detection mechanisms are done through antibiotic resistance. The host cell strain chosen is sensitive to a particular antibiotic and the plasmid is designed to contain a gene conferring resistance to this antibiotic.

Another approach for detection is through β-galactosidase gene complementation in which the host cells are mutants containing a β-galactosidase gene fragment and plasmid vector are designed to contain a different fragment of the same gene. By this way, after transformation functional complementation occurs and the host cells, which incorporate the plasmid are capable of β-galactosidase production.

The functional β-galactosidase activity can be accessed by conversion of a colorless substrate, Xgal (5-bromo, 4-chloro, 3-indolyl β –D- galactopyranoside) to a blue product [11]. The both methods are efficient for clone's selection, and their use depends on individual's preferences.

According to P. K. Gupta (2009) [15], there were three phases of plasmid development cloning vectors. The first included the plasmids pSC101, ColE1 and pCR1, which are naturally occurring plasmids, and not suitable for efficient cloning, since plasmid can transfer the gene through bacterial conjugation or can be integrated in the bacterial genome having no accessible detection system. Other disadvantage lies on having no more than two restriction sites for cloning.

The drawbacks of naturally occurring plasmids were overlapped by pBR313 and pBR322. pBR313 was too large having fifty percent of its sequences being non-essential. The size reduction brought the pBR322, which was largely used for many years.

The second phase relies on reducing the plasmids sizes, because the transformation efficiency and vector size have a proportional inverse relation. Thus, variations of the pBR322 appeared, including pAT153, pXf3, pBR327, etc. This plasmid vectors incorporate the selection mechanism of antibiotic resistance (described above).

The third phase involves incorporation of sequences for alpha-complementation selection (described above); incorporation of sequences from single strand M13 phage, for sequencing templates production; and, also integration of promoters' sequences, for *in vitro* transcription or expression of large amounts of foreign proteins. In this phase, plasmids like pUC, pGEM, M13, were developed.

Nowadays, there are a lot of plasmids commercially available that can be purchased depending on the application needs.

6.2.2. Lambda phage vectors

A bacteriophage lambda is a bacterial virus that infects *E. coli.* Its utility as a cloning vector depends on the fact that not all of the lambda genome is essential for its function [1]. The lambda genome has the left-hand region with essential genes for the structural proteins and the right-hand region has genes for replication and lysis, while the middle region has the genes for integration and recombination, which are non-essentials.

There are two possible types of lambda vectors: the insertion vector and the replacement vector.

The insertion vector has only a single recognition site for one or more restriction enzymes, enabling the DNA fragment to be inserted into the lambda genome. The lambda particle integrates DNA molecules between 37 and 52kb, and to adapt longer inserts is necessary to remove some of lambda genome. The region for replacement is the middle one where, more 23 kb of foreign DNA can be inserted. This vector is known as replacement vector [28].

The replacement vector cannot be integrated into the host cells chromosome being necessary to use a helper phage to provide integration and recombination functions. On the other hand, this vector has two restriction sites, having a whole section of phage genome being replaced during cloning [1].

6.2.3. M13 phage

M13 is filamentous bacteriophages that infect specific *E. coli*. Your attractive as a cloning vector consists in its genomes contain the desirable size for a potential vector (less than 10kb); does not kill the host when progeny virus particles are released and thus, is easily prepared from an infected *E. coli* cells culture. Besides, M13 is used as cloning vector to make single stranded DNA for sequencing and mutagenesis approaches.

The M13 genome is a single-stranded DNA molecule with 6407bp in length. This bacteriophage only infects bacteria carrying the F-pili (fragile protein appendages found on conjugation-proficient cells), being male-specific. When the DNA enters the cell, it is converted to a double-stranded molecule known as replicative form, which is a template for making about 100 copies of the genome. At this point replication becomes asymmetric, and single-stranded copies of the genome are produced and extruded as M13 particles. The property of do not lyse the host cell brings a DNA resource, although growth and division are slower than in non-infected cells [1, 11, 28].

6.2.4. Cosmids

Cosmids are plasmid particles into which certain specific DNA sequences, namely those for *cos* sites, are inserted. The goal of these vectors development is to cloning of large DNA fragments (up to 47kb in length). They are made up of plasmid sequences joined with lambda vectors sequences, trying to conjugate the properties of this both vectors in one (being transfected as a lambda vector by packaging/ infection mechanism and behaving as a plasmid when introduced into an *E. coli* cell).

The advantages consist of a highly efficient method of introducing the recombinant DNA and, a cloning capacity twofold greater than the best lambda replacement vectors. On the other hand, the gains of using cosmids instead of phage vectors are offset by losses in terms of ease to use and further processing of cloned sequences [1].

The methodology to use the cosmid cloning vectors consists in put together the cleaved vector and the target DNA for cloning, producing concatameric molecules. The concatameric

molecules are usually generated by first linearizing the cosmid so that each end has *cos* site. Then the linear cosmid is cut with a *Bam*HI, which generates sticky ends with the overhang sequence GATC. The foreign DNA is also digested with *Mbol*, which also generates a GATC overhang. Partial digestion leaves some site uncut and allows large segments of a genome to be isolated. These segments are mixed with the two halves of cosmid and joined using ligase. Thus, these molecules are packaged into phage heads by mixing with a packaging extract, becoming infectious. *E. coli* cells are infected with the cosmids, and after infection the cosmid circularizes and multiply as a plasmid vector [15, 28].

6.2.5. Phagemids

Phagemids combine desirable features of both plasmids and bacteriophages. The construct consists of a plasmid with a segment of a filamentous bacteriophage, such as M13, having two different origins of replication: the plasmid and the phage origin. The selected phage sequences contain all the *cis*-acting elements required for DNA replication and assembly into phage particles [11, 30].

These vectors allow successful cloning of inserts several kilobases. After *E. coli* suitable strain transformation with a recombinant phagemid, the bacterial cells are superinfected with a filamentous helper phage, activating the phage origin and the phagemid. The plasmid DNA creates single stranded DNA, which is secreted into phage particles. These particles contain a mix of recombinant phagemids and helper phage. The selection is usually done by β-galacto-sidase gene complementation and by antibiotic resistance.

Vector pairs that have the phage origin in opposite directions are available, and as a result single stranded DNA representing of both DNA strands are produced. This mixed single strand DNA population can be used directly for DNA sequencing, if the primer for initiating DNA synthesis is designed to bind specifically to sequences of phagemid adjacent to the cloning site [11, 30].

Both cosmids and phagemids are characterized as hybrid vectors.

6.2.6. Chromosome Bacterial Artificial (BAC)

A bacterial artificial chromosome (BAC) is a single copy bacterial vector based on a functional fertility plasmid (F-plasmid) of *E. coli*, which can accept very long inserts of DNA between 300-350kb and allows the maintenance of many structural characteristics of the native genome.

BAC vectors are superior to other bacterial system, due to the F factor, which has genes regulating its own replication and controlling its copy number. These regulatory genes are *oriS* and *repE*, mediating unidirectional replication and *parA* and *parB*, maintaining the copy number to one or two per cell. The cloning segment includes the lambda bacteriophage *cosN* and the P1 *loxP* sites; two cloning sites (*HindIII* and *BamHI*); and, several C+G rich restriction enzyme sites (*Not I, Eag I, Xma I, Sma I, Bgl I* and *Sfi I*) for potencial excision of the inserts. The cloning site is flanked by T7 and SP6 promoters for generating RNA probes for chromosome walking and for DNA sequencing of the inserted segment at the vector-insert junction. The

CosN and loxP sites provides convenient generation of ends that can be used for restriction-site mapping to arrange the clones in an ordered way [31].

Besides the maintenance of large DNA inserts, BAC has structural stability in the host, high cloning efficiency and easy manipulation of cloned DNA, being largely utilized for construction of DNA libraries from complex genomes and subsequent rapid analysis of complex genome structure [31].

For recombination with DNA inserts, after enzymatic digestion DNA ligase are used. Transformed suitable *E. coli* was carried out by electroporation, and the competent cells are cultivated first with gentle shaking on liquid medium and then spreading to LB plates. The selection of recombined cells is done by hybridization procedures.

6.2.7. Animal virus

Viral vectors are commonly used to deliver genetic material into cells for gene therapy due to specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect. This process can be performed inside a living organism (*in vivo*) or in cell culture (*in vitro*), being frequently used to increase the frequency of cells expressing the transduced gene [32].

The first use of vector virus for cloning was based on simian virus 40 (SV40), a polyomavirus originated of rhesus macaque, being a potent DNA tumor virus infecting many types of mammal cells in culture. The SV40 genome is 5.2 kb in size and contains genes coding for proteins involved in viral DNA replication, and genes coding for viral capsid proteins. Due to packing limitations, cloning with SV40 involves replacing the existing genes with the foreign DNA [32-34].

The other kinds of virus used for mammals' gene clones are adenoviruses, papillomaviruses, adeno-associated virus, herpes simplex virus (HSV), poxvirus and more recently retroviruses.

Adenoviruses came to solve the size of insert drawback of SV40, enabling the cloning of DNA fragments up to 8kb. On the other hand, due to its larger genome, adenoviruses are difficult to handle. Expression can be transient and the *in vivo* transfection can be impaired due to immune response.

Papillomaviruses also have a high capacity for inserted DNA with the advantage of stable transformed cell line.

Adeno-associated virus has this name because it is often found in cells that are simultaneously infected with adenovirus. To complete the replication cycle the adeno-associated virus uses proteins already synthesized by adenovirus, which acts like a helper virus. Lack of helper virus made the genome of adeno-associated virus integrate to host DNA. The major advantage of this vector consist of a defined the insertion site, always in the same position, being important in researches that cloning gene needs rigorously check such as gene therapy.

The herpesviruses include infections human viruses as herpes simplex virus (HSV), most used like a vector. The HSV is an enveloped double-stranded DNA, with 152kb, having advantages

like larger foreign DNA carrying; high transduction efficiency and, potential to establish latency.

Poxvirus vectors are double-strand DNA with 200kb in the core and carrying up to 25kb o foreign DNA. Gene is stably integrated into the virus genome resulting in efficient replication and expression of biologically active molecules.

Many viruses kill their host cells by infection, so special artifices are needed if anything other than short-term transformation experiments is desirable. Bovine papillomavirus (BPV), which causes warts on cattle, is particularly attractive because they have an unusual infection cycle in mouse cells taking the form of a multicopy plasmid with about 100 molecules present per cell. This infection does not bring the death of cell and, BPV molecules are passed to daughter cells during mitosis.

The most used viral vectors are the retroviruses, infectious viruses that can integrate into transduced cells with high frequency, inserting the foreign DNA at random positions but, with great stability. They can be replicated-competent or replication-defective.

Replication-competent viral vectors contain all necessary genes for virion synthesis, and continue to propagate themselves once infection occurs. These vectors can integrate an inserted about 8–10 kb, limiting the introduction of many genomic sequences. This made replication-defective vectors the usual choice. These vectors had the coding regions replaced with other genes, or deleted. These viruses are capable of infecting their target cells but they fail to continue the typical lytic pathway that leads to cell lysis and death.

The viral genome in the form of RNA is reverse-transcribed when the virus enters the cell to produce DNA, which is then inserted into the genome at a random position by the viral integrase enzyme. The vector, now called provirus, remains in the genome and is passed on to the progeny of the cell when it divides. The site of integration is unpredictable, which can pose a problem; therefore, the principal drawback of retrovirus vectors involves the require-ment for cells to be actively dividing for transduction, being widely used in stem cells. Great examples to overcome this disadvantage are lentiviruses vectors.

The lentivirus is a subset of retrovirus with the ability to integrate into host chromosomes and to infect non-dividing cells. Lentivirus vector systems can include viruses of non-human origin (feline immunodeficiency virus, equine infectious anemia virus) as well as human viruses (HIV). And for safety reasons lentiviral vectors never carry the genes required for their replication, preventing the occurrence of a wildtype-potentially infectious virus [32-34].

6.2.8. Transposons

DNA transposons elements are natural genetic elements residing in the genome as repetitive sequences that move through a direct cut-and-paste mechanism. This process is independent of previously recognized mechanisms for the integration of DNA molecules and occurs without need of DNA sequence homology. Thus, they can be used as tools from transgenesis to functional genomics and gene therapy.

Transposons are organized by terminal inverted repeats (ITRs) embracing a gene encoding transposase necessary for relocation. Transposons move through a "cut-and-paste" mechanism, known as transposition, which involves excision from the DNA and subsequent integration into a new sequence environment [35, 36].

The development of transposable vectors is based on a plasmid system, with a helper plasmid (expressing the transposase) and a donor plasmid (with terminal repeat sequences embracing the foreign gene) [36].

6.3. Importance of promoters

The promoters are defined as *cis*-regulatory elements responsible for the control of transcriptional machinery and determination of its level and specificity, marking the point at which transcription of the gene should start, and regulating the transcription. Promoters contain proximal elements, involved in the formation of the transcription complex; and, major elements that give cell specificity of protein expression [37, 38].

For long term transgenic expression *in vivo* or tissue specific expression, the transcription of the foreign gene should be controlled for promoters, which in this case are inserted on cloning vectors [37].

Approaches requiring a high ubiquitous expression of the transgene can be accomplished with non-tissue specific promoters. These promoters are actives in almost all of cell types, ensuring the foreign gene expression in all organism tissues. Examples of these promoters are metallothionein gene promoter, EF1 gene promoter, CMV early gene promoter, human H2K gene promoter, 3-methylglutaryl CoA reductase gene promoters, and others.

On the other hand, to restrict transgene expression to the target tissue the promoters used are tissue-specific. These promoters can direct the transgene expression to lung, epithelia, liver (albumin gene promoter), pancreas (amylase promoter), muscles (truncated muscle creatine kinase - MCK), neural cells (synapsin 1), mammary gland and cardiac cells (troponin T promoter), and so on [38]. Promoters used in cloning vectors should be sufficiently short to be cloned in a gene transfer vector.

Besides the use of tissue-specific promoters, another kinds of promoters are the inducible ones, which transcription can be selectively activated. These promoters respond to specific transcriptional activators are: transcriptional activators regulated by small molecules; intracellular steroid hormone receptors; and, synthetic transcription factors in which dimerization is controlled by antibiotics.

The promoters for transcriptional activators regulated by small molecules are based on the use of transcription factors that change their conformation upon binding one small chemical molecule (*e.g.* Tet repressor – TetR). The promoters for intracellular steroid hormone receptors act when hormone analogs are ligated to the hormone's modified receptors. Synthetic transcription factors in which dimerization are ones that in the presence of antibiotics tethers the transcriptional activation [37, 38].

7. Practical application of genetic Engineering and cloning: From transgenic animal models until cloning animal

Transgenic animal technology and the ability to introduce functional genes into animals are powerful and dynamic tools of genetic engineering. The genetic engineering field allows stable introduction of exogenous genetic information into any live organism, enhancing existing or, introducing entirely novel characteristics. The cloning technology is closely related with transgenic, being used as a tool for genetic engineering of an animal.

Together these technologies can be used to dissect complex biological process, like *in vivo* study of gene function during development, organogenesis, aging, gene therapy, and epigenetics studies. Besides, there are a lot of commercial applications like, model for human diseases, pharmaceutical biotechnologies development, and reproduction of a valuable animal.

7.1. A sheep named Dolly: Cloning

In 1997, Wilmut and coworkers announced Dolly production, which was the first mammal cloned from adult cells. In this experiment Dolly was born after reconstruction of 277 embryos with mammary gland cells.

Her birth at 5 July 1996 in Scotland brought huge excitement of the scientific world, beginning a biological revolution. The fact of Dolly has been created from adult differentiated cells showed the possibility not imagined before: dedifferentiation of already committed somatic cells, which brings a lot of repercussion. After Dolly, the differentiated cells cloning was achieved in a lot of species like, bovines, murines, caprines, swines, felines and canines [39-46]

7.2. What is cloning?

The definition of clone consists in the reproduction of genetically identical organisms, naturally or artificially, by asexual reproduction (without spermatozoa). The word "clone" comes from the greek word "klon", that means twig. With these characteristics clone for some organisms is a physiological asexual way of reproduction (e.g. bacteria and yeast). This conception, after Dolly's production went further, becoming the production of genetically identical live organism through nuclear transfer techniques. This defines clone to a process in which cellular material from a DNA donor is transferred to an egg whose own DNA has been removed, resulting after some procedures in embryo genetically identical to DNA of original cell clone.

The origins of nuclear transfer remount discoveries with amphibians by Spemann (1938), who demonstrated that nuclei of newt salamanders are pluripotent up to eight-cell stage, leading intensive studies with nuclear transfer in *Rana pipiens* and *Xenopus laevis*, attempting to understand the nuclei participation of differentiated cells in reprogramming. Studies by Brings and King (1952) showed that amphibian oocytes receiving blastula nuclei could be reared to maturity [47].

During 60 and 70 decades, nuclear transfer was done mostly in amphibian, leading to clones production from intestinal larvae cells, being the first evidence that differentiated cells keep the potential to form all tissues of an organism. [48].

In mammals the first nuclear transfer studies were done in mice, in which Illmensee and Hoppe (1981) reported that this technique could be used to produce mice clones from embryo cells. In domestic animals, Willadsen (1986) published the first report with lamb clones production. This accomplish was confirmed after with bovines, rabbits, swine, and others. And, at 1996 Dolly brought the accomplishment of mammal nuclear transfer form adult cells [48].

Nowadays, a lot of cloned animals could be produced, which besides the commercial interest of reproducing some valuable animal, made the technique used for research like reprogramming mechanism and epigenetics controls.

7.3. Producing a clone: Technical

Technically to produce a clone from nuclear transfer the majority of protocols are based on these steps: preparation of cytoplasm receptor; oocyte enucleation; preparation of nuclei donor cells; embryo reconstruction; artificial activation; embryos culture; and, embryo transfer. Each of them will be briefly described below.

7.3.1. Methodology, advantages and disadvantages

Initially to produce a clone animal by nuclear transfer is necessary to prepare the receptor cytoplasm. The receptor cytoplasm is a cell which nuclei was removed by in a process known as enucleation, being the most used cell the female egg: the oocyte. The oocyte can be used in a lot of division estate being, actually used in metaphase II.

To obtain the oocyte at metaphase II, first, or they are aspirated from ovaries from slaughterhouses or by Ovum Pick Up, ultrasound guided (used for domestic animals), being obtained before metaphase II, needing *in vitro* maturation or, being collected already at this estate, after *in vivo* maturation (mostly used for laboratory animals).

When maturation is needed, the oocytes are recovered from antral follicles at prophase I estate or germinate vesicle, and are matured in temperature, medium and time specific, inside incubators, having the control of CO_2 tension. The two kinds of procedure to obtain a metaphase II oocyte, have its advantages and disadvantages. The *in vivo* maturation, are capable of better quality oocyte production, but depending on the species, it cannot be achieved, needing the *in vitro* maturation. The *in vitro* maturation besides not be the natural way of reproduction, brings good and quality results too, being largely used.

The second step consists of oocyte enucleation. Besides a lot of methodologies have been developed, the most used way to remove the nuclei from the oocyte is micromanipulation procedures.

Before, to prepare the oocytes for enucleation, the cumulus cells from the matured ones are removed, which can be done mechanically by a tube agitator (vortex); with pipettes sized as thin as the oocyte; or, chemically by hialuronidase. After, the oocytes are carefully selected,

based on the presence of the first polar body (checking if the metaphase II was achieved), and cytoplasm morphology.

The micromanipulation procedure for enucleation involves first fixing the mature oocyte by a holding pipete. To remove the nuclei, the first polar body is used to reference of the metaphysary plate (nuclei). At metaphase II the chromatin remains at the oocyte periphery, close to the polar body. The enucleation is done by an enucleation pipete with a bevel-shaped tip, which penetrates the pellucid zone (PZ) aspirating the first polar body e part of the cytoplasm attached to this structure.

Another enucleation method is the oocyte bisection [49]. In this case the PZ is removed and the oocytes are sectioned by a micro-blade in two halves, being removed 50% of the cytoplasm. The half with the nuclei is discarded and the other one used for nuclear transfer.

To check the efficiency of enucleation, the enucleated oocytes can be stained with DNA fluorescent dyes using in most of the cases Hoechst 33342 (H342), which need exposure of ultra-violet (UV) light to be verified.

This procedure depending on the time of exposure can compromise the oocytes viability. Trying to minimize this effect the exposure only of the removed cytoplasm and polar body to UV light can be done. The presence of chromatin in this material indicates the success of enucleation. There are protocols not involving fluorochromes like the use of demecolcin, incubating the oocytes 1-2 hours, in a medium containing this substance. This procedure creates a protrusion at oocyte membrane where the chromatin is localized [48].

The amount of cytoplasm removed at enucleation process direct interferes in embryos development rates. The less amount of cytoplasm removed, better rates of embryo development are achieved. Usually, the enucleation for bovines has an efficiency rate between 50-70% [46].

The third step is preparation of nuclei donor cells, which depends of the cell type and the technique used for nuclei transfer. The donor cells can be originated from embryonic, fetal or adult cells. When using embryonic cells, the PZ of embryos are removed by enzymatic digestion, acid solution or mechanically. The embryonic mass should be held on a calcium and magnesium free solution, facilitating the blastomeres disintegration. If the donor cell was fetal or adult mostly fibroblasts are used due to easy culture. A primary culture is done by a biopsy from a skin fragment. The cells are held on culture until the third passage, at least, due to homogeneity and specific cells reaching on the culture, more than the third passage cells can be used as nuclei donor too.

Besides a lot of experiments have been realized to determine which somatic cell type would be the most appropriated for cloning, until now is not yet known if some kind of cell are most advantageous for nuclear transfer [48].

The fourth step is embryo reconstruction consisting of place the nuclei from the donor cell inside the enucleated oocyte. This can be achieved by microinjection or membranes fusion, whereas the first has low results [46]. Using the fusion method with micromanipulators help, each cell is introduced at the perivitelline space of the enucleated oocyte. Then the fusion can

be done by electric pulses (electrofusion), liposomes, polyethylene glycol, or still, by inactivated viruses. The electrofusion are the mostly used. In this method, the complexes receptor-donor nuclei are positioned at electrofusion chamber, where they are submitted to two electric pulses with low conductance, preventing heat dispersion. These pulses induce the membrane fusion incorporating the cell donor nuclei at the receptor cytoplasm.

The fifth step consists of artificial activation, which involves degradation of enzymatic complexes responsible for oocytes kept at metaphase II, being needed for accomplish the meiotic process initiating the embryonic development. At physiologic conditions, this is achieved with spermatozoa. But at cloning process, without the spermatic cell, chemically or physical methods are used (ethanol, electric pulses, calcium ionophore and, strontium chloride).

The activation moment of oocyte in relation with the nuclear transfer moment have important consequences at the chromatin integration and remodeling; viability and, embryo development [48].

The sixth step consists of embryo culture, in which the reconstructed and activated embryos are cultivated at CO_2 incubators, until the blastocyst stage (species time dependent). The culture conditions are similar with *in vitro* fertilization conditions, whereas cloned embryos are more sensitive to cryopreservation, and do not pass through expansion phase when blastocyst stage are achieved. The PZ rupture, done by enucleation process, made the expansion estate coincide with hatch estate, and at this estate the embryos are transferred to synchronized female receptors, and after gestation and parturition or caesarean, cloned animals are produced [46].

7.4. What is a transgenic animal?

A transgenic animal consists of an animal whose genetic material has been altered using genetic engineering techniques. Foreign DNA is introduced into the animal, using recombinant DNA technology, and then must be transmitted through the germ line so that every cell, including germ cells, of the animal contains the same modified genetic material [32, 50].

S. N. Cohen and H. Boyer generated a functional organism that combined and replicated genetic information from different species, creating the first genetic modified organism in 1973. In 1974 R. Jaenisch created the first genetically modified animal by inserting a DNA virus into a mouse embryo showing inserted genes was present in every cell. However the mice did not transmit the transgene. In 1981 F. Ruddle, F. Constantini and E. Lacy injected purified DNA into a single-cell mouse embryo and showed transmission to subsequent generations. During the early eighties the technology used to generate genetically modified mice was improved into a tractable and reproducible method [51, 52].

7.5. Producing a transgenic: Technical

Currently, the three most widely used procedures for creating transgenic animals are micro-injection of the cloned gene(s) into the pronucleus of a fertilized egg, injection of recombinant

embryonic stem cells into embryos, and the use of retroviruses. There are other methods like sperm cells mediated gene transfer; *in vivo* gene transfer and ICSI-transgenes. These methods will be briefly discussed below.

7.5.1. Methodology, advantages and disadvantages

The microinjection of foreign DNA directly into the pronuclei of fertilized zygotes is the most extensively and successfully used method of gene transfer in the mouse. This method was the first non-viral method for transgenic animal production. The DNA microinjection to pronucleus has low technical progress, but was disseminated for other species (rabbit, swine and goats) [53, 54].

To produce a transgenic animal a lot of zygotes are needed which is achieved by female superovulation before mating. For mouse, rats and rabbits the one cell embryos are transparent, being opaque in swine, goats, sheeps, and cow, due to lipid presence. In case of opaque embryos they should be centrifuge before the microinjection for concentration of lipids at one embryo side, allowing the pronuclei visualization.

The disadvantages of this technique are due to exogenous DNA introduced at the pronucleus is strongly mitogenic, leading a lot of embryos microinjected to death. Another disadvantage consists of integration of foreign DNA in a random manner, being not possible to predict the integration site and control the number of copies of transgenic integrated DNA. The transgenic production by microinjection to pronucleus are 2% for mouse, 0,1-0,5% for pigs, 0,01-0,1% for sheeps and goats and lower for cows [54].

Another used methodology is infection by retroviruses, in which the transgenes can be introduced by viral infection of preimplantation embryos. Retroviruses have the natural ability to infect cells and integrate its genome to infected cells. The retroviruses are modified, in which some of its genes are substituted by a target gene. Then these reconstructed viruses are transferred for cells, that after infected synthesize viral protein, secreting viral particles which can infect embryonic cells, or primordial germ cells, originating transgenic animals.

The disadvantages came from biosecurity, since this technique works with recombined viruses. This determines biosecurity rules to be carefully followed, preventing this vector dissemination. One advantage is the use of this method is for gene therapy, transferring the modified viruses for somatic cells of the patients. And another advantage came with lentiviruses uses that are successfully used for gene transfer (*see item 5 for advantages and disadvantages of this virus*).

The gene transfer by injection of recombinant embryonic stem cells into embryos is one of the most useful when is necessary to select for rare integration events or when is necessary an chimeric animal production [53].

Embryonic stem cells (EST) are cell lineages obtained from initial embryos estate, like morula or blastocyst. These cells have pluripotency capacity, being capable of participation actively of all organism tissue production, including the gametes. The technique for gene transfer consists in transfect EST with exogenous DNA; and the ones transfected after been selected,

are introduced into embryos. The resulting animals are chimeric and mosaics for the transgene, and if the germ cells have integrated the transgene, this can be passed for the progeny.

The disadvantages of this methodology are the impossibility of chimeric animals from other species besides mouse; transmit the transgene to their progeny. Being an advantage when the transgene requires homolog recombination.

Another ways of transgenic animals production are being used like, the use of gametes cells to transgenic animal production are already achieved. The oocytes uses do not generated enough transgenic animals, being the spermatozoon most appropriated cells for transgenesis [54].

The Sperm-mediated gene transfer (SMGT) enables the production of transgenic animals by exploiting the ability of sperm cells to bind and internalize exogenous DNA. The SMGT has being an easy and low cost method for transgenic animals production, in which the simple incubation of the exogenous DNA with the sperm, follow by artificial insemination or IVF procedures, can result in an transgenic animal. Although, a lot of transgenic animals produced by this technique, there are high number of studies with low reproducibility and the reason for this are unknown.

To pass through this low reproducibility the spermatic cell are been used for integration of the new genetic material by intracytoplasmic sperm injection (ICSI-transgenesis), method largely and successfully used for mammalian species other than mouse [53].

The *in vivo* transfer of a transgene can be done by the injection of the exogenous DNA to testicles or still blood veins, bringing good results. The revolutionary of this method came with production of *in vitro* sperm stem cell, from many species. After cultivated this primordial sperm cells are transfected with the exogenous DNA, then the recipient animal are treated for decrease its physiological sperm production, and the transfected cells are injected trough testicle efferent duct. Thus, after the spermatogenesis this male can be used for transmission of transgenic sperms.

7.6. Transgenic animals such as experimental models

Genetically modified animals currently being developed can be different broad intended purpose of the genetic modification: to improve animal production; xenotransplantation; to produce proteins intended for human therapeutic use; to improve animals' interactions with humans trough hypo-allergenic pets; to improve animal health by disease resistance animals; and, to research human diseases with the development of animal models [50].

The animal models production are used in pathologies or syndromes caused by inactivation or dysfunction of a determinate gene, being possible to delineate strategies and prepare the equivalent genetic modification in a homologous gene of the animal [55].

The use of animal models to research human disease, are done because of similarity to humans genetics, anatomy, and physiology or for being easier to have a lot of conditions developed in many transgenic animals, manipulating just one variable each time, which corroborate with statistical analyses of the results.

Extensive research for human diseases have been done with rats, mice, gerbils, guinea pigs, and hamsters, being the mice the mostly used due to genomic similarities to human and easy and developed handle and production methods; low cost; and, high reproductive rates.

Although these advantages sometimes the small size of mice leads to challenges in the design and application of instrumentation for physiological measurements, being possible to introduce transgenes into larger mammals and also fish like genetically modified zebrafish. The advantages using model experimental organisms larger than mice are easier physiological assessments and besides, provide alternatives when manipulation of the mouse genome does not produce the phenotype one wishes to investigate. Examples of this situation are provided by the hypertensive response of rats but not mice to forced expression of the *REN-2* gene and the more severe spondyloarthropathy produced by *B27* and b2-microglobulin transgenes in rats [56].

Multiple models of diverse pathologies have been generated, like diabetes, obesity, allergy, cancer, cardiovascular disorders, hypertension, embryo development abnormalities and, reproductive system abnormalities [57].

Sometimes, complex animal models are needed, using coexistent modification of genes trough techniques like knockout, knock in and knock down. Another approach is the use of news genetic modification variants, like inducible expression of transgenes and restriction of transgene expression at some organs. These techniques can generate animal models that better fit some human pathology [57].

7.7. How to join cloning with genetic modification: Complementary biotechnologies

The cloning technology together with genetic modification of organism originates a new method for animal transgenesis production. Between different areas of application of nuclear transfer, the transgenesis has the major benefits with its advances. Besides laborious, this technique allowed more efficiency transgenic production than pronuclear microinjection for ruminant animals.

The advantages of this methodology for animal transgenesis production are introduce, functionally delete or subtly genes of interest; produce embryos expressing the transgene constitutively not mosaic or chimeric; or still, achieve genetic modifications directed. This last approach can be done by insertion of genes in a determined chromosome position.

The genes inactivation by the cloning technique for transgenic production, are achieved by substitution from homolog recombination, which largely obtained just in somatic cells. Examples of this approach in pigs was the substitution of β-galactosiltransferase, making its kidneys being resistance to hyperacute rejection in to experimental transplant in non-human primates [56].

Another importance of this approach consists of the applications of transgenic farm animals. The major opportunity of this use is biopharming, mostly produced at the milk of transgenic female animals, in which cells modified containing important human genes under a promoter for mammary gland control (*for more information of promoters see topic 5*). After milk secretion this therapeutic protein are purified and used for clinical trials to evaluate their safety and

effectiveness treating human diseases and disorders before gaining regulatory approval. Other tissues examples, used for antibodies production are eggs of chicken or blood of transgenic cattle [58].

8. Future Prospects — Genetic engineering and cloning: A dream or a nightmare?

8.1. Emphasis on cloning technology

A clone is an identical copy of the parental material, which is development. Clones from a same cell type will have the same genomic properties, but in multicellular organisms different cell behavior and phenotypic are observed by the influence of environment. Cloning is not in itself a genetic engineering technique as transgenic but both techniques are strongly correlated. Cloning naturally may occurs after a single insemination and if a specific gene is not inserted into the host genome the resulting animal cannot be considered a genetically modified organism, transgenic [59]

It is well established in literature that clone embryos have a lower total number of cells when compared to embryos not cloned. Cloned bovine embryos have approximately 9% less cell, and this rate is 19, 43 and 55% charge respectively for pigs, rabbits and mice cloned embryos and this rate being positively correlated with the difficulty of performing cloning [60]. The discovery of cell types that offer better rates cloning is essential to the development and better understanding of the epigenetic molecular mechanisms, nuclear programming and reprogramming; thus, developing more secure and efficient cloning techniques [61]. Use races which have highest cloning success rates as a model, Ex. Japanese Black cattle, could help to find the key points to solve currently problems as high mortality rate of cloned offspring. In 2003, a study compared the size of spermatozoon chromosome telomeres obtained from cloned and not cloned animals. For both groups was observed that telomere length was maintained throughout animals age, fact which indicated that cloned animals could be used as breeders [62].

After Dolly´s birth, one glimpses in human medicine to use cloning technologies in gene therapy for tissues and organ replacement without risk of transplant rejection, since the donor would be the patient himself. For some people, the ability to clone differentiated cell is regarded as the long awaited immortality or as an insult to religious principles. Nowadays cloning assumes a prominent role in the world media among the most controversial issues. However, if would possible to produce healthy cloned offspring with low mortality and without genotypic and phenotypic changes, why would clone be so controversial? It is important remember that similar process naturally occurs, once the medical literature shows that one in 250 births produces the identical twins, the current human clone.

Currently cloning technology is in emphasis on science and media, and over the last few years has made important advances. However, more knowledge is needed to this technique able to exercise your full potential as a biotechnology in combination with gene therapy and engineering gene.

8.2. Ethical aspects of genetic engineering: Risks and benefits

The generation of the genetic engineering and his major advances in biotechnology as: sequence of complete genome for different plants and animals, creation of transgenic and cloning had the participation of different scientists.

Mendel is considered the father of modern genetics. Their findings were published in 1865 and just after 50 years (1909) were considered by Wilhelm Johannsen who discovered the genes, which were called before as "elements" by Mendel. In 1953, James Watson and Francis Crick published in Nature the discovery of the helical structure of DNA. At 1973, the concept of genetic engineering was created by S. Cohen and H. Boyer who published the ability to "cut and paste" the genetic material. However, it was Van Rensselaer Potter the responsible for the term Bioethics through his book Bioethics: bridge to the future (1971), which was an answer for the numerous innovations in science. The advances in this field culminated in the announcement of the complete sequencing of the human genome, being followed by other species sequencing.

The discoveries and advances of genetic engineering brought an ethical dilemma. For large population, this science has turned into a dangerous knowledge because we are accumulating information faster than ability to manage them. This creates a conflict between ethical principles and moral norms. Ethics is the part of philosophy that studies the moral ideals and principles for human behavior. However, morality is grounded in obedience to customs and habits. Ethics is different from moral because it is based on moral actions (good and bad) generated by reason. Thus, the concept of bioethics is the applicability of ethics in biological science, its discoveries and advances.

Using these definitions, genetic engineering is not in its bioethical concept harmful to society. Fear of the unknown by lack of information and knowledge has made us to come back at ancestors response. The modern society create new myths and rites. However, with technological advances in genetic engineering the natural boundaries were lost. Now it is the duty of man to establish and determine these new barriers. Genetic engineering should be rather limited and controlled by new ethical and technical barriers.

With industrial development and new technologies we are living in a new era, the biotechnology era. With advances, especially in genetics, a new science called genetic engineering that enables the manipulation (insertion and removal) of genetic material was created. This technique can be applied since one cell organism (bacteria) until complex multicellular organisms such as farm animals and humans. The genetic engineering, as the industry, searches for new products (life forms) that have greater production efficiency.

The welfare and environmental concerns for genetic engineering were very well discussed by Fox, MW (1998)[63]. According to the author, bio processing industry proposes the production of new forms of energy, synthetic and pharmaceuticals products. However, many promises of innovation are accompanied by profound discussions regarding the risks and ethical concepts. These aspects should not only be discussed by governments and industries, but also for the entire population and researchers. Generally genetic manipulation of bacteria and plants, which are transformed into real machines for the production of biological products

such as hormones, is considered moral and ethical acceptable. This is explained because plants and bacteria not have the ability to Suffer, to experience pain and emotional distress. Thus, would it be ethically acceptable to use more complex organisms, such as farm animals, as biological models and bioreactors? Had genetic manipulation any effect on your body structure and physiology? If any error occurs during the process, is the birth of animals with anomalies acceptable?

A study conducted in the UK in 1997 by Frewer et al.[64] consider public concepts on application of specific and general genetic engineering. The main concepts related to rejection of genetic engineering were: personal objections, immoral, unnatural, unethical, harmful, personal worry, negative welfare effects, dangerous risk, tampering with nature and creation of inequalities. The positive aspects were: beneficial, advantageous, necessary and progressive. The data closely relate applicability of genetic engineering with risks and benefits that are defined by the nature of their application. The activities considered more negative were associated with genetic manipulation of animals and humans, confirming results previously obtained by previous researchers. The most important for the establishment of a concept is the applicability of the technology, most humans and animals that plants and microorganisms. The results imply that the public attitudes are defined by the process associated with genetic engineering rather the product of this process. Unnaturalness is one of the most important concepts associated with animal and human genetic material. The medical risk is high in benefice and low in risk and it is considered acceptable. However, non-medical applications are low in benefice and high in risk and it is unacceptable.

The complexity of public concepts about genetic engineering generated a hierarchical model for the dissemination of information that was proposed in 1990 by Hilgartner [65], which uses concepts of public debates to enter public opinion. Thus, the ethical and moral concepts of society are fundamental to be associated at genetic engineering to it exerts your complete function and optimal use. The public ethical concept must be considered particularly to genetic engineering involving animals and humans.

Moreover, models of diffusion of knowledge about risks and benefits of genetic engineering to society should be applied. Only with more information and more knowledge you can get over rampant enthusiasm and ideological fear. So some paradigms about genetic manipulation will be clarified. The biggest problem in genetic is not to make changes in the DNA, but to be faithful to a principle which are common to all men, of all cultures and responsible for perpetuating human and natural environment; therefore more important than any gene is ethics [66]. So, to finish this chapter there is no better phrase than: ethics is responsible to regulate and coordinate the genetic engineering, as also occurs in the others branchs of science.

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