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SPECIFICS OF DEVELOPMENT AND TESTING OF BIOLOGICAL ORIGIN PRODUCTS

Study aid

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Study aid "Specifics of Development and Testing of Biological Origin Productsprovides information on basic concepts, types of biological drugs and the role of biotechnology in their production, stages of development of biological drugs, methods of quality control of biological products. In addition, the peculiarities of GMO products are also considered. Regulatory requirements for products of biological origin were also considered. The training manual "Specifics of Development and Testing of Biological Origin Products" is intended for improving the qualifications of specialists in biosafety and bioprotection.

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CONTENTS

INTRODUCTION

Biological medicinal products contain active substances of biological origin (human, animal, microbiological). Biological medicinal products include immunological medicinal products (i.e., vaccines), medicinal products prepared from human blood or plasma, advanced therapy medicinal products (cell therapy, gene therapy, tissue engineering), medicinal products produced by biotechnology and other medicinal products produced from biological sources. Biological medicines represent an important milestone in the treatment or reduction of symptoms of certain diseases such as cancers, metabolic diseases, musculoskeletal disorders, growth disorders, chronic autoimmune inflammatory rheumatic diseases. These medicines contribute significantly to successful treatment and patient's quality of life. The development and especially the production of biological medicines is more demanding compared to the chemical active substance products and this is also reflected in the price of these drugs. As original biological medicines lose their market exclusivity, biosimilar medicines (biosimilars), which are the copies of already existing biological medicinal products and are cheaper to develop, can enter the market.

Biological products include a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins. Biologics can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances, or may be living entities such as cells and tissues. Biologics are isolated from a variety of natural sources – human, animal, or microorganism – and may be produced by biotechnology methods and other cutting-edge technologies. Gene-based and cellular biologics, for example, often are at the forefront of biomedical research, and may be used to treat a variety of medical conditions for which no other treatments are available.

How do biological products differ from conventional drugs? In contrast to most drugs that are chemically synthesized and their structure is known, most biologics are complex mixtures that are not easily identified or characterized. Biological products, including those manufactured by biotechnology, tend to be heat sensitive and

susceptible to microbial contamination. Therefore, it is necessary to use aseptic principles from initial manufacturing steps, which is also in contrast to most conventional drugs.

Biological products often represent the cutting-edge of biomedical research and, in time, may offer the most effective means to treat a variety of medical illnesses and conditions that presently have no other treatments available.

CHAPTER 1. MODERN APPROACHES TO THE CREATION OF BIOLOGICAL PRODUCTS USING RECOMBINANT TECHNOLOGIES

Proteins are one of the most important classes of biological macromolecules and are involved in almost all cellular processes. Therefore, a significant amount of research in medicine is directed at the use of proteins as therapeutic drugs and diagnostic tools for diseases. Biopharmaceuticals in widespread use include blood proteins (for example, to treat hemophilia), thrombolytic enzymes, hormones, cytokines and growth factors, immune system proteins (interferons and antibodies used to treat infectious diseases and some cancers), and vaccines. In view of the widespread use of protein preparations, there is a growing interest in the development of effective and large-scale methods of their production. Currently, there are many methods of obtaining biologically active protein products: chemical synthesis, extraction from biological raw materials, synthesis using microorganisms. However, the use of these methods is not always efficient from an economic point of view and is not profitable for large-scale production. The use of biological raw materials of human and animal origin is not only ineffective from the point of view of technical and economic indicators, but may even be dangerous from the point of view of viral safety.

In 1973, Stanley Cohen and Herbert Boyer and colleagues developed a method of transferring genetic information from one organism to another [1]. This method, called recombinant DNA technology, allowed scientists to isolate specific genes and introduce them into the body of a new host. This new technology made it possible to create producers for the large-scale synthesis of many biologically active drugs (cytokines, hormones, enzymes, thrombolytics, anticoagulants, monoclonal antibodies, and vaccines), as well as to obtain drugs with improved properties (increased physiological activity, reduced likelihood of adverse reactions after administration and so on).

1.1. Biotechnological approaches to the creation of a producer strain for the production of recombinant drugs.

1.1.1. Justification of the producer's choice.

Referring to the modern achievements of molecular biology and biotechnology, the most optimal solution in the development of technology for obtaining biologically active substances is the creation of a recombinant producer for their synthesis. For more than 40 years since the discovery of recombinant DNA technology, scientists have created many recombinant expression systems of various biologically active substances based on prokaryotic and eukaryotic producers. Specific examples of which are described in detail in Table 1.

Table 1. Expression systems used for the synthesis of recombinant drugs [1-25]

Producer	**Vector**	**Regulatory sequence**	**Marker gene**	**Advantages of this system**	**Disadvantages of this system**
Prokaryotic systems					
Bacterial cells	Plasmid, virus, phage or cosmid	T7 RNA polymerase promoter (pT7), TAS promoter (pTAS), Trp promoter, Lac promoter, Tre promoter, PhoA promoter	Antibiotic resistance gene	A cost-effective and fast way to obtain many proteins. *Escherichia coli (E. coli)* is the most thoroughly studied organism compared to others, which makes it easier to work with it. High yields of target protein Economically beneficial and fast way	In most cases, the target protein is processed in the form of inclusion bodies in a chaotically unfolded form, which requires additional technological stages Requires consideration of virus security requirements when using a virus vector system.
Eukaryotic systems					
Yeast cells	Episomal or plasmid	Oxidase promoter I	Antibiotic resistance gene	Development of protein in	The target protein is

	vectors, integrating vectors, artificial yeast chromosomes			secreted form	processed in a glycosylated form, but the type of glycosylation does not always correspond to the human
Insect cell cultures	Baculovirus vector expression system	OrIE2 actin promoter	Zeocin resistance gene	Development of protein in secreted form	Precious media for cultivating the producer, Significant duration of the process of development of the target protein
Mammalian cell cultures	Plasmid vectors pC R, pcDNA-hPS, adenoviral vectors	Viral promoters, internal gene promoters and hybrid promoters	Selective marker genes encoding the synthesis of neomycin phosphotransferase, dihydrofolate reductase or glutamine synthetase	The target protein is humanized, The degree of purity of the obtained protein is much higher than that of the protein obtained using expression systems based on prokaryotic and yeast cells	High cost of developing the target protein Requires consideration of virus security requirements

Bacterial, yeast cells, plant, insect, animal and human cell cultures are used as a producer for the synthesis of recombinant drugs. To transfer the genetic sequence encoding the synthesis of the desired product to the organism of the producer, a transport system is needed, which uses vector DNA. A vector can be a plasmid, virus, phage, cosmid, or episome. The vector usually contains regulatory elements or sequences to control the level of expression. The regulatory sequence can be selected from promoters, enhancers, silencers, tissue-specific signals, peptide signals, introns, terminators, polyA sequences, or combinations thereof. These regulatory elements can be derived from animal, plant, bacterial, yeast, bacteriophage or viral genetic

material. The vector may contain not one promoter, but several. The vector must contain a replication start site and a marker gene [1, 2, 3].

The choice of an expression system depends on the given production goals. The nature of the biological system is extremely important for the biotechnological process. The main goal of gene cloning research, which is expected to be used in biotechnology, is the selection of conditions for effective expression in the desired host organism. Unfortunately, the very fact of embedding a particular gene into a cloning vector does not mean that this gene will be expressed. At the same time, in order for the production of the product to be economically justified, the level of its synthesis must be quite high. Many specific vectors have already been designed to achieve efficient expression; for this, manipulations are carried out with a number of genetic elements that control transcription and translation processes, protein stability, secretion of products from the host cell, etc.

Among the molecular and biological properties of expression systems, the most important are the following [3, 4]:

- type of transcription promoter and terminator;
- the strength of mRNA binding to the ribosome;
- the number of copies of the cloned gene and its localization (in the plasmid or in the chromosome of the host cell);
- final localization of the synthesized product;
- effectiveness of translation in the host's organism;
- stability of the product in the host cell.

There is no universal strategy for optimizing the expression of cloned genes. Most of such genes have unique molecular properties, and optimal expression systems for each of them have to be selected individually. The effectiveness of the expression of any foreign gene also depends on its affinity with the host organism. Despite the fact that many representatives of both pro- and eukaryotic organisms are capable of expressing foreign genes, *Escherichia coli* is mainly used to obtain important products for therapeutic and diagnostic purposes using recombinant DNA technology. This is primarily due to the fact that the genetic, molecular biological,

biochemical and physiological properties of this microorganism have been studied in detail. In addition, it is the cheapest and fastest way to obtain most proteins. But for the expression of some cloned genes, other host organisms are also used: Vacilius subtilis, yeast cells, mammalian cells, and plants, etc., although the strategies developed for *E. coli* systems are, in principle, used in these cases as well [5, 6, 7].

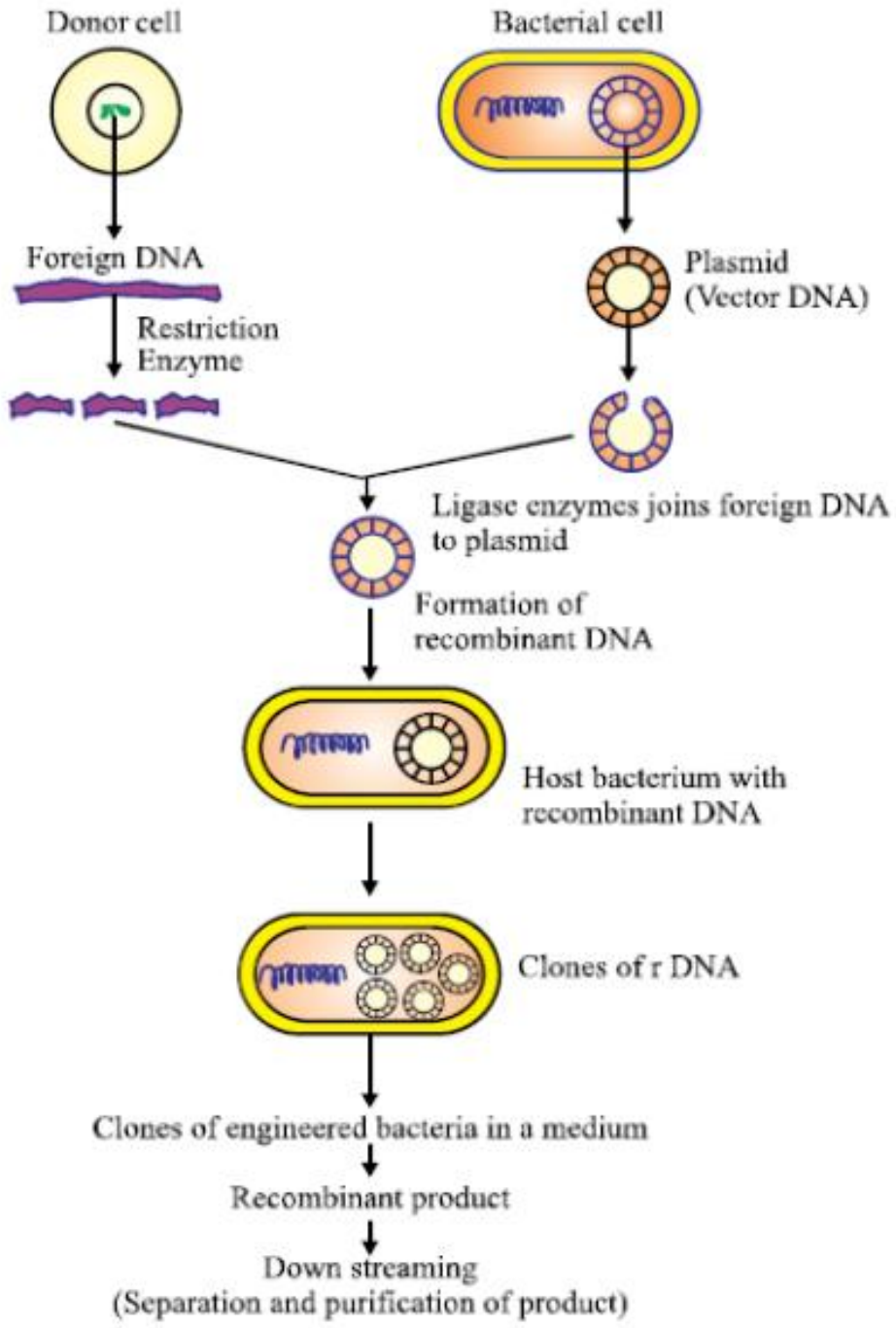

Figure 1.1. Schematic of the creation of a recombinant producer.

For all the differences between the types of eukaryotic organisms, the methodological approaches to culturing insect, plant, and mammalian cells have much in common. First, a particle of the necessary tissue of this organism is taken and subjected to processing with proteolytic enzymes that break down the proteins of the intercellular material. The released cells are placed in a complex nutrient medium containing amino acids, antibiotics, vitamins, salts, glucose, hormones and growth factors. Under these conditions, cells divide until a cell monolayer forms on the walls of the culture vessel. If after that the cells are not transferred to containers with fresh nutrient medium, growth will stop. It is usually possible to transfer and maintain up to 50-100 cell generations of the original (primary) cell culture, then the cells begin to lose the ability to divide and die. Cultured cells retain some properties of the original cell material, so they can be used to study the biochemical properties of various tissues [8].

Often, some cells of recultured primary cell cultures undergo genetic changes, as a result of which their growth is accelerated. Cell cultures that acquire selective advantages at the same time are capable of unlimited growth in vitro and are called stable cell lines. Some cell lines retain the basic biochemical properties of the original cells, others do not. In most cells capable of unlimited growth, there are significant chromosomal changes, in particular, there is an increase in the number of some chromosomes and the loss of others. In molecular biotechnology, stable cell lines are sometimes used to propagate viruses and to detect proteins encoded by cloned DNA sequences. In addition, they are used for large-scale production of vaccines and recombinant proteins [8].

To obtain heterologous recombinant proteins from cloned eukaryotic complementary DNA (cDNA), prokaryotic expression systems are usually used. However, in some cases, eukaryotic proteins synthesized in bacteria are unstable or biologically inactive. In addition, no matter how carefully the cleaning is carried out, the final product can be contaminated with toxic substances or substances that cause an increase in temperature in humans and animals (pyrogens). Protein preparations used for therapeutic purposes must meet the following quality indicators and

acceptance criteria: antigenicity, immunogenicity, purity, pyrogenicity, absence of bacterial endotoxins, biological activity and stability. To solve these problems, eukaryotic expression systems were developed to obtain recombinant proteins intended for therapeutic use. Which make it possible to obtain proteins identical to natural ones in terms of their biochemical, physical and functional properties. The inability of prokaryotes to synthesize authentic protein variants is mainly due to their lack of adequate mechanisms for introducing specific post-translational modifications [9-12].

Proteins in eukaryotic cells undergo the following post-translational modifications:

- formation of disulfide bonds. This reaction is catalyzed by the enzyme disulfide isomerase. An incorrectly folded protein is unstable and inactive;
- proteolytic cleavage of the precursor, removal of a certain section of the polypeptide chain with the formation of a functionally active protein;
- glycosylation is the main modification thanks to which proteins acquire stability, and in some cases - special properties. The most common reaction of glycolysis is the attachment of a specific sugar residue either to serine or threonine (O-glycosylation) or to asparagine (N-glycosylation);
- modifications of amino acids in the protein composition: phosphorylation, acetylation, acylation, gamma-carboxylation.

Of all these modifications, prokaryotic host cells are the least capable of performing the correct glycosylation and modification of specific amino acids of the heterologous protein. However, no prokaryotic system can carry out simultaneously all post-translational modifications in every potential heterologous protein. Thus, to obtain a protein with a complete set of specific modifications, it is necessary to test different eukaryotic expression systems and find one that would reproduce a biologically authentic product [13].

Eukaryotic expression vectors have the same structure as their prokaryotic counterparts and must contain: a eukaryotic selectable marker, a eukaryotic promoter,

appropriate eukaryotic transcription and translation termination sites, and an mRNA polyadenylation signal.

If the vector is a plasmid that replicates independently of the chromosome, then it must contain a replication initiation site that functions in the host cell. If the vector is intended for integration into the host's chromosomal DNA, then to ensure recombination it must carry a sequence complementary to a specific region of the host's chromosomal DNA (chromosomal integration site). Because many recombinant DNA operations are technically more difficult to perform in eukaryotic than prokaryotic cells, most eukaryotic vectors are designed as shuttles. In other words, these vectors carry two types of translation initiation sites and two types of selectable marker genes, one of which functions in Escherichia coli and the other in eukaryotic host cells. Such vector expression systems have been developed for yeast, insect and mammalian cells [14, 15].

A baculovirus expression system is usually used to create recombinant cells based on insect cell cultures. Baculoviruses infect only invertebrates, including many insects. During the infectious process, two of their forms are formed. One is represented by individual virions that are released from an infected host cell, usually cells of the midgut, and are able to infect other cells of this organ. The second consists of many virions enclosed in a protein matrix. The protein of this matrix is called a polyhedron, and the structure itself is a polyhedron. Synthesis of polyhedrin begins 36-48 hours after infection and continues for 4-5 days until the infected cells lyse and the host organism dies. After that, many such particles are released and enter the environment, where the protein matrix protects them from inactivation. If a susceptible host organism absorbs such a particle, the polyhedrin is solubilized and virions are released, capable of initiating a new infectious cycle [16].

The polyhedrin gene promoter is extremely strong, and the virus development cycle does not depend on the presence of the gene itself. Therefore, the replacement of the latter gene with a foreign protein followed by inoculation with the recombinant baculovirus obtained from an insect cell culture can lead to the synthesis of a large amount of heterologous protein, which, due to the similarity of post-translational

modification systems in insects and mammals, will be close (and possibly identical) to the native form of that protein, which must be obtained. Based on this, vectors were developed on the basis of baculoviruses for the expression of genes encoding proteins of mammals and animal viruses [17].

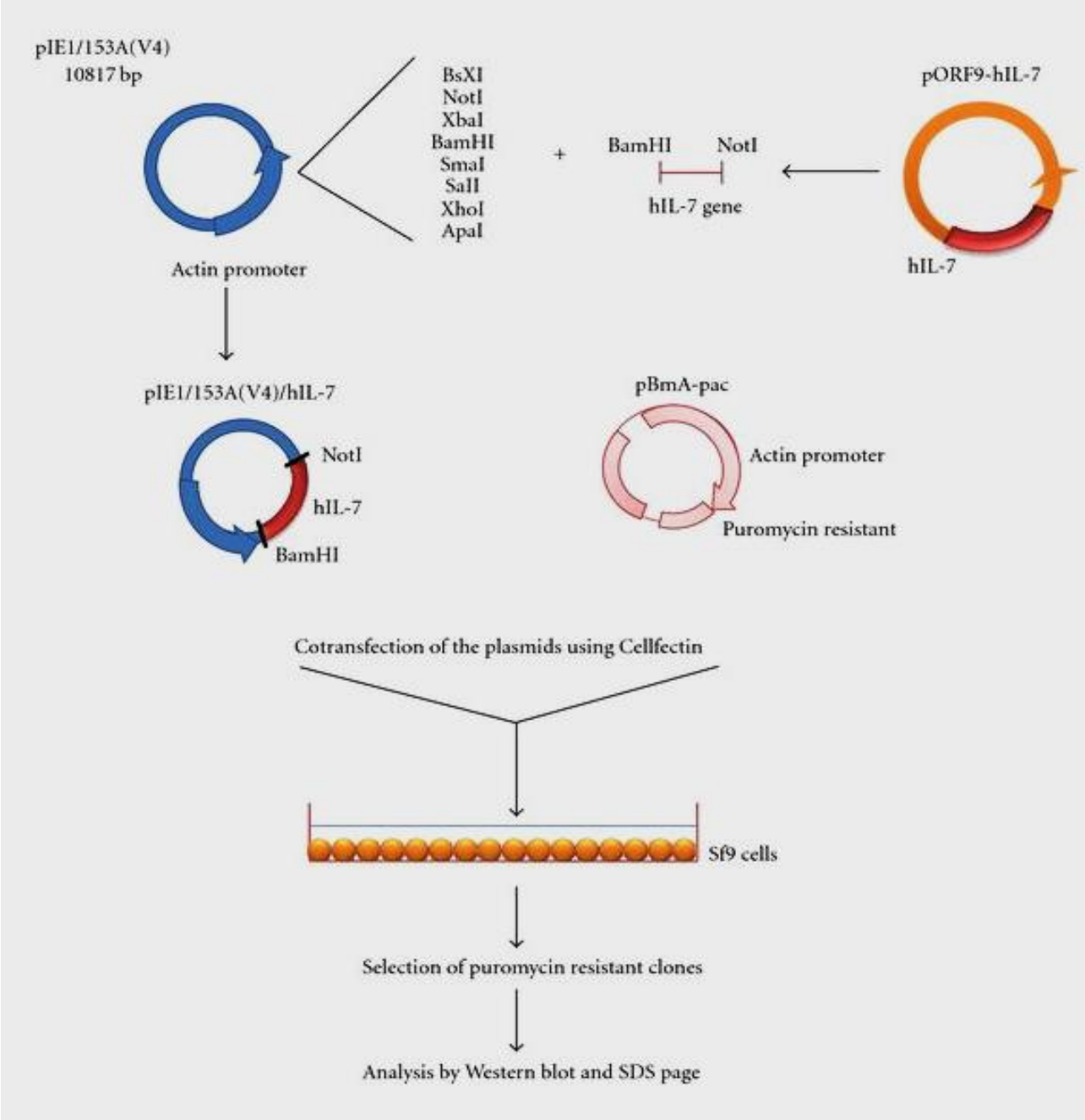

Figure 1.2. Creation of an expression system based on a baculovirus vector (BEVS) in *Spodoptera frugiperdera* cells (Sf9 cells) [18]

Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) is the most widely used. This baculovirus infects more than 30 other insect species and also grows well in culture in many cell lines. The cell lines commonly used to work with recombinant AcMNPV are derived from the gooseberry *Spodoptera frugiperda*. The polyhedrin promoter in these cells is extremely active, and when they are infected with wild-type baculoviruses, large amounts of protein are synthesized [18-21].

Extrachromosomal mammalian expression vectors are used to produce authentic recombinant proteins in mammalian cells that can potentially be used medically to treat human diseases. Some commercially valuable proteins in their active form consist of different polypeptide chains. For example, human thyroid-stimulating hormone is a heterodimer, and hemoglobin is a tetramer consisting of two subunits, two copies each (α2β2). To obtain an active multimeric protein, one can try to clone the gene or cDNA of each of the subunits, synthesize and purify the subunits, and then mix them in a test tube. However, it is possible to obtain only some multimeric proteins in this way, since the correct assembly of polypeptide chains *in vitro* is rarely carried out. The assembly of dimerized and tetrameric proteins *in vivo* proceeds quite efficiently. Therefore, strategies for the synthesis of two different recombinant proteins in one cell were developed. For this, host cells are simultaneously transfected with two mammalian expression vectors, each of which carried a gene or cDNA of one of the subunits and different genes of selective markers [22].

Two-vector systems have been successfully used to synthesize authentic dimerized and tetrameric recombinant proteins. Unfortunately, doubly transfected cells often lose one of the two vectors. In addition, the number of copies of each of the vectors is not always the same, so that one subunit may be synthesized in greater quantities than the other, and the yield of the final product may decrease. To solve these problems, vectors containing both cloned genes were constructed. In some cases, they have been placed under the control of independent promoters and polyadenylation signals. And in order to guarantee the synthesis of recombinant proteins in the same amount, so-called two-cistronic vectors were created, in which

the cloned genes shared a DNA segment containing an internal ribosome binding site. Such sites have been found in the genomes of mammalian viruses; they provide simultaneous translation of various proteins with polycistronic mRNA. Transcription of the "gene-internal ribosome-gene binding site" construct is regulated by one promoter and one polyadenylation signal. One transcript with two genes is synthesized, translation starts from the 5'-end of the mRNA and from the internal site, as a result, both subunits of the dimeric protein α and β are synthesized [23, 24].

In summary, mammalian expression vectors are as versatile and effective as vectors for other eukaryotic expression systems when it comes to obtaining authentic recombinant proteins for therapeutic and diagnostic purposes. However, industrial synthesis of recombinant proteins using modified mammalian cells is too expensive. In this case, less expensive expression systems are preferred, except in those situations where the authenticity of the recombinant protein can only be achieved using mammalian cell culture [25].

1.1.2. Fermentation and methods of induction of synthesis of recombinant drugs.

Depending on which system was chosen for the expression of the target protein, the selection of the strategy for the further technological stages of the production of recombinant drugs is carried out.

The fermentation process consists of two stages:

- receiving the inoculum;
- induction of target protein synthesis.

The inoculum is obtained by growing the producer on a medium that is optimal for growing the selected type of cells. Bacterial cells usually grow well on media with a simple composition containing Na^+, K^+, Mg^+, NH_4^+, Cl^-, HPO_4^{2-}, SO_4^{2-} ions, trace elements and a carbon source (for example, glucose). For the cultivation of bacterial producers of recombinant drugs, it is possible to use one of the media with a standard composition, which is usually used for growing bacteria, for example:

- Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract and 1 % NaCl);
- 2YT (1.7 % bactotryptone, 1 % yeast extract and 0.5 % $MgSO_4$);
- SOC (2 % bactotryptone, 0.55 % yeast extract, 1 % NaCl and 0.25 % KCl).

A carbon source – glucose and an antibiotic for selection of the transformed strain must be added to the standard media [26].

In this environment, the producer is usually incubated at a temperature of 36-38 °C, at a speed of rotation from 100 to 160 rpm. within 2-3 hours until the culture reaches the middle of the exponential phase of growth, which is determined by the culture liquid reaching a density of 0.6-1.0 OD_{590} [27, 28].

Nutrient media for growing yeast cells also have a fairly simple composition: molasses, malt extract, yeast wort with the addition of starch-containing products and sources of potassium, calcium, magnesium and iron. An antibiotic must be added to the medium for selection of transformed strains.

Cells of insects and mammals are grown in special media in the form of a monolayer on glass or in the form of suspension cultures using a wide variety of vessels-hemostats, fermenters, vials.

Cultivation of cells is carried out at a specified temperature (37 °C) and pH of the medium (6.8-7.5). The main components of media for culture are: mineral salts, amino acids, vitamins, antibiotics. According to their purpose, all environments are divided into growth and support. The composition of growth media should contain more nutrients to ensure active reproduction of cells to form a monolayer on the glass surface or a sufficiently high concentration of cellular elements in suspension (when obtaining suspension cultures). The supporting media should actually only ensure the survival of the cells in the already formed monolayer.

Their composition of growth and supporting nutrient media can include both natural products (amniotic fluids, animal serums) and substrates obtained as a result of partial processing of natural products (embryonic extracts, lactalbumin hydrolyzate, hemohydrolyzate, aminopeptide, etc.), and as well as synthetic chemically pure substances (amino acids, vitamins, salts).

As an example of a nutrient medium consisting entirely of natural components, we can name Buckley's medium, proposed for growing cell cultures. This medium includes cow amniotic fluid (85 %), horse serum (10 %) and cow embryo extract (5 %).

The components of nutrient media of natural origin cannot always be standardized, so their use is not always fully reproducible and is associated with a great danger of microbial and viral contamination of cell cultures. In connection with this, their gradual displacement by synthetic standard mixtures is taking place. The following synthetic media are most commonly used: 199 and Eagle's medium. Media containing strictly defined amounts of salts, amino acids and vitamins are widely used.

Regardless of the purpose, all nutrient media for tissue cultures are composed on the basis of some balanced salt solution with a sufficient buffer capacity. Most often, such solutions are Hanks' and Earl's. These solutions are a mandatory component of any nutrient medium for the cultivation of mammalian cell cultures. Animal serum (calf, bull, horse) is an integral component of most growth media, without 5-10 % of which cell reproduction and monolayer formation do not occur.

At the same time, the inclusion of serum prevents the creation of growth media of precise chemical composition, which is very important for the development of fundamental research in cellular physiology, since together with serum (or its derivatives) a whole complex of uncontrolled factors is introduced, which vary depending on the series of serum. Therefore, serum-free media of precise chemical composition were proposed. However, these media did not provide those indicators of proliferative activity of cells, which determine media with the addition of serums. To ensure the intensive growth of cells in serum-free media, the inclusion of sulfuric acid salts of Fe, Zn, Cu, as well as Mn is decisive.

Antibiotics must be added to the nutrient media, as well as to the buffer solution for washing tissues.

Induction of the synthesis of recombinant drugs can be carried out by two methods:

- addition of the chemical inducer Isopropyl-β-D-1-thiogalactopyranoside (IPTG) in a concentration from 0.1 mM to 2 mM;
- fermentation on a medium for autoinduction with lactose.

From a technical and economic point of view, the best technological solution would be method 2, as this approach significantly reduces the cost of the fermentation process. According to literature data, to ensure the process of autoinduction, with the output of the target product greater than in the case of IPTG induction, the following additional components should be included in the fermentation medium: $(NH_4)_2SO_4$, or NH_4Cl; KH_2PO_4, Na_2HPO_4, D-maltose, $MgSO_4$, glycerol, glucose, α-lactose, chloramphenicol, $NaHCO_3$, it is also possible to add trace metals (iron, manganese, cobalt, zinc, nickel, copper and molybdenum in the form of salts). The seed material is inoculated onto the medium of this composition in the ratio, and grown at the optimal temperature, with constant stirring for 16-22 hours, after which it is centrifuged, the biomass is collected and stored at a temperature of -20 ºC [29, 30, 31].

1.1.3. Isolation of recombinant proteins

In order to purify the recombinant product from the proteins of the producer cells, it is necessary to analyze the localization of the target protein in them. In bacterial expression systems, the target protein is usually produced in cells in the form of insoluble biologically inactive inclusion bodies. In this case, the further technological process includes the following stages [32]:

- destruction of cells and release of inclusion bodies,
- washing and centrifugation of inclusion bodies,
- renaturation, i.e. dissolution of inclusion bodies,
- refolding, accompanied by the formation of properly closed disulfide bonds, during which the protein acquires its native conformation

To isolate inclusion bodies, in which the synthesized recombinant product is located, it is first necessary to destroy the cell wall of the producer. Various chemical,

biological and physical methods are used to destroy cells. All procedures should be tough enough to destroy the cell wall, and at the same time gentle enough to exclude protein denaturation. In gram-negative bacteria, the cell wall is thinner and is covered with a layer of lipids on the outside.

Chemical methods of destroying cell walls include treatment with alkali, organic solvents or detergents. If the protein product is not destroyed at a pH of 10.5 to 12.5, large numbers of bacterial cells can be lysed quite effectively.

Treatment with organic solvents is a simple and inexpensive way of destroying cells, which is used to extract enzymes from microorganisms. However, to make sure that the protein product does not denature under the selected conditions, preliminary testing is usually carried out. Under the action of detergents, pores are formed in the membranes of bacterial cells through which proteins and other molecules leave the cells. Unfortunately, the use of detergents worsens the technical and economic characteristics of technologies, in most cases, proteins are denatured in their presence, and in addition, they can contaminate the final product.

The main biological method of destroying cells of microorganisms is lysis with the help of enzymes. Thus, egg white lysozyme easily hydrolyzes the cell walls of gram-positive bacteria. Lysozyme and ethylenediaminetetraacetic acid (EDTA) are used to destroy the cell walls of gram-negative bacteria. Enzymatic processing is highly specific, and lysis takes place in mild conditions.

Cells can also be destroyed by physical methods: non-mechanical (for example, using osmotic shock or rapid multiple freezing and thawing) or mechanical (using ultrasound, using a layer mill, homogenization under pressure). On the contrary, mechanical destruction is highly efficient, which makes it more attractive.

Physical non-mechanical methods, such as the method of repeated freezing and thawing, are not effective enough, since many cells remain intact after this procedure. and long-term.

Physical methods using ultrasound or a layer mill are quite effective in terms of cell destruction, but during treatment of the cell mass with these methods can lead to its heating, which can cause protein denaturation, and organize a procedure for

cooling the cell suspension during ultrasound treatment in large volumes rather problematic, therefore the use of these methods for industrial production is impractical [33].

For industrial production, in our opinion, the most appropriate in this case is the biochemical method of destroying cells with lysozyme and ethylenediaminetetraacetic acid (EDTA), followed by the addition of DNAse to destroy cellular DNA, and washing in a series of buffers containing detergents such as ethylenediaminetetraacetic acid (EDTA), triton X-100 and sodium deoxycholate to improve cleaning and subsequent dissolution of inclusion bodies [33, 16].

The next stage of the technology is the procedure for dissolving inclusion bodies, where recombinant proteins are in an aggregated, inactive form. The technological process of obtaining a recombinant protein may include dissolving inclusion bodies in the presence of a chaotropic agent (for example, high concentrations of urea, guanidine hydrochloride or thiocyanate salts) or detergents (sodium dodecyl sulfate, cetyltriethylammonium bromide, N-laurylsarcosine, Tween-80). If necessary, reducing agents (2-mercaptoethanol, dithiothreitol or cysteine) are added to the solubilizing buffer. To remove metal ions that lead to unwanted oxidation of thiol groups of proteins, chelating reagents such as EDTA in a concentration of 1 to 10 mM can be added to the composition of the solubilizing buffer. In addition, alkaline or acidic buffer solutions are used to dissolve inclusion bodies in combination with the above conditions [34, 35].

According to literature data, with an 8M solution of urea as a chaotropic agent, a large percentage of foreign impurities remains undissolved, which facilitates further cleaning [34].

The next stage, refolding technology (removal of the denaturing agent from the solubilization buffer) of the obtained protein. For this, it is possible to use several approaches, such as dilution, dialysis, diafiltration, as well as chromatographic methods.

When diluting, the renaturing buffer is usually introduced directly into the solubilized protein solution. In our opinion, this method has many disadvantages,

which consist in the need for subsequent concentration, as well as in the high yield of incorrect folding products, so this method is not used in industry.

Dialysis techniques are based on the relatively slow removal of the solubilizing reagent through a membrane with a fixed pore size. Since equilibrium in the system is established for a long time, this process is rarely used on an industrial scale. In addition, upon reaching intermediate concentrations of denaturing agents, protein aggregation is possible [34].

For industrial purposes, the diafiltration method is more widely used. With this approach, the removal of the denaturing agent is not limited by the speed of the diffusion process, but significant levels of accumulation of denatured protein on the membrane limit the widespread use of this method.

On an industrial scale, chromatographic methods such as metal chelating, ion exchange, reversed-phase chromatography, and gel filtration are usually used. The advantage of chromatographic methods lies in the possibility of carrying out the process in conditions where the protein is in a denatured state.

Gel filtration is a fast and efficient method that is widely used in industry. Usually, buffers containing amino acids (glycine, arginine), detergents in low concentrations (0.1-0.5 % NP-40, 0.005-0.1 % Tween-20), as well as certain anions are used for refolding by the gel filtration method. (phosphates, sulfates). These reagents actually do not affect the rate of refolding, but significantly reduce the probability of formation of aggregates, taking part in the destabilization of intermolecular and hydrophobic interactions [34, 36].

At the next stage, it is necessary to add an oxidizing / reducing pair of low-molecular-weight thiols to the obtained protein to form disulfide bonds, that is, to carry out the renaturation procedure. For example: reduced / oxidized glutathione, dithiotrietol / oxidized glutathione, dithioerythritol / oxidized glutathione, or cysteine / cystine. Recommended concentrations for the formation of disulfide bonds are 1-10 mM of reduced thiol and the ratio of reduced and oxidized forms is from 10:1 to 3:1 [34, 37].

If the product is processed in soluble form, in the culture medium, then the medium is concentrated, and the protein is subjected to further purification. If the product is a low-molecular compound found in the culture medium, then appropriate extraction methods are used. Finally, if the product has an intracellular localization, then before purifying it, the cells are destroyed, using one of the methods described above.

1.1.4. Purification of recombinant proteins

The further purification procedure consists in sequential chromatographic purification, using anion and cation exchange sorbents. For the first stage of purification, it is possible to use Q sepharose (strong anion exchanger) in combination with Diethylethanolamine (DEAE) sepharose (weak anion exchanger) – at this stage, the main purification from all foreign impurities is carried out, the second stage – purification on a column with a strong cation exchange sorbent in order to replace buffer with buffer for the finished dosage form [25, 37].

Different approaches can be used to isolate specific heterologous proteins from cell extracts and from mixtures of secreted proteins. One of them is based on joining to the cloned gene – without breaking the reading frame – a segment of DNA encoding a short amino acid sequence that specifically binds to some chemical element, compound or macromolecule. Such a construction is built into the expressing vector between the promoter and the transcription termination site. A short amino acid sequence in the recombinant protein synthesized in the host cell acts as an affinity tag. In one case, a DNA segment encoding six histidine residues (His6), a spacer region encoding seven amino acids, and a proteinase cleavage site of six amino acids was inserted before the cloned gene without breaking the reading frame; the obtained recombinant protein is purified by chromatography on a nickel-agarose column. A sequence of six histidine residues (hexahistidine) was bound to nickel ions, and the recombinant protein was retained in the column. It was eluted by adding a competing compound (for example, imidazole), which displaced the hexahistidine

of the recombinant protein from the complex with nickel ions, or by lowering the pH of the elution buffer. The affinity tag was cleaved with the help of a proteolytic enzyme (proteinase) and the recombinant protein was purified from it and from the proteinase by chromatographic methods. If the recombinant protein is not intended to be used for medical purposes, the hexahistidine sequence may not be cleaved, as it usually does not affect the structure and function of the protein.

Several affine labels have been developed. Among them – glutathione transferase, maltose-binding protein, and short amino acid sequences – antigenic determinants that bind to glutathione, maltose, and specific antibodies, respectively. Various cleavage sites specific for thrombin, enterokinase and other proteinases were also used. The affinity tag and the cleavage site can be located both at the N- and at the C-terminus of the recombinant protein and can be used in prokaryotic expression systems, as well as in insect, mammalian or fungal cell-based expression systems.

1.2. Cellular technologies for obtaining therapeutic monoclonal antibodies

Because of their exceptional specificity and high affinity, monoclonal antibodies (MAB) are considered particularly attractive molecules for the therapy of multiple diseases, and antibody-based drugs currently represent the fastest growing segment of all therapeutic proteins in the biotechnology industry.

There are five different classes of antibodies in humans, which are determined by the nature of the heavy chain (HC) C regions. The most common class of antibodies in the blood and the most common molecular format used therapeutically is IgG [38].

There are 4 types of monoclonal antibodies based on the ratio of their human and animal (most often mouse) nature. The ratio of the use of different types of monoclonal antibodies in clinical use is:

- Human – 51 %;
- Humanized – 34.7 %;
- whimsical – 12.5 %;

- mouse antibodies – 2.8 %.

As can be seen, human and humanized monoclonal antibodies (MABs) dominant in the field of therapeutic antibodies [39].

1.2.1. Mouse antibodies

The classic hybridoma technology is as follows: the antigen is injected into mice, and due to the natural immune response, antibodies of the required specificity are produced. Spleen cells with antibody-producing B-lymphocytes secrete and fuse *in vitro* with "immortal" myeloma cells. Fused hybridoma cells are selected with special selection media that kill unfused cells. Hybridoma cells contain information from both parent cells for antibody production and unlimited cell growth. A thorough screening process identifies those hybridoma cells that produce the desired monoclonal antibody. Cells can be mass cultured to obtain large quantities of antibodies and / or stored frozen in liquid nitrogen [40].

Disadvantages:

- inability to control the affinity and selectivity of the antibody;
- the mouse antibody is non-human due to the presence of other amino acid residues in certain parts of the constant part.

That is why the first commercial antibody, Muromonab-CD3, elicited an antibody response in many patients. Such antibodies are called Human anti-mouse antibodies (HAMA), i.e. "human anti-mouse antibodies". The antibody response greatly reduces the effectiveness of the drug, because it leads to its elimination from the body and makes it impractical to re-introduce it, which will cause an even stronger immune response [41].

1.2.2. Chimeric antibodies

Chimeric antibodies became the first type of antibodies close to human ones. These are monoclonal antibodies obtained from a non-human species (for example,

mice, rats), “humanized” to varying degrees with the help of engineered amino acid substitutions that make them more similar to sequences obtained from humans. This is done using recombinant DNA technologies [40].

In 1997, Rituximab was approved – an antibody against the CD20 protein on the surface of B-lymphocytes, intended for the treatment of B-cell lymphomas. To create it, the variable fragments obtained by the hybridoma method were combined with human constant domains, and in this way, the content of human areas was reached over 70 %. The inclusion of Rituximab in lymphoma treatment regimens made a revolution, allowing not only to increase the time to disease progression, but also to increase overall survival.

1.2.3. Genetic engineering of antibodies

Usually, the creation of a genetic engineering design for the expression of an antibody begins with the selection of an expression system, a vector, and the optimization of the nucleotide sequence encoding the antibody. It is necessary to adjust the composition of codons in such a way that the expression of the antibody is maximal. The amino acid composition of the protein is optimized in order to reduce the probability of sedimentation, aggregation, and immunogenicity . In addition, it is important to ensure the correct folding (folding) of chains and their connection to each other. Currently, there are programs that allow optimizing the nucleotide sequence of genetic engineering structures for the production of antibodies *in silico* (with the help of a computer *or* computer simulation) , although the last word remains according to experimental data.

As a vector, plasmids are used – circular DNA encoding both the protein gene itself and auxiliary elements. Thus, cytomegalovirus promoter is used for expression in the cell, which ensures high transcription efficiency.

Since the antibody consists of two polypeptide chains – light and heavy – either a two-plasmid expression system or one vector containing both genes is used. The latter option is more optimal, as it allows more accurate control of the ratio of

products of light (LC) and heavy (HC) chains. It was shown that under natural conditions in B cells, light chains are synthesized in greater quantities than heavy chains, and this ratio of synthesis rates is the most favorable for the production of antibodies [41].

The vector must also contain a gene for resistance to the antibiotic in the presence of which the cells are grown. This is necessary for the selection of cells containing the vector – when the antibiotic is added, the cells that do not carry the vector will die. Although the antibody is synthesized in mammalian cells, preliminary manipulations with the vector (for example, inserting a gene there and cloning, i.e. obtaining identical copies of the vector) are carried out in bacteria [42].

The process of obtaining a chimeric antibody using the example of infliximab – a drug against tumor necrosis factor (Tumor necrosis factor (TNF)), which is used to treat rheumatoid arthritis and Crohn's disease. To obtain it, mice are immunized with human TNF (hTNF) in combination with an adjuvant – a substance that enhances the immune response, then splenocytes are isolated and merged with myeloma cells. To select clones of cells expressing the required antibodies, a radioimmunoassay method with immobilized hTNF is used . After the construction of genomic libraries using phage vectors and screening by Southern blot, genes encoding light and heavy antibody chains are isolated. After that, a plasmid containing the genes of the mouse variable regions is obtained, combined with the genes of the human constant regions (obtained earlier by sequencing the genes of human B cells) and introduced into mouse myeloma cells for the production of a chimeric antibody [43, 44].

1.2.4. Humanized antibodies

Although the chimeric antibodies proved to be more effective and less immunogenic than fully murine antibodies, they still cause a Human anti-chimeric antibody (HACA) response – the formation of HACA – human antibodies against chimeric antibodies. Therefore, since the 1980s, the development of humanized

antibodies began, in which only the Complementarity-determining regions (CDR) and individual positions of the FR-regions of the variable domains are alien to humans, and the rest of the parts, including the frame, are almost completely human. CDR is a part of immunoglobulins with which they bind to their specific antigen. As the most variable parts of molecules, CDRs are crucial for the diversity of antigenic specificity generated by lymphocytes.

The first such antibody in development was alemtuzumab against the CD52 lymphocyte antigen. These drugs are used in the treatment of multiple sclerosis. It was obtained on the basis of a rat antibody whose CDR was transferred to the human VH and VL domains of an already existing human antibody [38].

Another approach was used in the development of the Daclizumab antibody against the CD25 receptor on T-lymphocytes, which became the first humanized antibody on the market. Daclizumab was also obtained by incorporating mouse CDRs into a human framework, but in this case the human parts of the antibody were selected by computer methods to maximize similarity to the mouse antibody from which the CDRs are derived. In addition, a computer model of a mouse antibody was built, and those amino acid residues of the frame that were in contact with the CDR were transferred to the frame of a human antibody, which allowed to improve the affinity [38].

Although humanized antibodies include an increased number of human sequences compared to chimeric ones, the possibility of producing antibodies against them remains – they are called HAHA (human anti-human antibody, human anti-human antibodies). HAMA-response can be directed to the entire antibody, HACA – against variable regions, and HAHA-response is even more focal – only against CDRs [39].

It should be noted that not all amino acid residues or groups of residues are similar in their immunogenicity. In addition, it is increasingly difficult to specify what constitutes a chimeric antibody compared to what constitutes a humanized antibody (eg, how many amino acid residues must be changed for an antibody to qualify as humanized) [40]. The invention of many methods of development and

humanization of antibodies has led to the fact that the line between chimeric, humanized and human antibodies is blurred in many cases. In this regard, in 2017, the World Health Organization (WHO) decided to cancel the previously existing nomenclature, according to which murine antibodies had the suffix – momab, chimeric – ximab, humanized – zumab, and fully human – mumab [45].

1.2.5. Fully human antibodies

As mentioned above, fully human monoclonal antibodies occupy more than half of the market for therapeutic drugs, and 3 main methods are used to obtain them. No single therapeutic drug developed by the method of single B cells has yet been approved, so we will consider the other two methods – phage display technology and the method of transgenic mice [39].

Technologies of phage display. Display methods use a wide repertoire of genes encoding antibodies as starting material. The most common method is phage display. The method of presenting the peptide on the surface of bacteriophage M13 was invented in 1985.

The first antibody created using phage display technology in 2002 was the drug “Humira” (adalimumab, Abbott company) against the TNF target for the treatment of rheumatoid arthritis and Crohn’s disease [46].

The quality of the obtained library of fragments depends on the ability to obtain a wide repertoire of diverse and at the same time high-quality antibodies. There are 2 fundamentally different approaches to obtaining libraries: natural and synthetic [47].

Natural libraries of variable domains of antibodies are obtained by polymerase chain reaction of natural genes with reverse transcription (RT-PCR) from lymphoid tissues or peripheral blood of humans and other animals. The advantage of this method is that the obtained antibodies will be in the correct conformation, as their genes encode functional antibodies. However, the disadvantage is that the variety of sequences is limited by the coverage of the natural immune system, in which there is

a certain unevenness in the use of certain sequences. Also, fragments from natural libraries vary widely in quality and are unpredictable in composition; many of them may be insufficiently stable or unsuitable for other reasons. The composition of natural libraries is 10^7 -10^{11} fragments [48].

Synthetic libraries are created by inserting artificially synthesized DNA into sequences encoding variable domains. DNA is synthesized in such a way as to introduce absolutely random mutations into the obtained antibody fragments and thereby expand the diversity of the area that determines complementarity – CDR. Synthetic libraries make it possible to use a certain cow (germline) structure of the variable domain, which is known to be the most represented in each individual, non-immunogenic and stable. However, the introduction of completely synthetic CDR regions can lead to incorrect formation of the structure and aggregation of the protein. It took a long time to work out the approaches to determine which CDRs are best to use. The composition of synthetic libraries, as a rule, reaches 10^9 -10^{11} fragments [49].

Selection of clones with the required properties. The antibody fragment gene shown in pink in figure 1.3 is inserted into phage DNA – inside the gene encoding the phage coat protein. This leads to the expression on the surface of the phage protein - a fragment of the antibody. The genes are then transduced into *Escherichia bacterial cells coli* and infected with an auxiliary phage, which causes the assembly of a bacteriophage containing an antibody fragment on its surface. As a result, a library of bacteriophages is obtained, each of which has an antibody fragment corresponding to the gene that entered this bacteriophage.

The phage library (fig. 1.3), consisting of 10^6 -10^{11} clones, is incubated with immobilized antigen (1). Unbound phages are removed by washing (2). Only bacteriophages containing an insert of a gene fragment of an antibody with high affinity to the antigen remain on the surface (3). In order to select the most high-affinity clones that have bound to the antigen, phages are removed from the surface and re-infected with *E. coli* (4). The process is repeated 2-3 times to enrich the population with antigen-specific antibody fragments. After several stages of selection, the DNA of the obtained phages is sequenced and the sequence encoding

the most affine fragments is learned. Cells are seeded on medium with an antibiotic and amplified (5) [50].

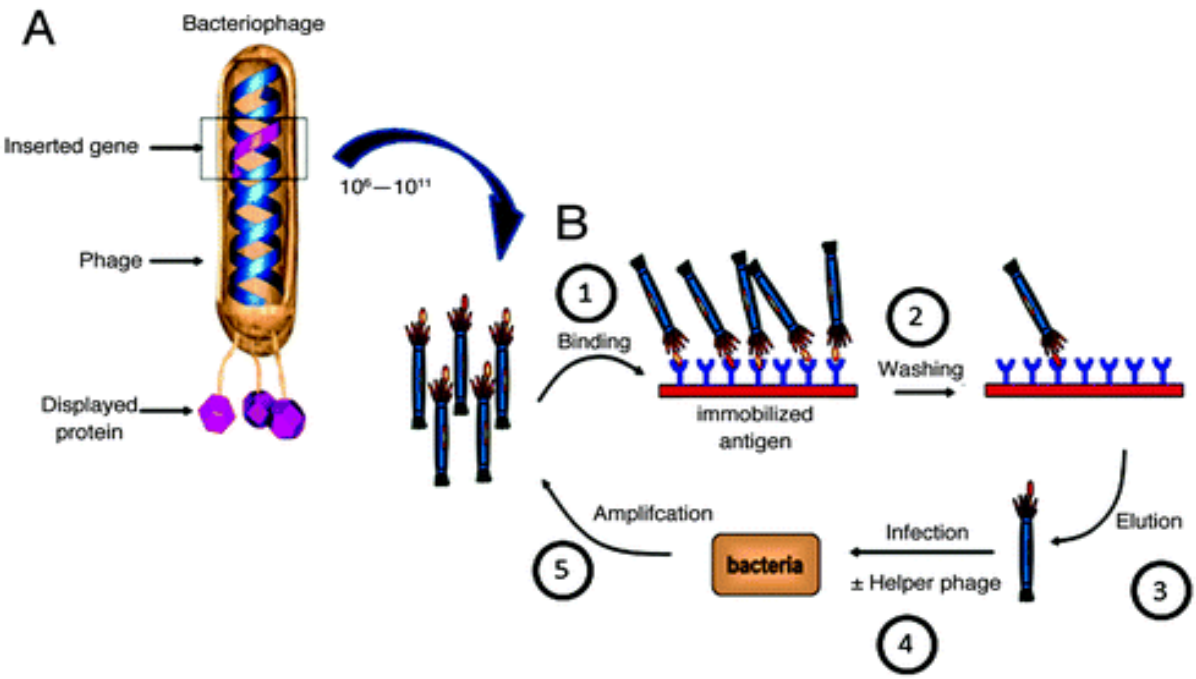

Figure 1.3. The selection process of the required genotypes according to antigen affinity [50]:

A – bacteriophage, which ensures the genotype-phenotype connection; b – phage display scheme.

An important advantage of using display methods compared to hybridoma methods is the ability to select only those antibodies that do not bind to unnecessary proteins, for example, those close to the target, but which do not need to be blocked in order not to cause toxicity. For this, it is enough to immobilize the unwanted protein and select those phages that did not bind to it.

It is also useful to create an antibody that binds well to mouse, monkey, and human variants of the target – this ensures rapid successful completion of preclinical studies and safety control before the introduction of a potential drug candidate into clinical trials.

There are other types of displays besides phage, for example, ribosomal or yeast. Their principle is the same: they ensure the connection of the gene encoding

the antibody fragment with its affinity to the target, that is, with the phenotype, and allow selection based on given properties from a huge number of variants in the library [51].

1.2.6. Technology of using transgenic animals

The method of transgenic animals has two important advantages over display methods [52]:

- a faster process of antibody formation;
- since the selection goes *in vivo*, not *in vitro*, the formation of antibodies with poor solubility and other problems in further development are excluded.

Using this method, one of the most famous antibodies, nivolumab, was obtained against the PD-1 target on activated T-lymphocytes, which revolutionized the treatment of metastatic malignant diseases: melanoma, lung cancer, and bladder cancer.

Nivolumab was developed using the technology of the Medarex company , which in 1993 obtained a line of mice expressing human IgM, IgG and Igκ and not expressing mouse IgM and Igκ . Mice were able to produce full-fledged B cells, in which variable-diversity-joining rearrangement (V(D)J-recombination) of human transgenic regions occurs (a mechanism of somatic DNA recombination that occurs at the early stages of lymphocyte differentiation and leads to the formation of antigen- recognizing regions of antibodies and T-cell receptors), and after the introduction of antigens – heavy chain class switching and somatic mutagenesis. Next, human antibodies are obtained from these mice using the usual hybridoma technology.

Thus, to obtain nivolumab, transgenic mice were immunized with recombinant human protein PD-1-Fc, consisting of the extracellular domain of PD-1 and the Fc fragment of IgG. At the same time, mice were injected with CHO Chinese hamster ovary cells expressing PD-1 on the surface. Splenocytes mice were fused with SP2/0 myeloma cells and hybridomas producing antibodies reactive against PD-1-Fc were

selected by enzyme-linked immunosorbent assay. The binding of nivolumab to CD4+ lymphocytes was determined by the flow cytofluorimetry method , and the kinetics of binding to the target was determined by the plasmon resonance method [38, 39].

Although the Medarex technology in mice generates a rather limited repertoire of genes encoding human VH and VL, even this is sufficient to generate high-affinity antibodies , although not in all cases. If the antigen to which antibodies are to be obtained is highly homologous to the murine counterpart, this will not be possible. In this case, you can use transgenic rats, chickens or display technologies. Also, display technologies are the only way out when the target is toxic in concentrations necessary for animal immunization [53].

1.2.7. Production of antibodies

Obtaining a producer clone. In order to obtain a stable antibody-producing clone, it is necessary to achieve the correct integration of the vector into the cell genome, to modify, if necessary, the apparatus of translation and post-translational modifications, to achieve high viability of the producer. In CHO cells (Chinese hamster ovary cells), for this purpose, the expression of pro- apoptotic factors was decreased and the expression of anti- apoptotic factors (for example, Bcl-2) was increased. Protein translation was increased by overexpressing chaperones to protect the cell from apoptosis under the influence of stress and ensure more correct folding of antibodies. In addition, the genome of the producer cell is modified to provide the desired glycosylation profile [49].

After the creation of the cell line, the selection and optimization of the cultivation process begins, first in 96-well plates, then in flasks and table-top bioreactors with a volume of 1-5 liters. As a result of the screening of hundreds and thousands of clones, the producer clone with the best characteristics is selected and transferred to pilot cultivation in reactors with a volume of 10-25 l, and then the process is scaled to larger sizes [38].

Cultivation. Usually, cells that produce antibodies are cultivated in bioreactors-fermenters. Traditional cultivation methods are batch and fed-batch processes . In the first case, all the components of the medium and the producer clone are loaded into the reactor for several days, after which the culture medium is collected for protein isolation. In the second case, nutrients are periodically added to the environment.

The most important factor in ensuring high protein yields is the culture medium. Moreover, progress in this area continues, and if in the 1990s the yields were 0.1 g/l, in 2007 – up to 5 g/l, now it is possible to achieve the target protein yields of 10 or more g/l. Such successes became possible thanks to the transition from media containing animal serum to serum-free media: first protein hydrolysates were used, and now – completely chemical media with a clearly defined composition. The fact is that the variability of the composition worsens the cultivation parameters. It also proved to be productive to change the composition of the environment depending on the stage of cultivation: different environments are used in the growth phase and in the product accumulation phase.

To increase productivity, fine-tuning of environmental parameters with feedback is also used: temperature, pH , salt composition, oxygen and carbon dioxide levels, which allows to achieve a reduction in the concentration of toxic by-products (lactic acid, ammonia) and a smoother consumption of nutrients.

Currently, there is a tendency to switch to continuous processes: to the so-called perfusion cultivation, in which fresh medium is continuously added to the cell culture, and the product is removed from it. This approach is better automated, gives a product of higher quality and with increased yields. Developers are also switching from steel bioreactors to disposable ones, as the latter have a number of advantages [48]:

- they are supplied to production clean and sterile, they do not need to be prepared for work;
- for the same reason, it is easier to validate the production process;
- they have more flexibility in case of need to change process parameters;

- they do not need to be put into operation for a long time and cleaned in case of contamination.

Isolation and purification of antibodies. The main stages here are various types of chromatography, diafiltration and inactivation of viruses. One of the features of antibody purification compared to other proteins is the use of chromatography with protein A. This is a protein of the cell wall of Staphylococcus aureus, which strongly and selectively binds to antibodies at normal pH and weakly at low pH. Due to the high yields and purity of the product, it is the preferred method in the first stage of antibody purification.

The most important requirement both at the production stage and at the antibody purification stage is the stability of the product composition. Since an antibody is a complex object and is produced in a biological system (in cells), the molecules are not the same, but with variations. The purpose of developing the production and purification process is to ensure that these variations do not affect the pharmacological characteristics of the product, its stability, and do not change much from one batch of the product to another. For this, validation of the production process is carried out – experimental confirmation that the process ensures the production of a product with the appropriate characteristics.

At some intermediate and final stages, the composition of the product is controlled by analytical methods. Mandatory for control are:

- bacterial endotoxins;
- protein A;
- presence of viruses;
- proteins and DNA of the producer, residues of the culture medium (antibiotics, serum, etc.), residues of the process (enzymes, reagents, inorganic salts, solvents, etc.);
- physical and chemical properties (carbohydrate composition, disulfide bonds, variability of N- and C-terminal residues, quaternary structure);

- presence of isoforms, extinction coefficient, electrophoretic, chromatographic, spectroscopic profiles, multimers and aggregates, heterogeneity of molecular charge.

Antibody production methods are constantly being improved: in more than 40 years since the first antibody was introduced to the market, many changes have taken place – antibodies have been learned to be humanized, methods of their artificial production have been developed, methods of studying affinity and other properties have been improved, great progress has been made in production and purification, which made it possible to reduce the cost of antibodies.

Despite all these successes, there is a need for faster and more accurate development of antibodies against a given target. Ideally, as many operations as possible should be carried out *in silico*, so as not to waste time and money on experiments, but we still do not have enough knowledge and capacity to model, for example, the pharmacokinetics and pharmacodynamics of antibodies.

As a result, monoclonal antibodies have emerged among the main class of therapeutic agents for the treatment of many human diseases, primarily oncological, immunological, infectious, nervous and metabolic diseases. With the development of humanized and then fully human MABs, the rate of new product approvals and sales increased rapidly, with total sales revenue of all MABs products reaching $115.2 billion in 2018. It is expected that the further growth of MABs products in the coming years will become a major factor in the overall sales of biopharmaceutical products.

Mouse technology have proven to be reliable methods for generating human antibodies. As vast repositories of genes encoding antibodies with unknown properties, high-quality phage antibody libraries are critical for the successful identification of therapeutic MABs. In addition, the optimal selection of phage display libraries depends on the quality of the target antigen, antigen immobilization and strict control of binding and washing conditions. Currently, there are nine fully human antibodies that have been identified from phage libraries approved for

therapy, and dozens more phage-derived antibody preparations are in clinical trials and awaiting commercialization [46].

In order to improve the quality of antibody preparations, researchers have developed several transgenic animals, including mice, fully human, and chimeric mice of the second generation. The continuous improvement and development of transgenic animals provides more opportunities for the development of antibody drugs in the world's pharmaceutical factories. Depending on the immunization protocol, high-affinity human antibodies can be obtained by selecting clones generated in animals. This selection is mainly carried out by hybridoma technology. Currently, there are 19 approved human MABs that have been discovered in transgenic animals.

Fully human monoclonal antibodies already account for more than half of the therapeutic market, plus a significant portion are currently undergoing clinical trials and awaiting approval. So, it is clear that the future lies precisely in this type of MAB, which no longer cause significant problems with immunogenicity and are significantly more effective.

1.3. Quality control of recombinant proteins.

The characterization of proteins obtained with the help of recombinant technologies should include the determination of their physicochemical properties, biological activity, immunochemical properties, purity and impurities using appropriate methods, and is a necessary condition for establishing and approving complete and appropriate specifications.

Detailed specification is usually performed during the development stages and, if necessary, during significant changes in the manufacturing process. At the time of filing the application for registration, the claimed product must be comparable to the corresponding standard drug, if one exists. When possible and appropriate, it should be comparable to a natural counterpart. Also, at the time of submitting the

application, it is necessary to develop properly characterized own standard materials that will be used for biological and physico-chemical testing of production batches.

Product-specific tests should be included in the list of mandatory methods for control of recombinant proteins. A justification for setting the acceptable range for the acceptance criteria must be provided. Acceptance criteria should be established and approved on the basis of data obtained for batches used in the course of research, batches used to confirm the constancy of the manufacturing process, as well as the results of stability studies and basic data from the development of the medicinal product.

Certain tests carried out during the manufacturing process may be sufficient to demonstrate compliance with the requirements of the specification if such tests are included in the specification and if the acceptance criteria are identical to those specified in the specification.

For all recombinant proteins, as a rule, the following quality indicators and acceptance criteria are considered generally accepted:

- Description – clearly indicate the physical state (eg solid, liquid) and color of the active substance;
- Identification – identification tests should be highly specific for the active substance and should be based on unique aspects of its molecular structure and/or other specific properties. Identification tests may include more than one component (physico-chemical, biological and/or immunochemical).

Molecular weight (or size). Molecular weight (or size) is determined using exclusion chromatography (State pharmacopoeia of Ukraine, SPhU, 2.2.30), electrophoresis in an SDS-polyacrylamide gel (under reducing or non-reducing conditions) (SPhU, 2.2.31), mass spectrometry (SPhU, 2.2.43) and other relevant methods of analysis.

Isoform composition. Determined by the method of isoelectric focusing (SPhU, 2.2.54) or other appropriate methods.

Coefficient of extinction (or molar absorption). In most cases, it is desirable to determine the coefficient of extinction (or molar absorption) for the target product

at a certain wavelength in the UV / visible region (for example, 280 nm). The extinction coefficient is determined by the method of UV / visible spectrophotometry (SPhU, 2.2.25) based on a solution of the product with a known protein content determined by such methods of analysis as analysis of amino acid composition or determination of nitrogen, etc.

Electrophoretic profile. Electrophoretic profile and homogeneity and purity data can be obtained using methods such as polyacrylamide gel electrophoresis (SPhU, 2.2.31), isoelectric focusing (SPhU, 2.2.54), SDS-polyacrylamide gel electrophoresis (SPhU, 2.2.31), immunoblotting, capillary electrophoresis (SPhU, 2.2.31) or other relevant methods.

Chromatographic profile. The chromatographic profile and data on identity, homogeneity and purity can be established using such methods as exclusion chromatography (SPhU, 2.2.30), reverse-phase liquid chromatography (SPhU, 2.2.29), ion exchange liquid chromatography (SPhU, 2.2.29), affinity chromatography, or other relevant methods of analysis.

Spectral profile. Ultraviolet and visible absorption spectra are determined as necessary. The higher-order structures of the product, if necessary, are studied using such methods as nuclear magnetic resonance spectrometry (SPhU, 2.2.33), circular dichroism, or other appropriate methods.

Purity and impurities. The absolute purity of the product obtained by recombinant means is difficult to determine, and the results largely depend on the applied method of analysis. Therefore, the purity of the active substance is usually assessed using a set of analytical methods. When choosing and optimizing analytical methods, the main thing is the ability to separate the target product from product-bound substances and impurities. Impurities found in such products are classified as process-related and product-related impurities.

Process-related impurities and contaminants. These impurities are formed during the production process and are divided into three main categories: obtained from the cell substrate, obtained from the cell culture, and obtained at further stages of production.

Impurities derived from the cellular substrate include, but are not limited to, proteins derived from the host organism, nucleic acids (host cell genome, vector, or complete DNA). For host cell proteins, as a rule, sensitive methods are used, such as immunoassay, capable of determining a wide range of protein impurities. In an immunoassay, the polyclonal antibodies used in the test are generated by immunization with a prepared cell product, excluding the gene-encoding product, by fusion (hybridization) of cells, or other suitable cell lines.

The level of host cell DNA can be determined by direct analysis of the product (such as a hybridization method). To avoid the need to establish acceptance criteria for impurities derived from the cell substrate, such as nucleic acids and proteins of host cells, precise studies involving laboratory-scale peak experiments can sometimes be performed to demonstrate the removal of these impurities.

Impurities derived from the substrate include, but are not limited to, inducers, antibiotics, serum and other components of the nutrient medium.

Impurities obtained at further stages of production include, but are not limited to, enzymes, chemical and biochemical reagents (for example, cyanogen bromide, guanidine, oxidizing agents and reducing agents), inorganic salts (for example, heavy metals, arsenic, non-metallic ions), solvents, carriers, ligands (for example, monoclonal antibodies) and other leachable substances.

For intentionally introduced endogenous and accidental viruses, the ability of the manufacturing process to remove and / or inactivate viruses must be demonstrated in accordance with the provisions of the ICH guideline Q5A(R1).

Product-related impurities, including degradation products Degradation products generated in significant quantities during production and / or storage must be tested and controlled for compliance with established acceptance criteria.

Truncated forms. Hydrolytic enzymes or chemicals can catalyze the cleavage of peptide bonds. They can be detected using high-performance liquid chromatography (SPhU, 2.2.29) or electrophoresis in an SDS-polyacrylamide gel (SPhU, 2.2.31). Depending on the properties of the molecular variants, peptide mapping can be applied (SPhU, 2.2.55).

Other modified forms. Deamidated, isomerized, with an unconjugated S-S bond (bridge), oxidized or transformed conjugated forms (for example, glycosylated, phosphorylated) can be detected and characterized using the methods of chromatography (SPhU, 2.2.29), electrophoresis (SPhU, 2.2.31).

Aggregants. This category includes dimers and more complex structures of the target product. As a rule, they are formed from the target product and the product of bound substances and are quantitatively determined using appropriate analytical methods (for example, exclusive chromatography (SPhU, 2.2.30), capillary electrophoresis (SPhU, 2.2.31)).

Degree of activity. Appropriate validated methods for measuring the degree of activity should be part of the specification. In certain cases, when appropriate methods for measuring the degree of activity are used for the analysis of a medicinal product, an alternative method (physicochemical and / or biological) may be sufficient to quantify the active substance. The determination of a specific activity can provide the necessary additional information in some cases.

Quantitative content. The quantitative content of the active substance, which is usually based on the determined protein content (mass), must be determined using an appropriate method. For quantitative determination, the spectrophotometric method can be used (SPhU, 2.2.25).

The pharmacopoeial requirements apply to the corresponding dosage forms. Typical pharmacopoeial tests include, but are not limited to: sterility (SPhU, 2.6.1), bacterial endotoxins (SPhU, 2.6.14), abnormal toxicity (SPhU, 2.6.9), microbiological purity (SPhU, 2.6.12, and SPhU, 2.6.13), container volume (SPhU, 2.9.17), pH (SPhU, 2.2.3), mechanical inclusions (SPhU, 2.9.19, and SPhU, 2.9.20, and SPhU, 2.9.21), uniformity of dosage units (SPhU, 2.9.5, and SPhU, 2.9.6) and water content for lyophilized medicinal products (SPhU, 2.2.32).

The pharmaceutical composition must be selected in such a way as to ensure stability and biological activity. Preference is given to the lyophilic injectable form of the finished medicinal product, as this form provides better stability, increases the shelf life, and simplifies storage conditions. To ensure stability and preservation of

biological activity, auxiliary substances are added to medicinal products. There are certain requirements for auxiliary substances used in the technology of preparation of medicinal products. They must be biologically harmless, non-toxic, chemically indifferent to the substances included in the composition of the drug, to the materials of technological equipment, packaging materials, to environmental factors in the process of manufacturing the drug and during storage. They should not cause allergic reactions, give the medicinal form the necessary properties. These substances must show the necessary functional properties at a minimum content in the preparation. They should contribute to the manifestation of the necessary pharmacological effect, not be subject to microbial contamination, withstand sterilization, not have a negative effect on the organoleptic properties of the drug or improve them, and be economically profitable.

Auxiliary substances used in the production of freeze-dried drugs include: solvents, fillers, preservatives, pH regulators, solubilizers, stabilizers, cryoprotectants.

Buffer systems (phosphate, citrate or acetate buffer), sodium hydroxide, various organic and mineral acids are used to ensure the required pH value of medicinal substances in solutions.

The role of molding filler is performed by compounds that are included in the composition of the dosage form, if the content of the active substance is small or the substance that is lyophilized is poorly soluble in water or a water-alcohol mixture. The following fillers are most often used: high molecular weight compounds, polyvinylpyrrolidone (PVP), dextran-70; carbohydrates-sucrose, lactose; hydrocarbon alcohols-mannitol, sorbitol? glycine

Depending on their properties, these substances perform other functions: they affect the freezing process (for example, sugars, polymers, alcohols, lower the freezing point of water and slow down its crystallization), change the eutectic point, drying speed, and final residual moisture.

The first technological step in the production of injectable lyophilized drugs is the preparation of a solution to be dried. In this connection, the physico-chemical

properties of the active substance (substance) and possible ways of its destruction in the water environment are studied, auxiliary components are selected.

Before the advent of recombinant DNA technology, many drugs based on human proteins could only be obtained in small quantities, their production was very expensive, and the mechanism of biological action was sometimes insufficiently studied. It was assumed that with the help of the new technology it will be possible to obtain the entire range of such drugs in quantities sufficient both for their effective testing and for use in the clinic. And these expectations were fulfilled. To date, more than 400 genes (mainly in the form of cDNA) of various human proteins have been cloned, which, in principle, can become drugs. Most of these genes have already been expressed in host cells, and their products are now being tested for possible use in the treatment of various human diseases. However, although more than 30 such biotechnological drugs have received approval in the United States, it will be several years before they are recommended for widespread use and go on sale; they must first undergo a series of rigorous pre-clinical and clinical trials. However, pharmaceutical companies are already showing interest in them. According to experts' estimates, the annual volume of the world market of medicines based on human proteins is about 150 billion dollars and is constantly growing. The volume of the world market of medicines based on recombinant proteins increases by 12-14 % per year.

The development of new methods of prevention and treatment of many human diseases made a huge contribution to the growth of people’s well-being in the 20th century. However, this process can never be considered complete. So-called “old” diseases (for example, tuberculosis) can make themselves felt again, as soon as preventive measures are weakened or resistant strains appear. The prospect of using specific antibodies as therapeutic agents looks very attractive; they can be used to neutralize toxins, fight bacteria, viruses, and treat cancer.

1.4. Biosafety of the use of drugs of biological origin

Biotechnology has many potential applications in environmental protection, energy production, and health care (eg, antimalarial drugs and gene therapy). In the chemical industry, microorganisms are used for the production of enzymes, biofuels and the creation of special bio-based products. For example, engineered bacteria that live on corn starch are used to produce high-tech fabrics. Another application of strains of modified bacteria capable of breaking down dichlorodiphenyltrichloroethane (DDT) into stable metabolites of 4-chlorobenzoic acid is for the purification of the polluted environment. In addition, synthetic biologists are working to create microbial biosensors for pollution monitoring. Engineered bacteria are used to control the concentration of naphthalene and organophosphorus pesticides in the water and gas phases.

Agriculture is a major beneficiary of synthetic biology, which improves sustainable farming methods, produces disease-resistant animals and plants, and reduces our dependence on traditional crops. Genetic modification is a set of approaches applied by synthetic biology, which allows to change the genetic apparatus of animals, plants or microorganisms. Combining genes from different organisms is known as recombinant DNA technology, and the resulting organism is called “genetically modified organisms” (GMO), “genetically engineered”, or “transgenic”. Genetic engineering technologies make it possible to increase the productivity of agriculture, reduce deforestation and avoid the loss of biodiversity in forests. It has been proven that biotechnological products increase productivity and reduce the use of insecticides in agriculture. The main transgenic crops grown commercially in the field are herbicide- and insecticide-resistant soybeans, corn, cotton, and canola. There are fruit and nut trees that yield faster, and plants that produce plastic with unique properties.

Despite a number of advantages of GMO crops, they have a potential negative impact on the environment:

- the spread of genes between transgenic plants and their sexually compatible relatives;
- changes in the levels of weediness or invasiveness of GMO crops or their wild relatives;
- horizontal transfer of constructed features to other species;
- non-target effects;
- development of resistance to pests or new secondary pests.

The main concern about the adverse effect of GMO products on human health concerns such dangerous phenomena as the spread of antibiotic resistance among microorganisms, toxicity, suppression of immunity, potentially oncogenic effect, allergenicity.

In medical and biological research, the assets of synthetic biology are used for the treatment, prevention and diagnosis of diseases. Microorganisms have been used for decades to produce drugs with pre-planned effects. For example, *Saccharomyces cerevisiae* is widely used in industry for the production of artemisinic acid, opioids, and some rare medicinal extracts. Synthetic biology will also offer was to use microbes (*Yersinia pseudotuberculosis* and some viruses) to treat cancer. The use of this approach is based on the use of so-called invading microbes, which are programmed to initiate the expression of substances to suppress tumor growth and immunotherapy of oncological diseases. In addition, scientists synthetically engineered a bacteriophage that produced enzymes capable of lysing biofilms and drug-resistant bacteria.

Another direction of synthetic biology is the development of vaccines. In particular, genetically modified bananas were created, which produce human vaccines against infectious diseases (hepatitis B). Another vivid example is the development of vaccines based on messenger RNA (mRNA) for the prevention of infection caused by SARS-Cov-2. In this type of vaccine, gene-engineered mRNA is used, which stimulates the formation of specific immunity to the S-protein of the COVID-19 coronavirus virus and prevents its attachment to the host’s cells. The

introduction of gene network biosensors into the practice of diagnosing infectious diseases allows for quick and inexpensive detection of the Ebola and Zika viruses.

In the European Union (EU) guideline “Manufacturing of biological medicinal products for human use” (“Rules governing medicinal products in the European Community”, Volume IV, GMP for Drugs, EU Commission, 1992), the following groups of drugs belong to biological drugs: vaccines, immune sera, antigens, hormones, cytokines, enzymes and other fermentation products (including monoclonal antibodies and rDNA products).

Their arsenal is constantly growing, and production technologies are constantly being improved. At the beginning of the 21st century, it is impossible to imagine a world without representatives of this group of drugs: insulin drugs, antithrombotic drugs, enzyme drugs, etc. In 2009 alone, 11 new active substances of biological drugs were approved in the EU, including golimumab, certolizumab pegol – for the treatment of rheumatoid arthritis, denosumab – for the treatment of osteoporosis, Chondrom Select (autologous chondrocytes) – for transplantation of autogenous chondrocytes in knee joint injuries; besilesomab – for osteomyelitis; epoetin theta – for the treatment of anemia, etc.

During the last decade, genetically engineered biological drugs capable of blocking the most important links of the immunopathogenesis of this disease have been introduced for the treatment of RA: TNF-α inhibitors (infliximab, adalimumab, etanercept, golimumab, and certolizumab), anti-B – cellular drugs (rituximab), T-lymphocyte coagulation blockers (abatacept), interleukin-6 (IL-6) receptor blockers-tocilizumab).

According to the data of randomized placebo-controlled studies, in terms of their effectiveness, the mentioned drugs are superior to the “gold standard” of the basic pharmacotherapy of RA – methotrexate. Recently conducted meta-analyses indicate that in patients in whom genetically engineered biological drugs were used, the effectiveness of therapy was higher in comparison with control groups of patients. The most effective and safe drugs suitable for the treatment of RA already in the early stages of the disease. This allows in the future to individualize the treatment of RA by

influencing both cellular and humoral immunity. However, the use of genetically engineered biological drugs sometimes caused the need to withdraw pharmacotherapy due to adverse reactions (ADRs) in a larger number of patients than during treatment with traditional basic drugs or placebo (especially when using adalimumab and infliximab).

Potential complications of therapy with TNF-α blockers – tuberculosis, hepatitis B, severe bacterial infections, autoimmune reactions, etc.; drugs of monoclonal antibodies to CD20 (rituximab) – infectious reactions, risk of fatal reactivation of the hepatitis B virus, submaximal response to vaccination, multifocal leukoencephalopathy, psoriasis; IL-6 inhibitor (tocilizumab) – gastrointestinal lesions, blood lipid profile disorders, neurolepsy, increased levels of alanine aminotransferase, aspartate aminotransferase, and bilirubin; anti-T-cell drugs (abatacept) – the risk of developing serious infectious lesions.

In recent years, another one has been added to the well-known pharmacological groups of drugs of biological origin – chondroprotector drugs. About 40 representatives of these drugs are allowed for medical use in Ukraine, by trade names. Numerous data from clinical observations testify to their effectiveness in the treatment of degenerative-dystrophic diseases of peripheral joints and spine (osteoarthrosis, osteochondrosis, spondyloarthrosis, etc.), osteopathies and chondropathies, chondromalacia, periodontopathy; for the purpose of prevention and treatment of joint damage due to physical exertion (including sports injuries); in the period of convalescence after bone fractures (to accelerate the formation of calluses), injuries, operations; as an additional remedy for joint pain.

However, among all drugs approved for medical use in Ukraine in recent years, adverse reactions have been registered in almost 50 cases, among which serious predicted general allergic reactions, anaphylactic shock, significant in terms of the area of skin damage and its derivatives, which accounted for 3 %, prevail.

In order to properly understand the reasons for the increase in the number of registered drug cases, it is necessary to reject the popular arguments “about high-quality and low-quality drugs”. This frequency of side effects is primarily explained

by the number of prescriptions of each individual drug and the attention paid to the negative effects of drugs by doctors and patients. It is known that the vast majority of representatives of drugs of biological origin are extremely widely prescribed to patients with various nosologies, from type I diabetes to various cardiovascular, infectious, digestive tract diseases, etc. In this message, we did not aim to analyze the safety issues of each drug in detail, but we tried to demonstrate the existence of the problem of PR in the medical use of drugs of biological origin in Ukraine.

The analysis of the obtained data on the systemic manifestation of adverse reactions during the medical use of drugs of biological origin in Ukraine (1996-2010) shows that, as among other groups of drugs (synthetic, phytopreparations), in biological adverse reactions the number of manifestations of adverse reactions registered by doctors in in different regions of Ukraine, allergic reactions are noticeably predominant. The second position is taken by cardiovascular disorders, the third by changes in the place of drug administration. This reflects the general trend regarding the nature of systemic disorders, which are found in the world and in Ukraine when using representatives of almost all drugs – the absolute dominance of allergic complications. However, the number of registered allergic reactions when using drugs of biological origin does not exceed ten times the frequency of allergic reactions to other drugs. Even the cases of death (0.56 %) registered in the period 2002-2008 during the medical use of the analyzed drug groups were associated with the development of anaphylactic shock against the background of the severe course of the main and concomitant diseases. It is known that proteins of biological / biotechnological origin, which are increasingly used as the main active substances of drugs, can cause humoral and cellular reactions. Their consequences can vary from the transient appearance of antibodies to life-threatening conditions (potential clinical consequences – serious hypersensitivity reactions, reduced effectiveness and induction of an autoimmune response with the formation of antibodies against endogenous forms of the injected protein, inclusive).

In addition, the immunogenicity of medicinal proteins can be influenced by factors from the patient’s side (genetic, immune), clinical and pharmacological

features of the drug (character of immunomodulatory therapy, method of administration, dose and dosing regimen), production technology (profile of impurities, contamination, metabolites, etc.).

In general, the obtained data indicate that, according to the distribution of the consequences of adverse reactions, the analyzed drugs are sufficiently safe for medical use, regardless of the manufacturer. At the same time, a significant number of cases related to the allergic status of patients should cause concern for doctors of relevant specialties. From the experience of the existence of the system of pharmacovigilance in Ukraine, it is known that a significant percentage of the cases of serious and non-serious adverse drug reactions are due to medical errors, in particular, inattentiveness to the anamnestic data of patients. Since almost one hundred percent of these drugs must be dispensed exclusively by a doctor's prescription, his control over the effectiveness and safety of pharmacotherapy must be strengthened, given that most of these drugs are vitally necessary or life-sustaining (insulin drugs, antithrombotic drugs, drugs that affect the gastrointestinal intestinal tract, joint apparatus, etc.).

The certain analysis of the safety problems of drugs of biological origin, which we have presented, shows that there are still limited observations of drug use on these drugs during their medical use in Ukraine. Various specialists who actively prescribe drugs of biological origin do not always pay attention to the need to control their safety or, under various circumstances, do not consider it appropriate to notify the pharmacovigilance system about complications of pharmacotherapy. On the other hand, manufacturers of these drugs (domestic and foreign), despite the recommendations of international organizations, the EU directives, and national regulations, do not conduct control studies on the safety profile of their drugs in the required volume during the period of validity of the registration certificate in Ukraine. According to the EU regulations, a previous (licensing) positive decision is not sufficient to identify all potential discrepancies between the reference product and its biosimilar version.

Therefore, the issue of the possibility of their reproduction, that is, the creation of biogenerics, has become extremely urgent. So far, obtaining biosynthetic molecules that are completely identical to the original is problematic. Representatives of this group of substances are more complex and heterogeneous products with a high molecular weight and three-dimensional structure than typical drugs with a clearly established chemical formula. The latter became the basis for the definition of reproduced biopharmaceuticals as “biosimilars”. Since biosimilars are complex protein drugs, they also have a complex effect on the organs and systems of the body, and their quantitative content in the body cannot be accurately determined. Therefore, the pharmacological activity of these drugs may differ even between series of the drug of the same manufacturer.

In connection with the above, the possibility of differences in efficacy and safety between original and reproduced biosimilars is not in doubt. Therefore, the manufacturers of these drugs must carry out full preclinical and clinical trials and provide comprehensive data (ordinary bioequivalence studies are not enough). Due to the impossibility of exact reproduction of the technology, biosimilars cannot be an exact copy of the original drug and have significant differences in the structure of the molecule, biological activity, efficiency and immunogenicity.

The above also applies to human insulin preparations. The best-known by the manufacturer, his original drugs differ in the technology of production: either it is a human genome, or a monkey genome, or it is synthetic DNA (currently, synthetic DNA is preferred). In addition, various insulin producers (*E. coli* and yeast) are used.

Significant differences were found when comparing almost 50 biosimilars of erythropoietin in 16 countries of the world. It turned out that almost 2/3 of them are not equivalent to the original drug and are other biotechnological products.

Based on this, in 2006, the Europe, the Middle East and Africa approved new requirements for the registration of biosimilars, and additional monitoring was introduced during their medical use.

That is why the problem of biosimilars – possible generic biotechnological products - is gaining importance. According to the definition of WHO (2010), a

biosimilar is a biotechnological product for which compliance with an already approved product has been proven in terms of quality, preclinical and clinical evaluation. However, true generic versions of many of the latest technological products are impossible, as it is unrealistic to reproduce their exact copy:

- the source material and / or technological process are different;
- complex products – large protein / sugar molecules.

In addition, even the smallest changes can lead to the manifestation of the main side effects of such products – immunogenicity, which can significantly affect the safety of their use.

The increase in the number of new artificially created protein molecules as the main active substance among the class of drugs of biological (biotechnological) origin excludes the possibility of the emergence of generic drugs among them in the usual sense for us. This is caused, first of all, by the need to solve problems related to the control over the immunogenicity of close (similar) substances, as well as to clarify possible genetic differences in their structure and arising from this is due to pharmacological differences in their effect on the body of patients. As stated in section 3 of the Guidelines CHMP/EWP/89249/2004 dated 31.07.2007, "...many factors that affect the immunogenicity of medicinal proteins remain unknown and unpredictable...". Therefore, "…it is impossible to predict the reaction of antibodies in humans based on animal studies...".

In this regard, a reliable pharmacovigilance program and risk management plan must be offered in each individual case of use of drugs of biological origin.

The further development and widespread application of advances in synthetic biology raises concerns about biosafety and biosecurity, as they may expose public health and the environment to unknown threats. Potentially negative effects of biotech products are associated with their ability to induce allergies, antibiotic resistance, oncogenicity, and human pathogenicity / toxicity. In addition, biosynthetic products contribute to the change or depletion of the environment due to unfair competition of synthetic organisms with local species, horizontal gene transfer and greater pathogenicity or toxicity.

Risks associated with advances in synthetic biology:

- obtaining unexpected results;
- simplification, cheapening, increasing availability for the general public of technologies for creating de novo microorganisms and methods of changing pathogenicity, mechanisms of transmission of dangerous viruses and bacteria;
- formation of antibiotic-resistant superbacteria after artificial application of plasmids.

To address the biosafety issues associated with artificially created microorganisms, efforts have been made to develop technical precautions at the laboratory level to limit the release and survival of synthetic microbes in the environment. One such protective strategy is the creation of genetic defenses to prevent the accidental release of genetically engineered microbes into the environment. This strategy involves the early development of bioretention systems using gene cassettes encoding toxins. It is an artificial suicide system for bacteria, in which the gene encoding autotoxins is used to kill a variety of bacteria after its expression. The combination of regulatable promoters and gene cassettes encoding autotoxins should arrest cell growth when released from laboratory media. Another variant of protective systems that will allow control of synthetically created microorganisms and ensure their uncontrolled spread is a system based on bacterial toxin / antitoxin pairs, where the main mechanism includes the neutralization of the toxin by the antitoxin at the level of transcription or translation. In addition, genetic mechanisms can be built on the basis of auxotrophic mechanisms. For example, a biological control system was created in *Salmonella enterica*, which is based on the expression of genes encoding arabinose-dependent growth. Transcription of genes regulated by arabinose is stopped in this microbe due to the lack of arabinose when the bacteria penetrate the host cells. In addition, suppression of these genes activates the synthesis of mRNA, which encodes the main genes responsible for the lysis of bacterial cells.

Another approach introduced to contain the negative effects of synthetic biology is the genetic firewall, or xenobiology. This is an important branch of synthetic biology aimed at the development and synthesis of xenonucleic acids or at the creation of protein with non-canonical amino acids. There are no xenonucleotides or non-canonical amino acids in nature. Therefore, synthetic organisms dependent on such artificial molecules will not survive outside the created environment. This strategy can effectively eliminate the risks associated with the exchange of genetic information and prevent horizontal gene transfer between synthetic and existing natural organisms. In addition, genetic materials released by dead synthetic cells cannot be incorporated into natural organisms because they cannot be recognized by natural DNA polymerase.

Another approach that provides bioprotection against synthetic microorganisms is the use of “watermarks” or DNA barcodes that label synthetic DNA or organisms. Scientists have developed several watermarks for encrypting information using the DNA crypt algorithm, which are effective for coding and regulatory regions of DNA. Watermarks or barcodes not only aid in the tracking and identification of synthetic organisms, but can also provide proprietary protection for engineered strains.

Regulatory policy regarding the achievements of synthetic biology. Legislative policies that regulate issues related to synthetic biology in different countries are formed according to the fields of research and application of synthetic biology. State acts on synthetic biology are similar in the European Union and the USA. Modern synthetic biology still uses methods covered by Directives 2009/41/EC and 2001/18/EC. These legislative acts regulate the use and intentional release of genetically modified organisms into the environment. The legislation of the European Union regarding the use and regulation of obtaining GMOs is based on Directive 90/219/EC, which regulates the activities of genetic modification of microorganisms, their cultivation, storage, transportation, destruction and disposal.

The manufacture of drugs in the United States is regulated by the Federal Food, Drug, and Cosmetic Act (FDCA). The use of toxic substances is controlled by the Insect Pest Act, the Public Health Security and Bioterrorism Preparedness Act of

2002, the Bioshield Act, the Biodefense and Pandemic Vaccine and Drug Development Act, the National the bioengineered food disclosure standard, the USA Government's Life Regulation Policy, the Dual-Use Research Act, and other laws and regulations.

China also has laws and regulations in place to ensure biosafety and biosecurity in synthetic biology laboratories. These regulations include the Biosafety Law of the People's Republic of China (2020), the Biotechnology Research and Development Safety Management Regulations (Biotechnology Research Regulations), infectious disease prevention and control policies, and laboratory management.

In the European Union, three separate pieces of legislation have been developed that regulate the use of GMOs in food products: Directive 90/220/EEC, Regulation on novel foods (258/97) and Regulation 1139/98 (labelling of certain food products). The Directive focuses on issues related to the protection of human health and the environment. This document covers the environmental risk assessment and approval for the release of all GMOs at the research and development stage, as well as the placing on the market of products containing or consisting of GMOs.

In order to solve the problems of regulating the use of GMOs and ensuring the safety of food products in Ukraine, a national legislative framework was created. The Law of Ukraine "On the State System of Biosafety in the Creation, Testing, Transportation and Use of Genetically Modified Organisms" is aimed at ensuring a systematic approach to the settlement of issues related to GMOs. In addition, the resolutions of the Cabinet of Ministers of Ukraine "Issues of labeling of agricultural goods produced with the use of genetically modified organisms" dated November 21, 2007, "On the approval of temporary safety criteria for handling genetically modified organisms and the implementation of genetic engineering activities in a closed system" dated October 16, 2008; Order of the Ministry of Health of Ukraine "On approval of the List of food products subject to control of the content of genetically modified organisms" dated November 9, 2010; Order of the Ministry of Agrarian Policy and Food of Ukraine "On Strengthening State Control over the Safety of

Agricultural Products and the Presence or Absence of Genetically Modified Organisms" dated February 24, 2011, etc.

CHAPTER 2. GENETICALLY MODIFIED ORGANISMS AND BIOSAFETY ISSUES

Genetically modified organism is any organism, with the exception of the human organism, that has a new combination of genetic material and obtained thanks to the use of modern biotechnology methods.

The main purpose of obtaining GMOs is to improve the beneficial characteristics of the recipient organism (for example, increasing the plant's resistance to herbicides, insect pests, pathogenic microorganisms, etc.) to reduce the cost of the final product.

The creation of GMOs is the result of the “gene revolution” associated with the development of methods of selection, reproduction, transfer and expression of genes of one organism in the cells of another [54].

The main stages of the “gene revolution” that led to the creation of GMOs:

- 1953: Scientists J. Watson (USA) and F. Crick (England) proposed lived a model of the structure of DNA, allowing to give a chemical explanation of its biological properties substance as a carrier of genetic information;
- 1970: H. Korana (USA) first synthesized a complete double-stranded DNA molecule, including a sequence of 77 nucleotides (components that make up nucleic acids) and proved that it can serve as a matrix for the construction of alanine transport RNA;
- 1970: H. Smith (USA) isolated enzymes from cells – restrictases capable of selectively cut DNA and RNA molecules into separate fragments. The discovery of restriction enzymes was important a practical step towards the creation of recombinant DNA molecules;
- 1972: The first recombinant DNA molecule (recDNA) was obtained in the laboratory of P. Berg (USA), in which fragments of phage lambda were joined (phages are viruses that infect bacteria);

- galactose operon (a set of genes responsible for splitting the milk sugar lactose) bacteria Escherichia coli with circular DNA of monkey virus SV 40;
- 1973: The first functionally active one was obtained in the laboratory of H. Boyer and S. Cohen (USA) a molecule of recombinant DNA, due to the connection of plasmids (small circular DNA molecules, hacharacteristic for bacterial cells and capable of independent reproduction) *E. coli* and a fragment of DNA plasmid of another bacterium. The resulting hybrid plasmid could successfully function in *E. coli* cells, reproduce and be transmitted to other cells both naturally and with the help of humans.

With the advent of methods for creating recombinant (chimeric) DNA, it became possible to change living organisms for practical purposes by introducing genes of other organisms into them. A whole system of methodological techniques has been developed to create such chimeras. Protein enzymes are widely used as a "tool" for manipulating DNA: restrictases; ligases; polymerases. With their help, DNA molecules are cut, stitched or their copies are obtained. The main tool of genetic engineering work is vectors. These are DNA molecules capable of transferring, reproducing and storing genetic information. A vector is a kind of vehicle for transferring foreign DNA into recipient cells. Small DNA molecules of plasmids, viruses and phages, as well as mitochondria and plastids (cellular organelles) are used as vectors in genetic engineering.

The procedure for obtaining GMOs includes several main stages.

Isolation and identification of individual genes (corresponding fragments of DNA or RNA) that are going to be transferred to other organisms. For this purpose, nucleic acids are isolated from organisms that have such genes using special chemical methods. They are cut into individual fragments using sets of restriction enzymes. The most important are restriction enzymes that are capable of cutting nucleic acids to form so-called sticky (complementary) ends. The resulting fragments have short single-stranded ends consisting of several nucleotides. If you combine DNA fragments of any origin (e.g., fragments of bacterial plasmids and fragments of animal or plant DNA) in one test tube, obtained using the same restriction enzyme

that produces sticky ends, and add an enzyme – ligase, these fragments will join together. The result is chimeric (recombinant) DNA, which can contain DNA fragments isolated from different organisms or synthesized artificially. The described technology makes it possible to create complex genetic constructs based on plasmids (or other types of vectors) intended for transfer to the cells of other organisms [55].

Cloning (reproduction) of the transferred gene. In order to multiply the few chimeric DNA molecules created in the test tube, the vectors with the fragments built into them must be transferred to the recipient cells. Plasmid vectors are usually introduced into recipient cells by genetic transformation. Transformation of *E. coli* cells, based on the joint incubation of "competent" bacterial cells (cells capable of transformation) and DNA, has become especially widespread for cloning vector DNA. As a result of transformation, DNA is "absorbed" by bacterial cells and autonomously reproduces in their cytoplasm (the internal environment of the cell). On a selective medium, transformed bacterial cells are selected that carry a certain selective marker that was already on the vector or should have appeared during the formation of the recombinant molecule. If, for example, the vector contained a gene for resistance to the antibiotic ampicillin, then this antibiotic is added to the selective medium and all surviving cells will contain this vector. In order to determine whether the transformed cells carry recombinant DNA, the vector plasmid is isolated from the cells and subjected to electrophoresis. The electrophoresis method is based on the principle of movement of substances in an electric field from one pole to another at a speed depending on their size. Using this simple technique, it is possible to separate, identify and purify fragments of vector DNA of different molecular weights in an agarose gel.

Transfer of a gene (or transgene construct) into a cell and its incorporation into the DNA of the recipient organism. The main method of transferring genes (gene constructs) from the cells of a donor organism to the cells of a recipient organism is the transformation process. Transformation includes several main stages and requires a number of conditions: the presence of transforming DNA; "competent" cells; integration of donor (transforming) DNA into the recipient's DNA and expression

(work) of the transferred genes. There are various methods of transformation: by hybridization of somatic cells; incubation of recipient cells with foreign genetic material; microinjection of genetic material into the nuclei of animal cells, etc. Their use depends primarily on the biological characteristics of the recipient organism. For example, two main methods are used to transform plant cells.

Transformation of a plant using the so-called Ti-plasmid, which carries the "target" gene, which is delivered to the cells by a soil bacterium (*Agrobacterium tumifaciens*). Ti-plasmid is a ring-shaped DNA molecule contained in the cells of *Agrobacterium tumifaciens*, which causes the formation of tumors in plants when they are infected with this bacterium. When plants are infected with bacteria, a small fragment of the Ti-plasmid is integrated into the genome of plant cells, causing a hormonal imbalance and a transition to uncontrolled division and growth, which leads to the formation of a tumor. The "target" gene, capable of changing a particular plant property, is genetically engineered into a Ti plasmid, which is then transferred to an agrobacterium. During the joint cultivation of an agrobacterium and a host plant cell culture, the Ti plasmid enters the plant cells, and the "target" gene with additional DNA fragments is integrated into the plant genome. Each such cell can then be regenerated into a whole transgenic plant, which will contain genetic information from two or more different organisms. This method is used to transform dicotyledonous plants.

The method of biological ballistics. In this case, DNA containing the "target" gene is sprayed onto tiny particles of tungsten or gold. These particles with DNA are then placed in a so-called gene "gun". As a result of the "shot", they "bombard" plant cells at a tremendous speed, penetrating their cytoplasm and nuclei. Some of these cells integrate the "target" gene into their DNA. A new transgenic plant can be regenerated from each such cell.

Identification of transgenic cells (organisms). The process of transferring and incorporating foreign DNA into the genetic material of plant cells occurs with a fairly low frequency, in the best case 1 cell in 1000 is transformed. Therefore, it is necessary to somehow separate such cells from the rest, to create the most favorable

conditions for their division and development. In this case, together with the “target” gene (e.g., resistance to herbicides, viruses and insect pests), a second, so-called selective gene is introduced. Most often, antibiotic resistance genes are used for this. If, after the introduction of foreign DNA, the cells are placed on a nutrient medium with an antibiotic, then only transformed cells will be able to grow on it.

2.1. Creation and use of GMOs

Stages of GMO creation:

- identifying a donor organism for the target gene;
- isolation and identification of the gene or its artificial synthesis;
- synthesis of the genetic construct and its ligation with a cloning vector resulting in a “vector-target gene” construct;
- genetic modification – transferring the transgene into living cells;
- selection of transformed cells;
- cloning (multiplication) of GMOs and their subsequent use for the intended purpose.

Methods for Creating Recombinant DNA Molecules.

Artificial synthesis using DNA synthesizers. Recombinant technology which involves cutting the DNA molecule into specific fragments and joining them in the desired combination. The tools for genetic engineering manipulations are enzymes: restriction enzymes, ligases, terminal transferases, etc. (see Table 2.1 and Figure 2.1). In the creation of recombinant DNA, linkers (artificial restriction sites) play an important role. These are oligonucleotides with a restriction site for a specific restriction enzyme. They can be attached to the "blunt" ends of a DNA fragment using DNA ligase. After treatment with the appropriate restriction enzyme, the DNA fragment will have “sticky” ends compatible with the ends of DNA cut by the same restriction enzyme (see Figure 2.2).

Table 2.1. Nucleotide Sequences Recognized by Some Restriction Enzymes and the Nature of the DNA Ends Obtained Using Them

Restriction enzyme	Restriction site	Nature of DNA ends cut off
EcoRI	G ↓ A-A-T-T-C C-T-T-A-A ↑ G	"Sticky" ends with a 5'-phosphate group
BamHI	G ↓ G-A-T-C-C C-C-T-A-G ↑ G	-»-
PstI	C-T-G-C-A ↓ G G ↑ A-C-G-T-C	-»-
Sau3AI	↓ G-A-T-C C-T-A-G ↑	-»-
NotI	G ↓ C-G-G-C-C-G-C C-G-C-C-G-G-C ↑ G	-»-
PvuII	C-A-G ↓ C-T-G G-T-C ↑ G-A-C	"Blunt" ends
HpaI	G-T-T ↓ A-A-C C-A-A ↑ T-T-G	-»-
HaeIII	G-G ↓ C-C C-C ↑ G-G	-»-

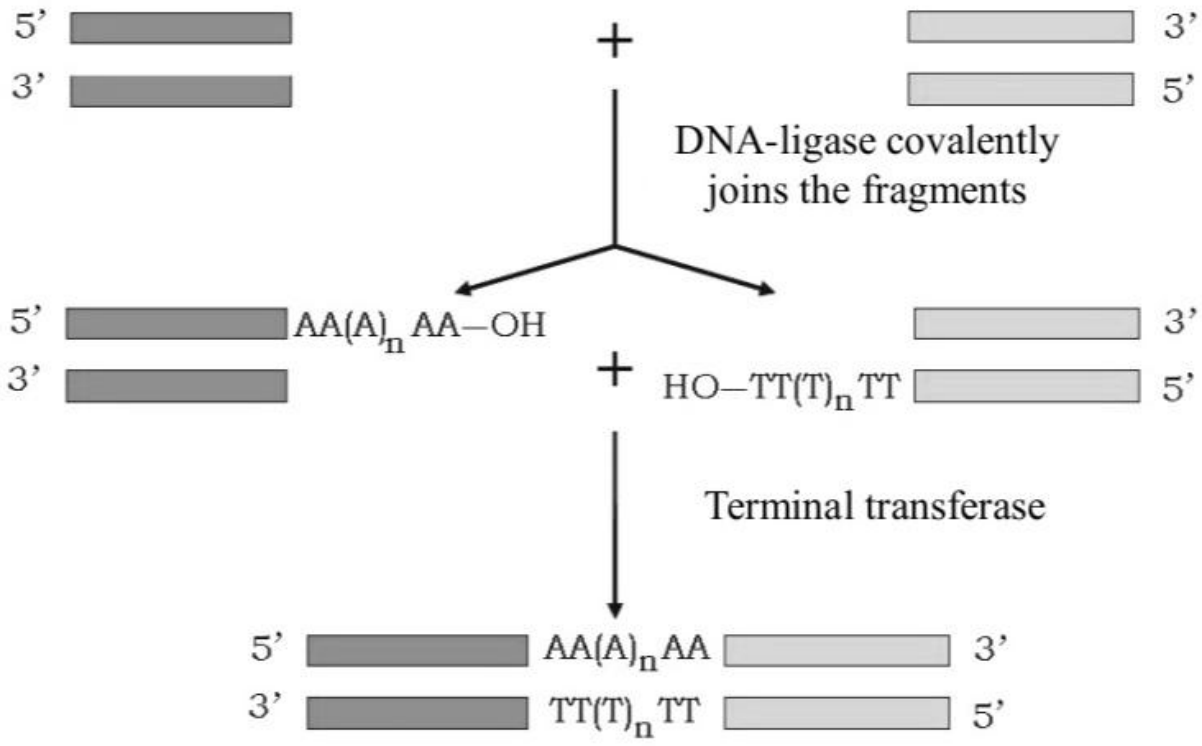

Figure 2.1. Ligation of Two DNA Ends with "Blunt" Ends by Attaching poly-dA or poly-dT [56]

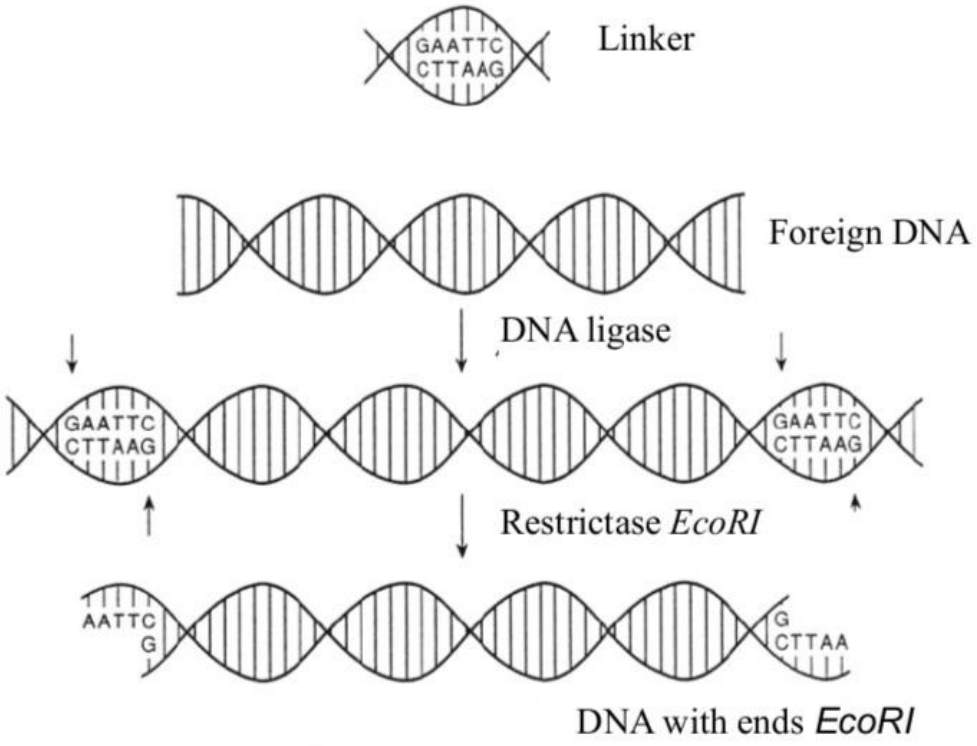

Figure 2.2. Use of Linkers for Creating Recombinant DNA [56]

Electrophoretic Spectrum of Restriction Fragments. A restriction enzyme makes a specific number of cuts at defined locations in the DNA molecule of the analyzed organism (strict specificity). During electrophoresis, these fragments remain intact and can be isolated (eluted) from the gel as biologically active double-stranded molecules. An example of an electrophoretic spectrum of restriction fragments is shown in Figure 5.3.

Figure 2.3. Electrophoretic spectrum of DNA restriction fragments [56].

Vectors (carriers) of the target gene. Bacterial plasmids. Requirements for a plasmid vector: small size (for *E. coli*, no more than 15 kbp); presence of a unique restriction site for insertion; presence of one or more selective genetic markers for

identifying transformed cells. Typically, the cleavage site of plasmid DNA is located within the structural sequence of one of its marker genes, which allows an increased efficiency in selecting transformed cells (Fig. 2.4).

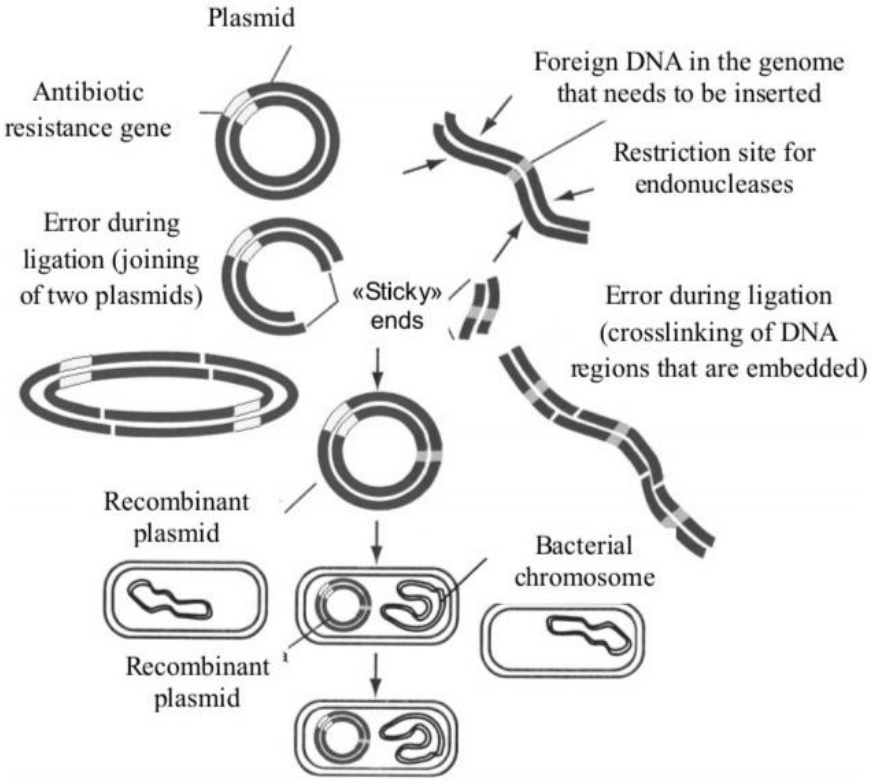

Figure 2.4. Introduction of a recombinant construct into a bacterial cell using a plasmid [56].

Ti-plasmids of Agrobacterium bacteria. This phenomenon refers to the irreversible transfer and incorporation of a specific fragment (T-DNA) of the *A. tumefaciens* or *A. rhizogenes* plasmid into the chromosomal DNA of a plant cell. Genes located on the T-DNA fragment of the bacterial plasmid are actively expressed in the plant cell, leading to the synthesis of large amounts of specific compounds called opines – octopines and nopalines. Additionally, various phytohormones are produced in high concentrations in the cells, sharply stimulating growth processes. The structure of Ti-plasmids of various types is shown in Fig. 2.5.

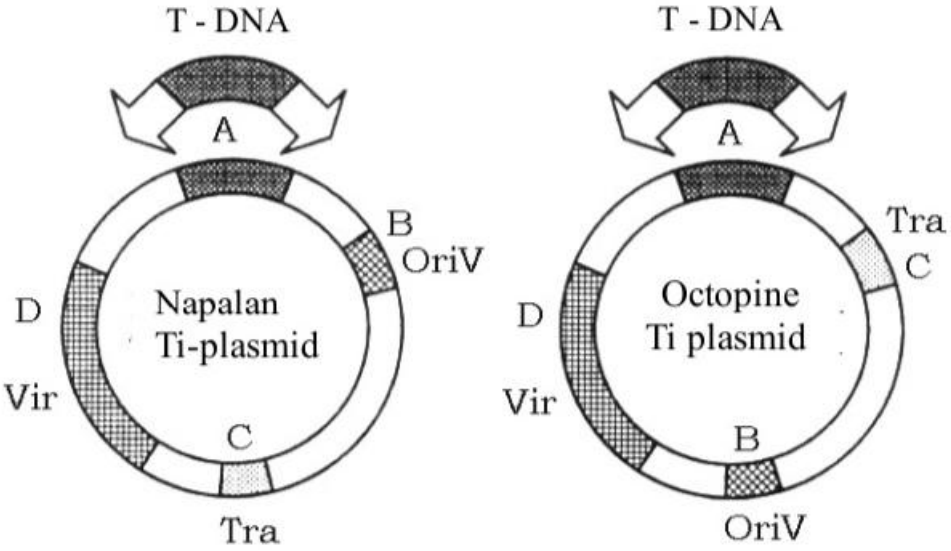

Figure 2.5. Nopaline and octopine Ti-plasmids (*A. tumefaciens*). Nopaline and octopinae plasmids are 30 % homologous. T-DNA (≈2 3 kbp) – the region transferred into the plant, carries oncogenes and genes for the synthesis and catabolism of opines (A); Vir – the virulence region (D); Tra – genes for conjugative transfer (C); OriV – the plasmid replication region.

Viruses and phages. Some viruses do not lead to cell death but integrate into the host cell's genome and replicate with it, or cause uncontrolled cell proliferation. Such viruses include the SV-40 DNA virus and the polyoma virus. Integration of certain oncogenic RNA viruses leads to the budding of viral particles from the cell without its lysis. Examples of such viruses include retroviruses. This tool is mainly used in gene therapy research rather than in molecular biology. Bacteriophages are often used as vectors for bacterial cells. DNA fragments up to 20 kbp can be cloned using such vectors [57].

Cosmids and phasmids. There are hybrid vectors that contain both phage and plasmid DNA, namely cosmids and phasmids. Cosmids are plasmid vectors into which a segment of the λ phage genome has been inserted allowing the DNA molecule to be packaged into a phage particle. Phage particles provide efficient penetration of the hybrid DNA into the cell (via injection), after which the DNA is circularized at the "sticky" ends and replicates in a plasmid-like manner. Phasmids are also hybrids between phages and plasmids; after inserting foreign DNA, they can

develop as phages under certain conditions and as plasmids under other ones. Using such vectors, DNA fragments up to 40 kbp can be cloned.

Chimeric constructs, which are plasmid vectors based on phage P1, allow the cloning of DNA fragments ranging from 100 to 300 kbp.

Gene transfer methods.

Electroporation – the application of an electric current to bacterial cells, which creates temporary pores in the cell wall, allowing DNA to enter the cell. This increases transformation efficiency by approximately 100 times.

Biolistic transformation. The smallest particles (0.4-1.7 μm in diameter) made of tungsten or gold are coated with recombinant DNA. These particles are accelerated to speeds of 300-600 m/s in a special pneumatic device ("gene gun") and directed at plant tissues or cell cultures. As a result, the particles penetrate the cells, and foreign DNA integrates into the chromosomes or organellar DNA.

DNA microinjection (for animal cells). There are several variations of this method:

- microinjection of DNA into the pronucleus (nucleus) of a single-cell embryo (zygote) – this method is inefficient and very expensive;
- injection of DNA into the cytoplasm of embryonic cells, which is characterized by low efficiency due to the destructive action of endonucleases but is relevant for birds and vertebrates due to the opacity of their pronuclei;
- use of germ cells (gametes), involving the incubation of isolated sperm in a solution of foreign DNA, followed by their use for fertilization *in vivo* or *in avitro*.

The basic scheme of animal transgenesis using DNA microinjection is shown in Fig. 2.6.

Agrobacterium-mediated transformation. *A. tumefaciens* and *A. rhizogenes* are natural genetic engineers of plants. The process of Agrobacterium transformation is initiated by plant injury. The wounded cells release various chemical compounds into the environment, attracting Agrobacteria (positive chemotaxis). The bacteria attach to the plant at the wound sites using cellulose fibrils (Fig. 2.7).

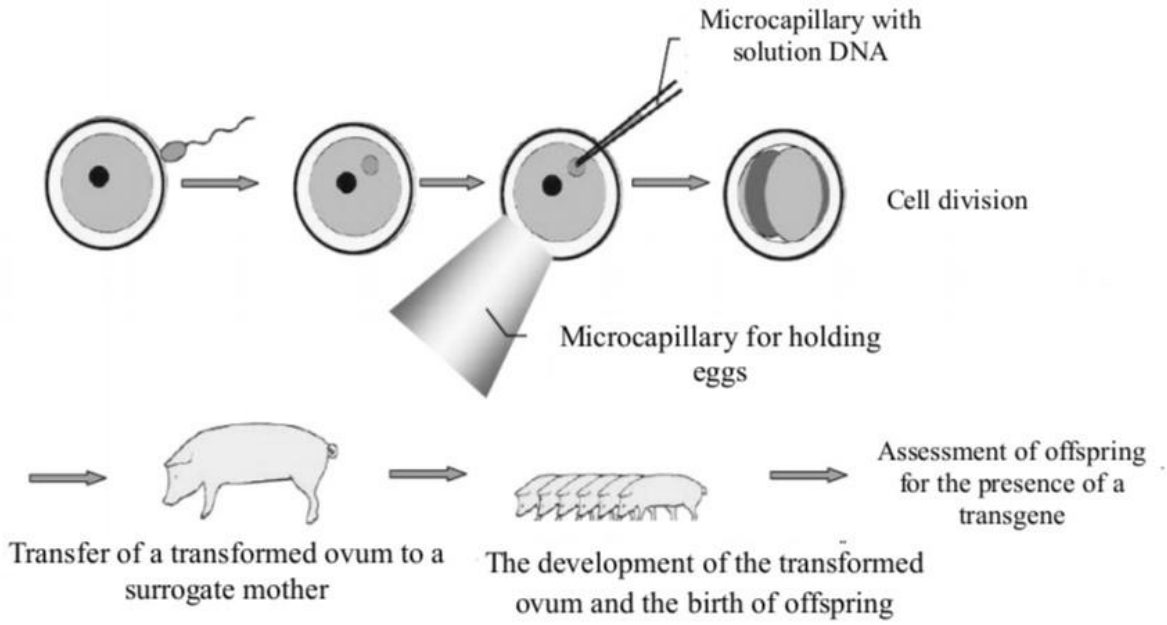

Figure. 2.6. Scheme of animal transgenesis using DNA microinjection [59].

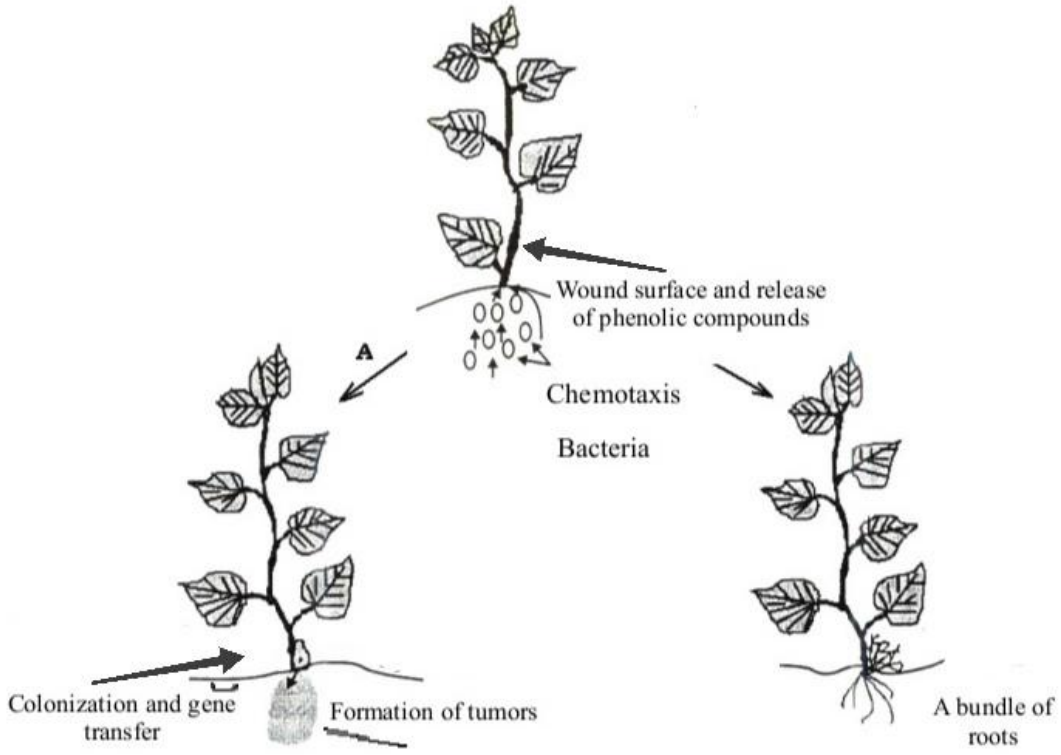

Figure. 2.7. Genetic transformation of plants: A – *A. tumefaciens*, B – *A. rhizogenes*

T-DNA contains genes for the synthesis of unusual amino acids and carbohydrates (opines), which serve as nutrients for Agrobacteria, as well as phytohormone genes that induce tumor-like growth in plant tissues. Receptors on the

surface of the bacterial cell (Fig. 2.8a) respond to the breakdown products of the plant cell wall, leading to the activation of vir genes (Fig. 2.8b), whose products (Fig. 2.8c) replicate (Fig. 2.8d) and transfer T-DNA into the recipient cell (Fig. 2.8e). Agrobacterium-mediated transformation is widely used in plant genetic engineering, but the so-called binary vector system is more commonly used. This system consists of a plasmid containing T-DNA, where the genes for opine and phytohormone synthesis have been replaced by the target transgene, and a separate helper plasmid carrying only vir-genes [60].

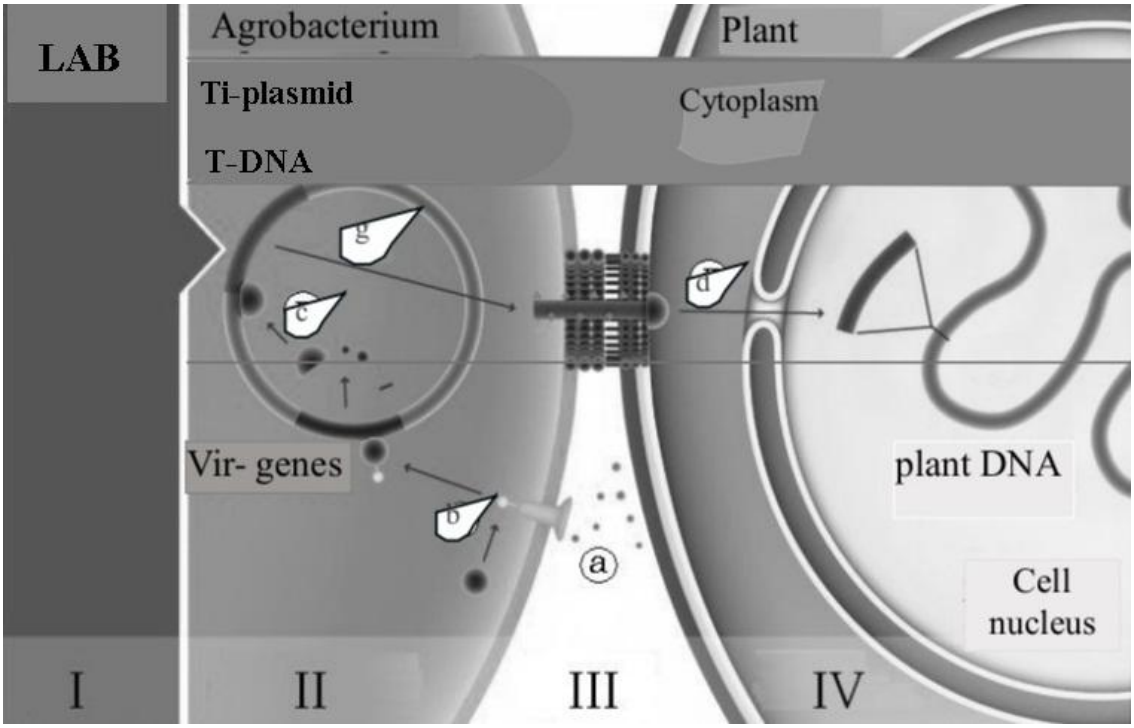

Figure. 2.8. Scheme of Agrobacterium-mediated transformation: I – Creation of a Ti-plasmid with the desired gene, II – Copying of T-DNA, III – Transfer of T-DNA into the plant cell, IV – T-DNA integrates into the genomic DNA (additional explanations in the text).

Viral transduction is the process of transferring bacterial DNA from one cell to another by a bacteriophage. Both temperate and virulent phages are capable of transduction; however, virulent phages destroy the bacterial population, so transduction with their help is of little significance in nature or research. There are two types of transductions: general (nonspecific) and specific.

General (nonspecific) transduction. This process is carried out by phage P1, existing in bacterial cells as a plasmid, and by phages P22 and Mu, which integrate into any region of the bacterial chromosome. After prophage induction, with a probability of 10^{-5} per cell, there may be an erroneous packaging of a bacterial DNA fragment into the phage capsid, in which the phage DNA itself is absent. The length of this fragment equals that of the normal phage DNA, and its origin can be random: a segment of the chromosome, a plasmid, or other temperate phages [61].

When this DNA fragment enters another bacterial cell, it can integrate into the genome, usually through homologous recombination. Plasmids transferred by the phage can circularize and replicate within the new cell. Sometimes, the DNA fragment does not integrate into the recipient's chromosome, nor does it replicate, but it is retained in the cell and transcribed. This phenomenon is called abortive transduction.

Specific transduction. The most well-studied example of specialized transduction involves phage λ. This phage integrates only into one region (att site) of the *E. coli* chromosome with a specific nucleotide sequence (homologous to the att site in phage DNA). During induction, its excision may occur with an error (probability 10^{-3} to 10^{-5} per cell), where a fragment of the same size as the phage DNA is cut, but starting from the wrong location. As a result, some phage genes are lost, while some *E. coli* genes are captured by the phage. The likelihood of gene transfer decreases as the distance from the gene to the att site increases.

For each temperate phage that specifically integrates into the chromosome, its own att site is characteristic, and accordingly, the genes located near it are capable of transferring. Some phages can integrate at any location on the chromosome and transfer any genes through the mechanism of specific transduction. Moreover, the chromosome usually contains sequences partially homologous to the att site of the phage's DNA. When the fully homologous att site is damaged, it is possible to achieve phage integration into the chromosome through these sequences and transfer genes located next to them during specific transduction.

When a temperate phage carrying bacterial genes integrates into the chromosome of a new host bacterium, it contains two identical genes – the host's own and the one introduced from outside. Since the phage lacks some of its own genes, it often cannot induce itself and reproduce. However, when this same cell is infected by a "helper" phage of the same type, the induction of the defective phage becomes possible. Both the DNA of the normal "helper" phage and the DNA of the defective one, along with the bacterial genes it has transferred, exit the chromosome and replicate. Therefore, about 50 % of the formed phage particles carry bacterial DNA. This phenomenon is called high-frequency transduction [62].

2.2. Identification and selection of GMOs

The following methodological techniques are used:

- use of marker genes of the first, second, and third generations: NPT gene (neomycin phosphotransferase and other antibiotic resistance genes), GUS gene (β-glucuronidase), GFP gene (green fluorescent protein);
- selection of cells in media with antibiotics;
- detection of the expression products of reporter genes and / or their activity. The production of the target gene product serves as a proof of its expression. At the final stage of recombinant technology, cloning (multiplication) of the genetically modified organism occurs, allowing its further use.

Hopes associated with genetic engineering and GMOs:

- GMOs as models for fundamental research;
- medical applications: disease prevention (vaccines, including "green" or plant-based vaccines), disease diagnosis, treatment of infectious and genetic diseases (antibiotics, hormones, immunoglobulins, enzymes, etc.);
- breeding: creation of animal breeds, plant varieties, and strains of microorganisms with improved inherited traits, such as increased productivity in plants and animals; improved quality of biomass, raw materials, or final products; creation of microorganisms producing biologically active substances;

development of organisms resistant to pathogens and environmental factors that are beneficial to humans;

- environmental applications: waste recycling to reduce environmental pollution, bioremediation, and bioremediation of contaminated environments.

2.3. Examples of aspects of GMO use

Herbicide resistance allows a transgenic plant to be unaffected by lethal doses of chemicals. As a result, fields are cleared of weeds, and herbicide-tolerant crops survive. Often, companies selling seeds of such plants offer the corresponding herbicides in a bundle. Insect resistance significantly reduces crop losses caused by insect pests. For example, when a Colorado potato beetle eats a leaf of GM potatoes, it dies. Almost all such plants contain an inserted gene of a natural toxin from the soil bacterium *Bacillus thuringiensis* (Bt gene). Pathogen resistance, including resistance to fungi, bacteria, and viruses, is achieved, for example, through chitinase genes or viral coat protein genes. This is one of the most important areas of transgenesis. Forms have been created that are resistant to phytopathogens, *Erwinia carotovora*, potato viruses, and more [63].

Resistance to abiotic stress factors, such as plants with increased cold resistance, salt-tolerant forms, heat-stable plants, and those resistant to heavy metals, etc.

Product quality (improved soybean protein, rapeseed oil with low erucic acid content, cottonwood without “fluff”, forestry cultures, fruits of the correct shape, increased shelf life of fruits, and flowering crops with large flowers and unusual petal colors).

“Green vaccines” and medicines (vaccines against hepatitis B and C, bananas with introduced analgesics, etc.).

2.4. Prerequisites for the emergence of risks associated with the use of GMOs

Genetic engineering fundamentally differs from traditional methods of developing new varieties and breeds. The artificial addition of foreign genes significantly disrupts the finely tuned genetic control of a normal cell. Gene manipulation is fundamentally different from the combination of maternal and paternal chromosomes that occurs during natural crossbreeding.

Currently, genetic engineering is technically imperfect because it cannot fully control the process of integrating a new gene. Therefore, it is impossible to predict the site of integration and the effects of the added gene. Even if the location of the gene can be determined after its integration into the genome, the available information about DNA is very incomplete for predicting the outcomes.

As a result of the artificial addition of a foreign gene, dangerous substances may unexpectedly form. In the worst-case scenario, these could be toxic substances, allergens, or other harmful substances to health. The knowledge about such possibilities is incomplete [64].

There are no completely reliable methods for testing safety. More than 10 % of serious side effects of new drugs cannot be detected despite thoroughly conducted safety studies. The degree of risk that harmful properties of new foods modified through genetic engineering will go unnoticed is likely significantly greater than that of pharmaceuticals.

Knowledge about the effects of GMOs on the environment is insufficient. It has not been proven that GMOs will not have a harmful impact on the environment. There are many opportunities for the uncontrolled spread of potentially dangerous genes used in genetic engineering, including gene transfer by bacteria and viruses. Complications caused in the environment are likely impossible to rectify, as released genes cannot be retracted.

Knowledge about hereditary material (DNA) is incomplete. Only about 3 % of human DNA functions are known. It is risky to manipulate complex systems about

which knowledge is incomplete. Extensive experience in biology, ecology, and medicine shows that this can lead to serious unforeseen problems and disorders.

New and dangerous viruses may emerge. Experimental evidence has shown that genes from viruses integrated into the genome can connect with genes of infectious viruses. Such new viruses may be more aggressive than the originals. Viruses can also become less species-specific. For example, plant viruses could become harmful to beneficial insects, animals, and even humans.

Possible negative consequences of GMO use:

- unpredictability of the consequences of transgene expression due to the pleiotropic effects of genes;
- potential danger (toxicity, allergenicity, etc.) of products whose biosynthesis is controlled by transgenes that have undergone mutagenesis;
- shortage of transgenic plant seeds and their significant price increase due to terminator technologies (geopolitical conflicts);
- decrease in the varietal and species diversity of cultivated crops due to the mass introduction of higher-yielding, and often more profitable, varieties of transgenic plants;
- emergence of harmful mutant organisms (e.g., weeds) with unpredictable properties due to spontaneous transgene transfer in the agrosphere;
- impact on non-target insects and other animals (including those beneficial to humans). Negative effects of expressed entomotoxins on the rhizosphere microbiota of transgenic plants;
- horizontal transfer of transgenes to cultivated and wild plants;
- appearance of new, more dangerous viral pathogens due to the evolution of phytoviruses as a result of interaction with the genetically modified product of a virus-resistant transgenic plant.

Biosafety of GMOs as a clear necessity. GMOs and bio-threats raise issues of biosafety. These issues are particularly pressing at this time, as biotechnology and bioengineering are becoming some of the most important scientific and technical priorities of the 21st century. GMOs have been grown for sale since 1996. The total

area of GM crop cultivation exceeds 100 million hectares. The activities of corporations producing GMOs are not limited to agriculture; they are gradually taking over the pharmaceutical market, the forestry business, and are being introduced into aquaculture and other areas [66].

Biosafety refers to the state of protection for humans, society, and nature from potential harmful effects caused by GMOs and other biological objects, as well as the products and substances derived from them. Ensuring the biosafety of humans and the environment requires the development and implementation of a reliable system of state measures, encompassing scientific, technological, legislative, and legal frameworks.

2.5. Risk assessment of GMOs and legal regulation

2.5.1. Principles of risk assessment of possible undesirable effects of GMOs on human health and the environment

Principles of risk assessment for potential undesirable effects of GMOs.

Scientific basis of the assessment. The assessment must be scientifically grounded. It should take into account information published in scientific and technical literature, data from specialized databases, test results, information regarding the previous use of GMOs, expert conclusions, and methodological recommendations developed by international and national organizations.

Individual risk assessment. The risk assessment is conducted on a case-by-case basis. The information required to conclude about the safety of a GMO may vary in nature and level of detail, depending on the specific GMO, its intended use, and the potential receiving environment.

Substantial equivalence principle. Risks associated with GMOs should be considered in the context of risks that exist with the use of unmodified recipient organisms in the same potential receiving environment. Traditional breeding methods can also produce varieties, breeds, or strains that may pose risks to human health and

the environment. To determine the specific effect of genetic modification, GMOs must be compared with the original, conventional variety.

Methodology for assessing the risk of potential negative effects of GMOs. The methodology includes the following steps:

- identification of any new genotypic and phenotypic characteristics associated with the presence of transgenes that could cause undesirable effects of GMOs on human health and the environment;
- assessment of the likelihood of negative consequences based on the intensity, duration, and nature of the impact of the GMO on humans or the potential receiving environment;
- assessment of consequences in the event that negative (undesirable) effects occur;
- assessment of the overall risk posed by GMOs based on the evaluation of the likelihood of occurrence and consequences of undesirable effects;
- formulation of recommendations on whether the risks are acceptable or manageable; and defining strategies to regulate such risks.

2.5.2. Nature of risks to human health and the environment related to GMOs

Risks associated with transgenic products. A typical new transgenic construct is shown in Figure 2.9.

A transgenic plant variety differs from the original only in that a relatively small DNA fragment, encoding information for 1-2 new genes and their regulatory elements, is added to its genetic material, which contains 25-30 thousand existing genes. The activity of the introduced genes manifests in the biosynthesis of 1-2 new proteins for the organism – either enzymes or structural proteins. Since genetic engineering can work with any genes that exist in nature, not only with genes from organisms evolutionarily close to specific crop species, which is characteristic of

traditional breeding, the products of the introduced genes (enzymes, proteins) may appear in the genetically modified organism as unusual, foreign, and unnatural for that species. These gene products, which do not occur naturally in the species, represent the most significant and tangible risk factors associated with genetically engineered organisms [67].

LB	Promoter of a selective gene	Selective gene	Terminal sequence of a selective gene	Promoter of a trans gene	Trans gene	Terminal sequence of a transgene	RB

Figure 2.9. A typical transgenic construct used in plant genetic engineering: LB - left border, RB - right border: DNA fragments containing 25 nucleotide pairs from the Ti-plasmid of *Agrobacterium tumefaciens*, which are necessary for transferring transgenic constructs into plant cells through the *Agrobacterium transformation* method. In many genetic constructs, the selective gene and its regulatory elements are absent.

At the same time, not all recombinant proteins in GMOs are entirely foreign or unnatural for a particular species. *Firstly*, there is a fairly large group of transgenic plant varieties that were created through genetic manipulation of their own genes (e.g., tomatoes with extended shelf life; soybeans and canola with improved oil composition; potatoes with enhanced starch quality; coffee with reduced caffeine content; tobacco with lower nicotine content). *Secondly*, many organisms that are quite distant from each other in evolutionary terms share a large number of identical metabolic pathways, and accordingly, the composition and structure of the enzymes responsible for these processes are also identical. *Thirdly*, modern scientific data on the structure of the genetic material of humans, some animals, and plants have significantly expanded our understanding of the similarities and differences between the genes of various taxonomic groups and the likelihood of their horizontal transfer. It was discovered that the genome of the plant *Arabidopsis* contains about a hundred human genes, including genes such as the breast cancer gene! The soil bacterium *Agrobacterium tumefaciens* regularly transfers part of its genes into plants, causing

them to form a tumor known as a crown gall. This is a completely natural process, which genetic engineers successfully use [68].

Thus, the exchange of genetic information between distant species occurs constantly in nature. In some cases, it takes millions of years, while in others (such as Agrobacterium transformation), it can happen daily and even hourly. Nevertheless, when planning to introduce a new gene into a plant, microbe, or animal, it is necessary to carefully study the gene itself, as well as the product of its activity, and ensure their safety.

Risks associated with the insertion of transgenes into an organism's genetic material. There are reasons to believe that the insertion of transgenes may occur randomly, meaning they could integrate into almost any region of the DNA molecule within a cell. *This poses a risk that the introduced gene could disrupt areas of DNA that code for structural or regulatory elements of other genes* (see Fig. 2.10). *However, the probability of this happening is generally not very high*. The genetic material of higher organisms is organized in such a way that less than 10% of the DNA molecule's length is occupied by genes and their regulatory elements, which increases the stability and resistance of the DNA molecule to external influences. This means that genes within the DNA molecule are not densely packed one after another but are separated by large gaps filled with non-coding nucleotide sequences. Moreover, even within the coding sequences of genes, there are introns, which also carry no genetic information. These introns are spliced out during the "maturation" of the messenger RNA molecule formed during gene transcription.

Nevertheless, there is still a chance that a transgene could insert into a region of DNA already occupied by another gene. If the insertion disrupts the coding region of the damaged gene (arrow 1 in Fig. 2.10), the product of that gene will not be produced. Obviously, a similar outcome would occur if the promoter or terminal sequences were damaged (arrows 2 and 3 in Fig. 2.10). If the disrupted gene plays a crucial role in the organism, the absence of its product could have serious consequences, potentially leading to loss of viability. Clearly, genotypes with damaged genes cannot reach the level of commercial crop varieties.

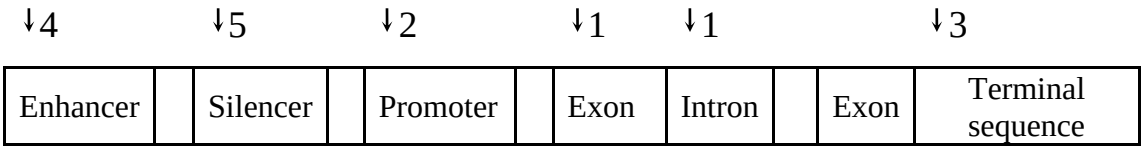

Figure. 2.10. Possible sites for transgene insertion into the plant genome

If other regulatory elements, such as enhancers (which increase gene activity) or silencers (which decrease it), are disrupted during the insertion process, this could lead to changes in the activity of the affected genes. For instance, plant varieties that produce toxic compounds (such as solanine in potatoes) at concentrations harmless to human health could, as a result of genetic modification, increase the synthesis of these compounds to levels exceeding safe limits. Such genotypes then become hazardous to health. The risks associated with the negative effects of vertical or horizontal gene transfer. Genes and their products, harmless in GMOs, may prove to be quite dangerous in a different genetic and ecological environment. For instance, if pathogenic bacteria in the digestive tract acquire antibiotic resistance, it could significantly complicate the treatment of diseases they may cause [69].

The risks associated with the negative effects of vertical or horizontal gene transfer. Genes and their products, harmless in GMOs, may prove to be quite dangerous in a different genetic and ecological environment. For instance, if pathogenic bacteria in the digestive tract acquire antibiotic resistance, it could significantly complicate the treatment of diseases they may cause.

2.4. Potential negative effects of GMOs on human health, methods of assessment, and prevention strategies

Commercial use of GMOs has only recently begun. It is very difficult to assess the impact of GMOs on the environment in such a short period of time. The behavior of new genes in open ecosystems, their response to parasites and diseases are completely unpredictable. However, the relationship between agricultural and

environmental risks is very clearly visible. This relationship is determined by the same sources of potential and real risks. Such sources can be considered the increasingly widespread use of pesticides and genetically modified organisms in agricultural production. The most significant agricultural and environmental risks include.

Reduction in the diversity of traditional (native) plant varieties and animal breeds. The spread of GMOs leads to the displacement of other varieties and breeds, and therefore to a decrease in varietal (breed) biodiversity. This diversity is the basis of sustainable agriculture.

Reduction of species diversity. The production of GMOs leads to a reduction in the species diversity of plants, animals, fungi and microorganisms living in and around the fields where they are grown. Fast-growing species of transgenic organisms can displace ordinary species from natural ecosystems [70].

Uncontrolled transfer of genes, especially genes determining resistance to pesticides, pests and diseases, due to cross-pollination with wild relatives and ancestral species. As a result, a decrease in the biodiversity of wild ancestral forms of crop plants and the formation of “superweeds”.

The widespread use of broad-spectrum herbicides (e.g., glyphosinate or glyphosate) will lead to the depletion of the species composition of beneficial entomo- and avifauna (insects and birds) and the destruction of agrobiocenoses.

Depletion and disruption of natural soil fertility. GMO crops with genes that accelerate plant growth and development to a much greater extent than conventional ones deplete the soil and disrupt its structure. As a result of the suppression of the vital activity of soil invertebrates, soil microflora and microfauna by GMO plant toxins, the natural fertility of the soil is disrupted.

For a better understanding of the methodology for assessing the impact of genetically modified organisms on the environment and health, a two-volume manual can be recommended.

One of the most discussed and controversial issues related to the use and distribution of GMOs is the problem of the potential impact of GMO products

(products obtained from GMOs or ingredients containing them) on human health. The situation is aggravated by the fact that a full range of studies on the impact of GMOs on the human and animal body has not yet been conducted. An assessment of the nutritional risks from consuming GMO products is currently possible only on the basis of fragmentary data and scattered scientific facts. According to experts, in order to identify all the risks associated with GMOs, it is necessary to study the consequences of growing and breeding GMOs in all conditions, as well as the impact of GMO products on all groups of living organisms (animals, plants, fungi and protozoa), to track possible genetic, teratological, immunological and endocrinological changes in all organ systems, all ethnic and age groups of people. It is impossible to conduct such studies either theoretically or practically.

That is why many scientists fear that the use of GM Oproducts in food increases the risk of food allergies, poisoning, mutations, promotes the formation of tumors, and also causes resistance to antibiotics.

GMO products can be divided into three categories [71]:

- products containing GMO ingredients (mainly transgenic corn and soybeans). These additives are added to food products as structuring, sweetening, coloring agents, and also as substances that increase protein content;
- products from the processing of transgenic raw materials (for example, soy curd, soy milk, chips, corn flakes, tomato paste);
- transgenic vegetables and fruits, and in the near future, possibly, animals directly consumed for food.

In addition, food products made from animals fed on GMO products should probably be classified as a separate category. The most likely potential food risks associated with GMO products include [72, 73].

Direct action of toxic and / or allergenic transgenic GMO proteins on humans and other warm-blooded animals.

Food allergies are quite common and are steadily growing among the population of developed countries. This is primarily due to the unfavorable environmental situation, changes in traditional diets and modern food industry

technologies, leading to an increased content of chemical additives and preservatives in food. As a rule, transgenic proteins that provide resistance to various types of insects, fungal or bacterial diseases have an allergenic or toxic effect.

Thus, a number of publications discuss the allergenic effect of transgenic chitinase proteins that are capable of destroying the chitinous walls of pests (insects and fungi). Chitinase genes have modified various varieties of rice, potatoes, wheat and other crops. At the same time, the so-called “banana allergie” are well known, the main allergens in which are chitinases of avocado, bananas, and chestnuts.

Thus, narcissus lectin, which has pronounced insecticidal properties, is a mutagen, and the strongest mutagenic effect has been shown on lymphocyte cultures (cell elements of the blood) of human embryos.

Lectin proteins were among the first transgenes in the formation of resistance to insect pests. By binding to the surface of cells, they lead to their adhesion and disruption of the physiological functions of the body. This property of plant lectins is associated with a 40-year history of their use as cytotoxic drugs in cancer chemotherapy. The work carried out with transgenic insecticidal lectins of Brazil nuts (*Bertholletia excelsa*) was stopped due to their high allergenicity. Chitin-binding lectins from wheat and bean sprouts have enormous insecticidal potential, but are toxic to mammals.

A number of transgenic varieties of corn, tobacco and tomatoes that are resistant to insect pests produce lignin, a substance that prevents plant damage. It can decompose into toxic and mutagenic phenols and methanol. Therefore, an increase in the lignin content in the fruits and leaves of plants is dangerous for humans.

Potential health risks associated with GMOs. Synthesis of new proteins: transgenic organisms may produce proteins that are novel to the recipient organism, and these proteins could be toxic and / or allergenic.

Altered gene activity: the insertion of foreign DNA may change the activity of certain genes, leading to diminished consumer quality of food products derived from these organisms. For example, genetically modified (GM) products could have higher

levels of toxic or allergenic substances compared to their non-modified counterparts, exceeding established safety limits.

Horizontal gene transfer: transgenes, particularly antibiotic resistance marker genes, could potentially be transferred to other organisms, including gut microorganisms, leading to unintended health consequences.

When discussing human health risks associated with GMOs, the primary concern is the risks posed by consuming products derived from or produced by GMOs (e.g., milk from genetically modified cows).

The safety assessment strategy for genetically modified food is based on the principle of **"substantial equivalence"**, developed by the OECD (Organization for Economic Co-operation and Development). Based on this principle, it is not the safety of new food products that is assessed, but the difference between the same and traditional food analogues, which is the basis for the history of safe food production.

A new product (plant variety) can be:

1) equivalent in essential features to the selected analog;

2) equivalent to the analogue, with the exception of one (several) significant features that are well defined;

3) not equivalent to the analogue in essential features.

In the 2nd and 3rd cases, the safety assessment of GMO features different from the original analogue is carried out according to the following indicators:

- potential toxicity;
- potential allergenicity;
- the possibility of transferring transgenes (primarily antibiotic resistance genes) from GMOs to the microflora of the digestive tract of humans and animals;
- the possibility of potential deterioration of the nutritional value and assimilation of nutrients.

Assessment of potential toxicity of new food products. If the non-analog test substance is a plant food component with a long history of safe use, new product toxicity studies are not required. In other cases, the following are carried out:

- determination of the concentration of potential toxins in edible parts of plants;

- establishing the specific gravity of this product in the diet of certain population groups;
- comparison (for proteins) of their amino acid sequence with that of known toxins and food antagonists (for example, protease inhibitors) according to electronic databases;
- assessment of the stability of new substances before heat treatment;
- determining the rate of destruction of potential toxins in the gastrointestinal tract (in model systems);
- analysis of the level of toxicity of new substances in model systems (cell culture *in vitro*);
- toxicity analysis in experiments on forced feeding of laboratory or domestic animals with food containing products obtained from the researched GMO or its new components for a long time (chronic experiment – duration of 1-2 years) or for a short time, but using high concentrations researched products (acute experiment – duration of about 2 weeks, concentration of the researched transgene product up to 5 g per 1 kg of animal weight).

Assessment of the allergenic potential of transgene products. The scheme developed by WHO and FAO (Food and Agriculture Organization of the United Nations) experts is used (Fig. 2.11).

Among the wide variety of transgenic crops, only a few produce compounds that are truly novel and not typical for conventional varieties of the same species due to genetic modification. Examples include the enzymes phosphinothricin acetyltransferase and neomycin phosphotransferase, which deactivate the herbicide glufosinate ammonium and aminoglycoside antibiotics (such as kanamycin and neomycin), respectively. However, even substances with a long history of safe use undergo thorough testing. Table 2.2 presents some characteristics of transgenic proteins widely found in genetically engineered crops approved for use in agriculture.

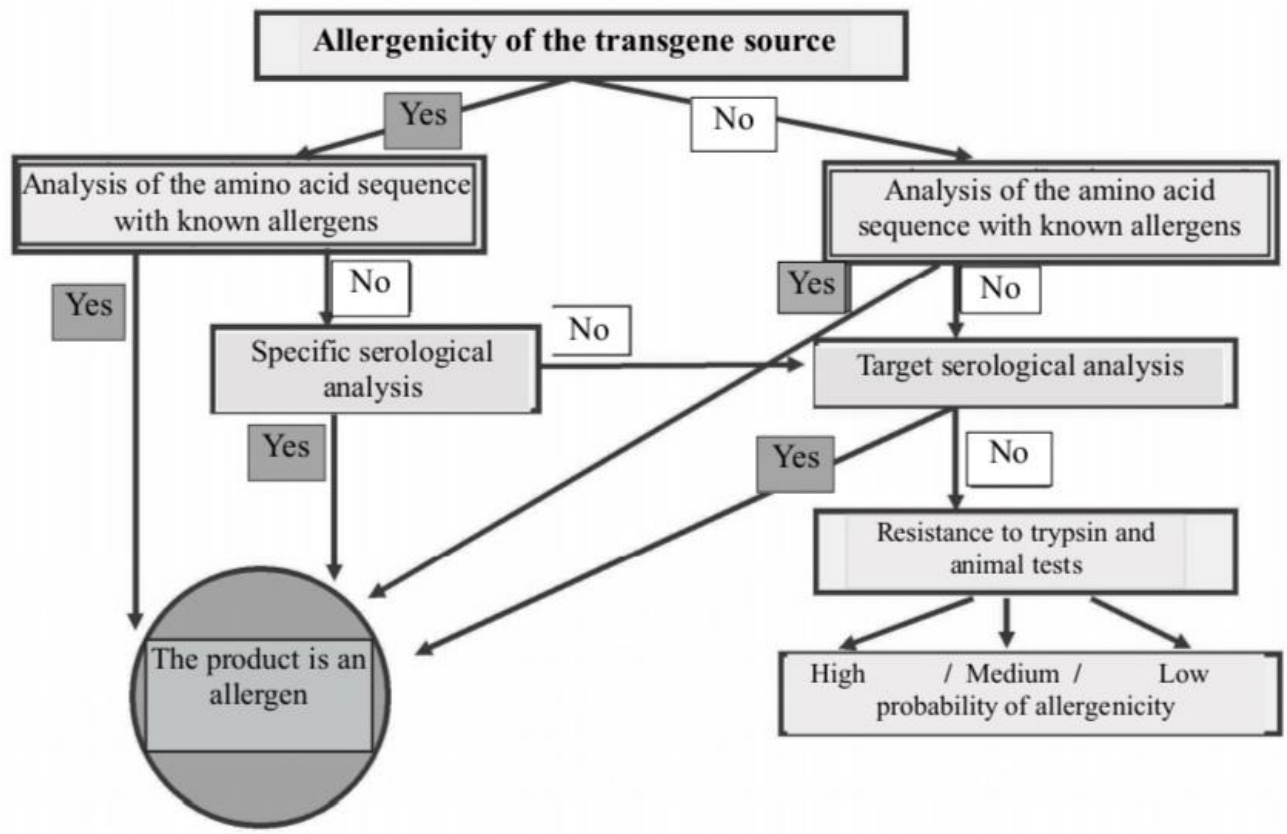

Figure 2.11. Scheme for Assessing the Allergenicity of Transgenic Products

Most transgenic proteins are unstable compounds: they easily denature at relatively low temperatures (breaking down during the processing of plant materials) and in acidic environments. This indicates a low likelihood of these proteins causing allergic reactions. After all, allergens are characterized by the following characteristics: resistance to the action of digestion, to processing, molecular weight 10-70 kDa, content in food more than 1 %. In order for an allergic reaction to develop, the protein must enter the small intestine in an almost unchanged state (it is absorbed into the blood there)

Table 2.2. Characteristics of proteins produced by some transgenes [74]

Protein	pH of denaturation	Temperature of denaturation	Concentration in tissues	Hour of digestion	
				in the gastric juice	in the duodenal juice
NPT II	–	–	Potatoes (potatoes) – 2.7 μg/g	<10 c (50%); 20 min (100%)	2-5 min (50%)
EPSPS	5	65 °C, 15 min	Cotton (seed) – 60-70 μg/g	–	<10 min (50%)
PAT	4	75 °C	–	1 min; 10 min (pH 4)	–
CP PVY	–	–	<2 μg/g (12-244 times lower than the natural level)	–	–
CRY I A(b)	–	–	Corn: at the time of flowering 8-16 g/ha; at the end of the growing season <0.8 g/ha; not detected in grain and silage	10 min (1:1000 dilution); 5 min (1:100 dilution); 2 min (90%)	Not digested
CRY IIIA	–	–	Potatoes: leaves - 20-63 μg/g; tubers - 0.1-0.6 μg/g	-»-	-»-

Remarks. The table shows characteristics of the attack proteins: NPT II (neomycin phosphotransferase II) – the product of a selective gene for resistance to antibiotics kanamycin, neomycin, geneticin, seen in the Tp 5 transposon of *E. coli*; EPSPS (5-enolpyruvilshikimate-3-phosphate synthase) is a product of the mutant cp4 gene of Agrobacterium sp. (or aroA for Aerobacter; sm for Salmonella), which provides resistance to the herbicide glyphosate; PAT (phosphinotricin acetyltransferase) is a product of the pat gene from Streptomyces viridichromogenus, which provides resistance to the herbicide glucosinate ammonium; GP PVY (potato virus Y capsid protein) – ensures potato resistance to virus Y; CRY I A(b) (crystalline protein, 5-endotoxin, Bt-toxin, Bt-protein) is a product of the truncated gene of the soil bacterium Agrobacterium tumefaciens v. kurstaki, which ensures the resistance of sprouts to Lepidoptera comas, for example to the larvae of the corn borer Ostrinia nubilalis; CRY IIIA (crystalline protein, 5-endotoxin, Bt-toxin, Bt-protein) is a product of a truncated gene of the soil bacterium Agrobacterium tumefaciens v.Tenebnonis, which ensures the resistance of growths to Coleoptera comas, such as the Colorado potato beetle.

The "–" sign means that there is no data available.

Assessment of the likelihood of potential deterioration of nutritional value and nutrient absorption. In practice, a large number of transgenic forms are usually obtained from samples without visible mutations, which are selected during subsequent traditional breeding. The safety of the selected forms for human health and the environment is then carefully studied. In particular, the content of both nutrients (proteins, fats, carbohydrates, mineral elements, vitamins, etc.) and potentially harmful substances for health in the plant raw materials is analyzed. For a transgenic variety to be allowed for agricultural use, it must not differ significantly from the original variety, except for the trait introduced as a result of transgenesis or for the traits that were the goal of genetic modification (the concept of substantial equivalence) [75].

Horizontal transfer of transgenes from GMOs to the microbiota of the human and animal gastrointestinal tract. In any transgenic construct, in addition to the transgene itself and its regulatory elements, there is usually also a so-called selective or marker gene necessary for the selection of transformed cells. Genes conferring resistance to specific antibiotics (kanamycin, ampicillin, streptomycin) are commonly used as selective genes, which have already lost their significance as antimicrobial agents due to the widespread resistance of microorganisms to these antibiotics. Therefore, the presence of antibiotic resistance selective genes in transgenic constructs is not considered hazardous to human health and the environment; however, alternative selective systems are currently being developed. Increasingly, herbicide resistance genes are being used as selective genes (although ecologists fear this may lead to an increase in herbicide-resistant weeds), non-toxic carbohydrates (such as xylose, mannose, 2-deoxyglucose), and genes for induced expression of phytohormones, among others.

2.6. Negative consequences of the release of GMOs into the environment and methods for their assessment

When assessing environmental risks, it is essential to consider the various complex interactions between organisms and their environment, many of which are difficult to evaluate precisely or may even be unpredictable. It is particularly challenging to predict distant consequences and various cascading effects [76].

Negative effects of GMOs on the environment:

- **destructive impact on biological communities and the loss of valuable biological resources** due to the contamination of local species with genes transferred from GMOs;
- **creation of new parasites**, primarily weeds, and an increase in the harmfulness of existing ones, either based on the GMOs themselves or as a result of transgene transfer from other species;
- **production of substances derived from transgenes** that may be toxic to organisms that live on or feed on genetically modified organisms and are not targets of the transgenic traits (e.g., bees, other beneficial or protected species);
- **adverse effects on ecosystems from toxic substances** resulting from the incomplete degradation of hazardous chemicals, such as herbicides (a significant number of currently created GMOs are herbicide-resistant forms);

Risk assessment of possible negative effects of GMOs on the environment. The general methodology includes the following stages:

- **reviewing information about the biological characteristics of the recipient and donor organisms** (systematic position, method of reproduction and dispersal, survival in the environment; geographical distribution, description of natural habitats; potentially significant interactions with organisms other than plants (toxicity));
- **reviewing information about the nature of the genetic engineering modification**: a description of the fragment of DNA (size and source) inserted into the genome (plasmid), the expected function of each component or region

of the inserted DNA, including regulatory and other elements affecting the functioning of the transgenes, the presence of known potentially hazardous sequences, localization of the insertion, stability of incorporation, and the number of transgene copies.

Consideration of information on the biological characteristics of GMOs and their interaction with the environment. This involves data on new traits and characteristics that have either emerged or ceased to manifest in GMOs compared to the recipient organism, especially those that may influence survival, reproduction, and spread in the potential receiving environment [77]. It includes:

- information on the genetic stability of the GMO, the degree and level of transgene expression;
- activity and properties of proteins encoded by transgenes;
- the potential for genetic information transfer (the presence of wild or cultivated related species in the potential receiving environment capable of hybridizing with the GMO, and the likelihood of transgene transfer from the GMO to such organisms);
- the probability of competitive advantage of the GMO compared to the intact recipient organism, as well as the potential for a sharp increase in the GMO population in the receiving environment;
- information about target and non-target organisms, and the anticipated mechanisms and outcomes of GMO interactions with them.

Characterization of the Potential Receiving Environment. The geographical location of the site where the release will take place, its proximity to reserves and other conservation areas; its size, climatic, geological, and soil characteristics, as well as flora and fauna [78].

2.7. The state biosafety system for the creation, testing, transportation, and use of genetically modified organisms

The main document regulating biosafety issues is the Law of Ukraine "On the State Biosafety System for the Creation, Testing, Transportation, and Use of Genetically Modified Organisms" (adopted on May 31, 2007). This Law regulates the relationships between executive authorities, producers, sellers (suppliers), developers, researchers, scientists, and consumers of genetically modified organisms and products produced using technologies that involve their development, creation, testing, research, transportation, import, export, marketing, release into the environment, and use in Ukraine while ensuring biological and genetic safety. However, the Law does not apply to humans, tissues, and individual cells within the human body [79, 80].

Main principles of state policy in the field of genetic engineering activities (GEA) and handling of GMOs:

- prioritizing the preservation of human health and the environment protection over obtaining economic benefits from the use of GMOs;
- ensuring measures to comply with biological and genetic safety during the creation, research, and practical use of GMOs for economic purposes;
- monitoring the importation of GMOs and products derived from their use into the customs territory of Ukraine, as well as their registration and circulation;
- public accessibility of information about potential risks associated with the use of GMOs intended for use in an open system and measures to ensure biological and genetic safety;
- state support for genetic engineering research and scientific and practical developments in the field of biological and genetic safety during the creation, research, and practical use of GMOs for economic purposes.

Areas of activity subject to regulation when dealing with GMOs:

- Genetic engineering activities (GEA) conducted in a closed system;
- GEA conducted in an open system;

- state registration of GMOs and products produced from their use;
- circulation of GMOs and products produced from their use;
- export, import, and transit of GMOs.

Powers of the Cabinet of Ministers of Ukraine in the field of genetic engineering activities and handling of GMOs:

- ensures state regulation and control in the field of handling GMOs and GEA;
- ensures the implementation of measures for state support of GEA;
- directs and coordinates the work of central executive bodies and other executive authorities in the field of handling GMOs and GEA;
- organizes international cooperation to ensure the safe handling of GMOs and the development of scientific knowledge in this field;
- approves the procedure for the state registration of GMOs and products obtained from their use;
- approves the procedure for the importation of GMO sources for food products, feeds, and food products and feeds produced from GMOs;
- approves the procedure for granting permission for the transit movement of GMOs across the territory of Ukraine;
- approves the procedure for licensing GEA in closed and open systems;
- approves the procedure for conducting state testing (trials) of GMOs in an open system and obtaining permission for their conduct;
- approves safety criteria for handling GMOs in a closed system.

Powers of the central executive authority in the field of education and science:

- ensures the development of scientific and scientific and technical potential in the field of GMOs;
- ensures the protection of international and national patents and other types of intellectual property in the field of GMO handling, genetic engineering, and biotechnology;
- develops safety criteria for GMO handling and genetic engineering activities in closed systems;

- develops and improves the control system for compliance with biotechnology safety rules;
- conducts licensing of biotechnology in closed systems;
- based on the results of state ecological and state sanitary and epidemiological expertise regarding the biological and genetic safety of GMOs, carried out in accordance with Ukraine's international treaties, provides permits for the importation of unregistered GMOs if they are used exclusively for research purposes in closed systems and open systems, as well as for their state testing.

Powers of the central executive authority in charge of ecology and natural resources:

- conducts state ecological examination of GMOs intended for use in an open system;
- based on scientific principles and international experience, develops criteria for assessing the risk of potential impacts of GMOs on the natural environment;
- carries out state registration of plant protection products obtained using GMOs;
- exercises state supervision and control over compliance with biological and genetic safety measures regarding biological entities in the natural environment during the creation, research, and practical use of GMOs in an open system;
- grants permits for the release of GMOs in an open system.

Powers of the central executive authority in charge of health protection:

- based on scientific principles and international experience, develops criteria for assessing the risk of potential impacts of GMOs and products obtained from their use on human health, including food products;
- conducts state sanitary and epidemiological examination of GMOs used in open systems to substantiate conclusions regarding their biological and genetic safety for humans for the purpose of their state registration;
- exercises state supervision and control over compliance with biological and genetic safety measures as to humans during the creation, research, and practical use of GMOs in an open system;

- conducts state sanitary and epidemiological examination of products obtained from the use of GMOs to substantiate conclusions regarding their safety for human health and life;
- carries out state registration of GMOs used as sources for food products, as well as state registration of food products, cosmetics, and pharmaceuticals that contain GMOs or are obtained from their use;
- approves the list of food products subject to GMO content monitoring and the corresponding methods for detection and identification of GMOs;
- monitors food products obtained using GMOs based on the criterion of containing only registered GMO sources.

Powers of the central executive authority in charge of agricultural policy:

- provides state approval (testing) and state registration of agricultural plant varieties, animal breeds, and microbiological agricultural and veterinary products created basing on GMOs;
- exercises state supervision and control over compliance with biological and genetic safety measures regarding agricultural plants and animals during the creation, research, and practical use of GMOs in open systems at enterprises, institutions, and organizations of the agroindustrial complex, regardless of their subordination and ownership forms.

Powers of the central executive authority in charge of veterinary medicine:

- carries out state registration of GMO sources for animal feed, as well as registration of feed additives and veterinary products that contain GMOs or are obtained from their use;
- approves the list of feeds subject to GMO content monitoring and the corresponding methods for detection and identification of GMOs;
- monitors feeds obtained using GMOs based on the criterion of containing only registered GMO sources.

Commission on Biosafety and Biological Protection under the National Security and Defense Council (NSDC) of Ukraine. Main Tasks of the Commission

(according to the Decree of the President of Ukraine dated June 10, 2009, No. 423/2009):

- analysis of existing and possible threats of biological origin to the national security of Ukraine;
- analyzing the implementation of state target programs, the status of measures implementation in the field of biosafety and biological protection;
- generalization of international experience in the formation and implementation of state policy in the field of biosafety and biological protection;
- development and submission to the NSDC of Ukraine of proposals regarding:
    - determination of Ukraine's national interests in the field of biosafety and biological protection;
    - mprovement of legal regulation in the field of biosafety and biological protection.

Features of regulating genetic engineering activities in closed systems:

- genetic engineering activities in a closed system are subject to licensing;
- licensing of such activities is carried out based on a risk assessment for handling GMOs in a closed system;
- the procedure for such licensing is approved by the Cabinet of Ministers of Ukraine upon the proposal of the central executive authority in the field of education and science.
- Enterprises, institutions, and organizations conducting genetic engineering activities establish a Commission on Biological and Genetic Safety for conducting genetic engineering work that carries out a preliminary risk assessment when planning and preparing genetic engineering work.

Features of regulating genetic engineering activities in open systems:

- GMOs used in open systems must comply with biological and genetic safety requirements, provided that the prescribed technology for their use is followed;
- a mandatory condition for the use of GMOs in an open system is the availability of methods and techniques for their identification, developed

according to international standards and approved in the established manner in Ukraine;

- the release of GMOs into the environment is prohibited until their state registration.

Prior to state registration, the release of GMOs into the environment is only possible for the purpose of state approval (testing). Exercising of state approval (testing) of GMOs in open systems is carried out exclusively, based on a permit issued by the central executive authority on ecology and natural resources. The permit is issued once for the state approval (testing) of a specifically defined GMO.

The permit for conducting state approvals (testing) of GMOs in open systems may be revoked in cases when scientifically substantiated information is obtained, that could lead to a reassessment of the risks associated with the impact of GMOs on human health and the environment, increasing those risks, as well as violations of the permit conditions.

2.8. Labeling of food products that contain GMOs or are produced using them

Labeling requirements for food products are established by the Law of Ukraine "On Food Safety and Quality" (Article 38). The procedure for labeling food products that contain GMOs or are produced using them and placed on the market is approved by the Resolution of the Cabinet of Ministers of Ukraine dated May 13, 2009, No. 468.

Food products that contain GMOs in amounts exceeding 0.9 % or are produced from agricultural products with GMO content exceeding 0.9 % must carry the label "with GMOs". In the list of ingredients of the food product, after the name of each ingredient that contains GMOs or is produced using them, the inscription (genetically modified), (contains genetically modified organism), or (produced from genetically modified organism) must be included in parentheses, specifying the name of the organism. The inscription must be printed in the same font as the list of ingredients.

For food products containing a single ingredient, the corresponding inscriptions must be printed on the label in a font size of no less than 2 mm. Labeling of food products that contain GMOs or are produced using them and are sold without packaging or with packaging that has a maximum surface area of less than 10 cm^2, is carried out by the seller by placing the appropriate label.

Labeling of food products that do not contain GMOs or whose content is less than 0.1 % is carried out with the inscription “without GMOs”. The specified information is subject to verification in accordance with the procedure established by Derzhspozhivstandard (State Service of Ukraine on Food Safety and Consumer Protection). Food products that contain more than 0.9 % GMO or are produced from agricultural products “with a GMO” content of more than 0.9 %, on which the corresponding inscription is not made, are subject to withdrawal from circulation.

By-laws regulating the circulation of GMOs and products produced using GMOs:

- procedure for state registration of genetically modified organisms as sources for fodder, feed additives and veterinary drugs containing such organisms or obtained with their use (Resolution of the Cabinet of Ministers of Ukraine dated 12.10.2010, No. 919);
- procedure for conducting state approval (testing) of genetically modified organisms of agricultural plant varieties in open systems (Resolution of the Cabinet of Ministers of Ukraine dated July 23, 2009, No. 808);
- procedure for issuing permits for conducting state approval (testing) of genetically modified organisms in open systems (Resolution of the Cabinet of Ministers of Ukraine dated April 2, 2009, No. 308);
- procedure for issuing permits for the importation of unregistered genetically modified organisms into the customs territory of Ukraine for research purposes or state approvals (testing) (Resolution of the Cabinet of Ministers of Ukraine dated August 20, 2008, No. 734);

- procedure for issuing permits for the transit movement of unregistered genetically modified organisms in Ukraine (Resolution of the Cabinet of Ministers of Ukraine dated April 28, 2009, No. 423).
- procedure for labeling food products that contain genetically modified organisms or are produced using them and placed on the market (Resolution of the Cabinet of Ministers of Ukraine dated May 13, 2009, No. 468);
- criteria for assessing the risks of the potential impact of genetically modified organisms on the environment (Order of the Ministry of Ecology and Natural Resources of Ukraine dated February 7, 2011, No. 36);
- procedure for licensing genetic engineering activities in a closed system (draft).

Testing products to determine the presence of GMOs in Ukraine is carried out in accordance with national standards:

- DSTU ISO 21569:2008 “Food Products. Methods for detecting genetically modified organisms and their derivatives. Qualitative method based on nucleic acid analysis”;
- DSTU ISO 21570:2008 “Food Products. Methods for detecting genetically modified organisms and their derivatives. Quantitative method based on nucleic acid analysis”;
- DSTU ISO 21571:2008 “Food Products. Methods for detecting genetically modified organisms and their derivatives. Nucleic acid extraction”;
- DSTU ISO 21572:2006 “Food Products. Methods of analysis for determining genetically modified organisms and derivative products. Methods based on protein analysis. (ISO 21572:2004, IDT)”;
- DSTU ISO 24276:2008 “Food Products. Methods for detecting genetically modified organisms and their derivatives. Basic requirements and definitions”;
- DSTU ISO/TS 21098:2009 “Food Products. Methods for detecting genetically modified organisms and their derivatives. Additional procedures and information regarding nucleic acid-based analytical methods described in ISO 21569, ISO 21570, ISO 21571 (ISO/TS 21098:2005, IDT)”.

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