N. S. GOLYAK

OBJECTS AND METHODS OF PHARMACEUTICAL BIOTECHNOLOGY

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МИНИСТЕРСТВО ЗДРАВООХРАНЕНИЯ РЕСПУБЛИКИ БЕЛАРУСЬ
БЕЛОРУССКИЙ ГОСУДАРСТВЕННЫЙ МЕДИЦИНСКИЙ УНИВЕРСИТЕТ
КАФЕДРА ФАРМАЦЕВТИЧЕСКОЙ ТЕХНОЛОГИИ

Н. С. Голяк

ОБЪЕКТЫ И МЕТОДЫ ФАРМАЦЕВТИЧЕСКОЙ БИОТЕХНОЛОГИИ

OBJECTS AND METHODS OF PHARMACEUTICAL BIOTECHNOLOGY

Учебно-методическое пособие

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Описаны основные этапы создания рекомбинантной ДНК, продукты, получаемые с использованием технологии рекомбинантных ДНК, векторы на основе плазмид, селекция трансформированных клеток.

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INTRODUCTION

Pharmaceutical biotechnology is a relatively novel and advancing field of applied science the core principles of which are applied for drugs development.

Most of the pharmaceutical biotechnology companies use recombinant DNA (rDNA) technology to design more effective protein-based drugs.

Proteins are already used for more than 100 years to treat or prevent diseases in humans. It started in the 19 century with «serum therapy» for treatment of diphtheria and tetanus by Emile von Behring and others. Behring received the Nobel Prize for Medicine in 1901 for his pioneering work on passive immunization.

The next big step in the development of therapeutic proteins was the use of purified insulin, isolated from pig or cow pancreas for treatment of diabetes type I by Banting and Best. In 1923 Banting received the Nobel Prize for this work.

Thanks to advances in biotechnology we have moved completely from animal-derived proteins to proteins with the complete human amino acid sequence. Such therapeutic human proteins are less likely cause side effects and immune responses.

In 1982, human insulin became the first recombinant human protein approved for sale in the USA. There are now almost 200 human proteins marketed for a wide range of therapeutic areas.

Biotechnology makes use of findings from various research areas, such as:

- molecular biology;
- biochemistry;
- cell biology;
- genetics;
- bioinformatics;
- microbiology;
- bioprocess engineering;
- separation technologies.

Therapeutic proteins differ in many aspects from classical, small molecule drugs (size, composition, production, purification, contamination, side effects, stability, formulation, regulatory aspects).

These fundamental differences justify paying attention to therapeutic proteins as a family of medicines with many general properties different from small molecules.

ADVANTAGES OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA (rDNA) technology has a positive impact upon the production of pharmaceutically important proteins:

It overcomes the problem of source availability. Many proteins of therapeutic potential are produced naturally in the body in minute quantities (e.g. interferons, interleukins and colony-stimulating factors). But it is impossible to extract them from native source material in quantities sufficient to meet clinical demand. While recombinant production allows manufactures to produce any proteins in whatever quantity it is required.

It overcomes problems of product safety. In the past direct extraction of product from some native biological sources has led to the transmission of diseases. Examples include the transmission of blood-borne pathogens such as hepatitis B and C and human immunodeficiency virus (HIV) via infected blood products and the transmission of Creutzfeldt–Jakob disease to persons receiving human growth hormone (GH) preparations derived from human pituitaries.

It provides an alternative to direct extraction from inappropriate/dangerous source material. A number of therapeutic proteins have traditionally been extracted from human urine. Folliclestimulating hormone (FSH) is obtained from the urine of postmenopausal women, and human chorionic gonadotrophin (hCG) is extracted from the urine of pregnant women. Urine is not considered a particularly desirable source of pharmaceutical products.

It facilitates the generation of engineered therapeutic proteins displaying some clinical advantages over the native protein product. Techniques, such as site-directed mutagenesis, facilitate the logical introduction of predefined changes in a protein's amino acid sequence. An overview summary of some engineered products is provided in Table 1.

Table 1

Selected engineered biopharmaceutical products that have currently gained marketing approval

Product	Alteration introduced	Results
Faster acting insulins	Modified amino acid sequence	Generation of faster acting insulin
Slow acting insulins	Modified amino acid sequence	Generation of slow acting insulin
Modified tissue plasminogen activator (tPA)	Removal of three of the five native domains of tPA	Generation of a faster acting thrombolytic (clot degrading) agent
Modified blood factor VIII	Deletion of 1 domain of native factor VIII	Production of a lower molecular mass product
Chimaeric/humanized antibodies	Replacement of most/virtually all of the murine amino acid sequences with sequences found in human antibodies	Greatly reduced/eliminated immunogenicity. Ability to activate human functions

Despite the undoubted advantages of recombinant proteins, many protein-based products extracted directly from native source material, are still on the market. In certain circumstances for economic reasons, direct extraction of native source material can prove to be equally/more attractive than recombinant production (e. g. human serum albumin).

To understand rDNA technology it is required to know the structure of DNA and proteins.

DNA STRUCTURE

The information encoded in genetic material is responsible for establishing and maintaining the cellular and biochemical functions of an organism.

In most organisms the genetic material is DNA:

– The process of DNA synthesis is called replication.

– A specific order of deoxyribonucleotides determines the information content of an individual genetic element (gene).

Some genes encode proteins while others encode only ribonucleic acid (RNA) molecules.

The protein-coding genes (structural genes) are decoded by two successive major cellular processes (fig. 1):

1. Transcription — RNA synthesis

2. Translation — protein synthesis. A protein consists of a precise sequence of amino acids essential for its activity.

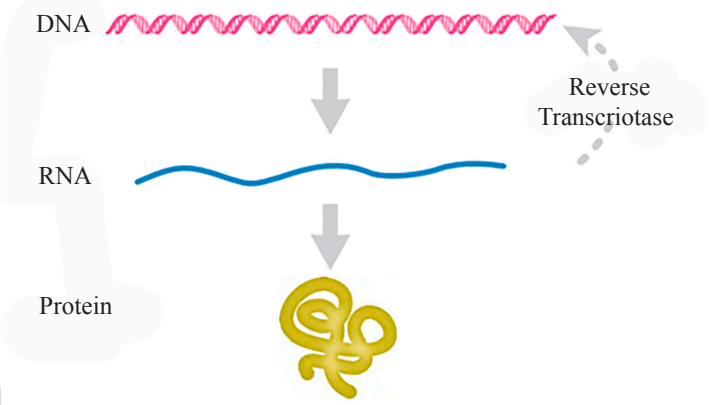

Fig. 1. The central dogma of molecular biology [4]

DNA is made up of individual units called nucleotides linked to each other to form long chains. A nucleotide consists :

– base;
– five-carbon sugar;
– phosphate group.

The term «base» denotes any of the four bases found in DNA: Adenine (A), Guanine (G), Cytosine (C), Thymine (T).

The sugar of DNA is 2′-deoxyribose because it does not have a hydroxyl (OH) group on the 2′ carbon.

In DNA the phosphate group and base are attached to the 5′ carbon and 1′ carbon atoms of the sugar.

The nucleotide subunits of DNA are joined by phosphodiester bonds with the phosphate group of the 5′ carbon of one nucleotide linked to the 3′ OH group of the deoxyribose of the adjacent nucleotide.

A polynucleotide strand has a 3′ OH group at one end (the 3′ end) and a 5′ phosphate group at the other (the 5′ end). The two polynucleotide chains of DNA are held together by hydrogen bonds between the bases of the opposite strands. Base pairing occurs only between specific complementary bases.

Adenin pairs only with Thymin, and Guanin pairs only with Cytosine.

– The A · T base pairs are held together by two hydrogen bonds, and
– the G · C base pairs are held together by three hydrogen bonds.

Structure of DNA see fig. 2.

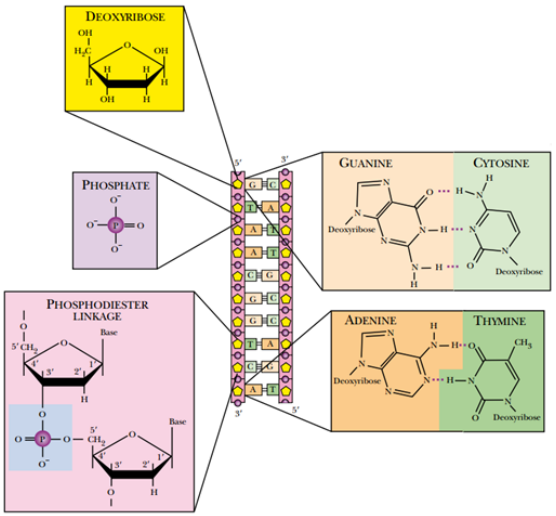

Fig. 2. Structure of DNA [4]

RNA STRUCTURE

RNA molecules are linear polynucleotide chains that differ from DNA in two important respects.

First, the sugar part of the nucleotides of RNA is ribose, which has hydroxyl groups on both 2′ and 3′ carbons of sugar.

Second, instead of thymine, the base uracil (U) is found in RNA.

Structure of RNA see fig. 3.

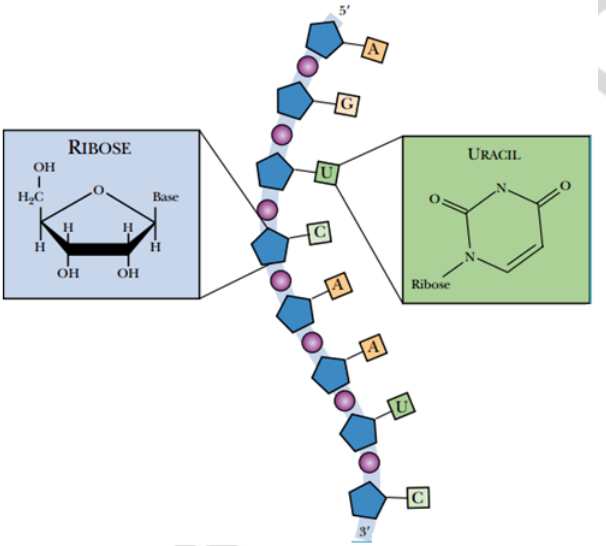

Fig. 3. Structure of RNA [4]

The major kinds of RNA molecules that are essential for the decoding of genetic information are mRNA, ribosomal RNA (rRNA), and tRNA.

The production of RNA from DNA is called transcription.

– In most prokaryotes a single RNA polymerase is responsible for the transcription of all RNA types.

– In eukaryotic organisms mRNA, rRNA, and tRNA are each transcribed by a different RNA polymerase.

Genetic material has two major functions.

– It encodes the information for the production of proteins, and

– it is reproduced (replicated) with a high degree of accuracy to pass the encoded information to new cells.

PACKAGING OF NUCLEIC ACIDS

Bacteria have just a few thousand genes, each approximately 1000 nucleotides long. These are carried on a chromosome that is a single giant circular molecule of DNA. In bacteria DNA is condensed in supercoiling in order to take up less space. The supercoiled DNA forms loops connected to a protein scaffold (fig. 4).

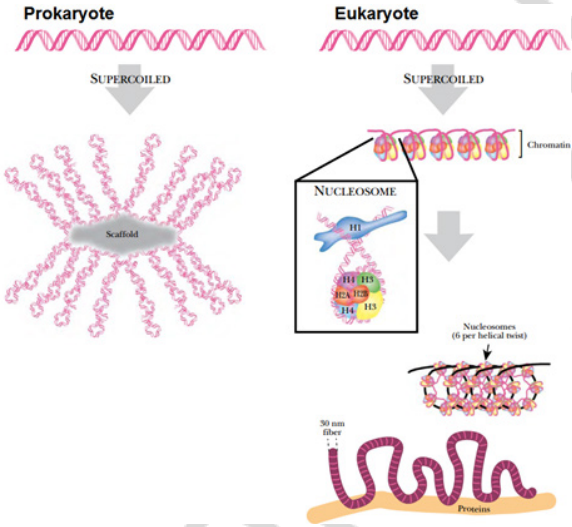

Fig. 4. Packaging of DNA in Prokaryotes and Eukaryotes [4]

In humans and plants more DNA must be packaged so just adding supercoils is not sufficient. First eukaryotic DNA wounds around proteins called histones. Histones have a positive charge to them, and this neutralizes the negatively charged phosphate groups. DNA plus histones looks like beads on a string and called chromatin. Each bead or nucleosome has about 200 base pairs of DNA and nine histones, two H2A, two H2B, two H3, two H4, and one H1. H1 connects the beads by holding the DNA in the linker region. Histone tails are important in regulation.

In regions of DNA that are expressed, the histones are loose, allowing regulatory proteins and enzymes to have access to the DNA. In not expressed regions histones are condensed, preventing other proteins from accessing the DNA (the structure is called heterochromatin). Chromatin is not condensed enough to fit the entire eukaryotic DNA genome into the nucleus. It is coiled into a helical structure, the 30-nanometer fiber, which has about six nucleosomes per turn. These fibers loop back and forth, and the ends of the loops are attached to a protein scaffold or chromosome axis.

DNA REPLICATION

Each strand of an existing DNA molecule acts as a matrix for the production of a new strand. DNA synthesis of prokaryotes and eukaryotes includes a large number of different proteins. Of these, DNA polymerases are responsible for binding deoxyribonucleotides.

In bacteria DNA replication is initiated at a specific region of the chromosome called the origin of replication (or origin). Prokaryotic transription entails the binding of RNA polymerase to a promoter region and cessation of transcription at a terminotion sequence (fig. 5).

In eukaryotes, a chromosome has many different sites of replication initiation. Part of the eukaryotic replication process includes enzymatically joining segments of newly synthesized DNA together with phosphodiester bonds.

DECODING GENETIC INFORMATION

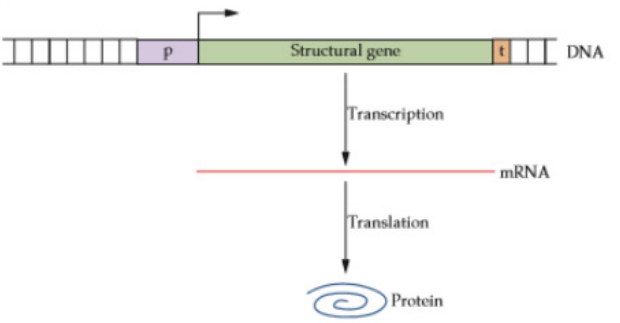

Fig. 5. Schematic representation of a prokaryotic structural gene [2]
The promoter region (p), the site of initiation and transcription direction and the termination sequence for RNA polymerase (t)

In eukaryotic organism a structural gene usually consists of several coding regions (exons) separated by noncoding regions (introns) (fig. 6). After RNA polymerase has bound to the promoter and the entire eukaryotic structural gene is transcribed, the introns are removed from the primary transcript, and the exons, in the correct order, are linked (spliced) together to form a functional mRNA (fig. 7).

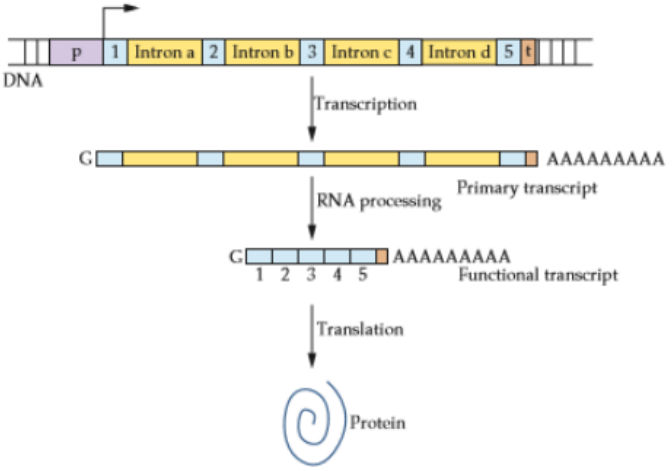

Fig. 6. Schematic representation of a eukaryotic structural gene [2]

The promoter (p), the site of initiation and direction of transcription and the terminator (t). The primary transcript is polyadenylated at the 3′ end and capped with a modified guanine (G) nucleotide at the 5′ end

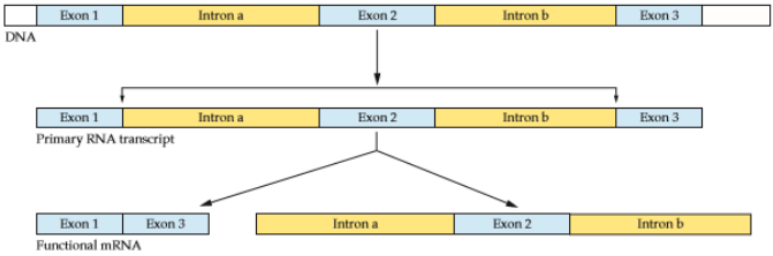

Fig. 7. Splicing of a eukaryotic primary RNA transcript [2]

The arrows mark the sites spliced together after the introns removal. In this example, introns a and b are spliced out of the primary transcript, and exons 1, 2, and 3 are spliced together to form a functional mRNA

Processing of the primary transcript removes the introns. The functional RNA is translated into protein. In general, exons tend to be 150 to 300 bases in length, and introns can vary from 40 to over 10.000 bases.

The introns in a primary tronscript moy be removed in more than one way in a process know as alternative splicing (fig. 8).

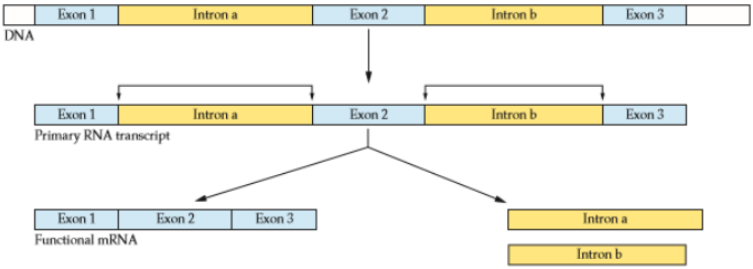

Fig. 8. Alternative splicing of a eukaryotic primary RNA transcript [2].
The arrows mark the sites spliced together after the intervening RNA region removal. In this example exon 2, surrounded by introns a and b, is spliced out of the primary transcript, and exons 1 and 3 are spliced together to form a functional mRNA transcript

In prokaryotes the processes of transcription and translation are not spatially separated (fig. 9).

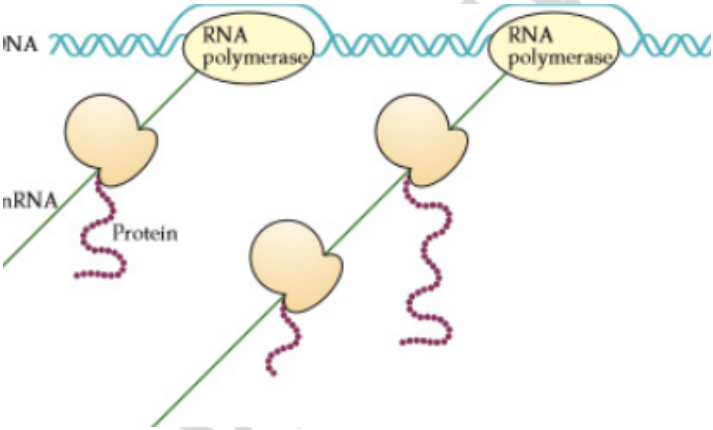

Fig. 9. Concurrent transcription and translation in prokaryotes [2].
In prokaryotes translation and transcription in cytoplasm occurs simultaneously

In eukaryotes transcription and translation are spatially separated (fig. 10).

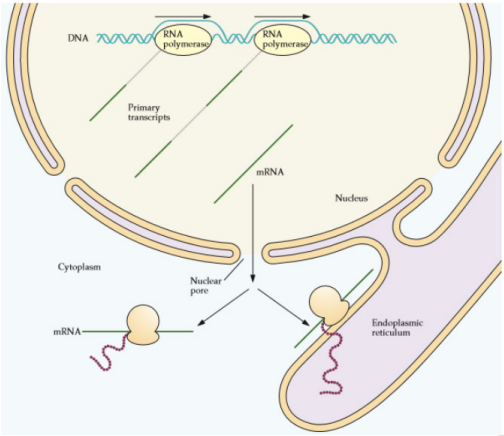

Fig. 10. Transcription and translation in eukaryotes [2]
Transcription occurs in the nucleus, while translation occurs in cytoplasm

RECOMBINANT MOLECULAR CLONING/ RDNA TECHNOLOGY

Recombinant molecular cloning means the transfer of genetic information from one organism to another.

Genetic engineering describes the process of manipulating genes. It generally involves isolation, manipulation and subsequent reintroduction of DNA stretches into cells and is usually undertaken in order to confer on the recipient cell the ability to produce a specific protein. «rDNA technology» is a term used interchangeably with «genetic engineering». rDNA is a piece of DNA, artificially created in vitro, which contains DNA (natural or synthetic) obtained from two or more sources. When developing a new biopharmaceutical protein, one of the earliest undertaken actions entails identifying and isolating the gene (or complementary DNA (cDNA)) coding for the target protein, the generation of an appropriate piece of rDNA, containing the protein's coding sequence and introduction of this rDNA into an appropriate host cell so that the target protein is made in large quantities by that engineered cell.

Thus far, the vast majority of approved recombinant proteins have been produced in the bacterium *E. coli*, the yeast *S. cerevisiae* or in animal cell lines (most notably Chinese hamster ovary (CHO) cells or baby hamster kidney (BHK) cells.

Recombinant DNA experiment often has the following format:

1. The DNA (**cloned DNA, insert DNA, target DNA, or foreign DNA**) from a donor organism is extracted, enzymatically cut, and joined to another DNA entity (a cloning vector) to form a new, recombined DNA molecule (**cloning vector–insert DNA construct, or DNA construct**).

2. This cloning vector–insert DNA construct is transferred into and maintained within a host cell. The introduction of DNA into a bacterial host cell is called transformation.

3. Transformed cells are identified and selected (separated, or isolated) from the cells without DNA construction.

4. If required, a DNA construct can be created so that the protein product encoded by the cloned DNA sequence is produced in the host cell (fig. 11).

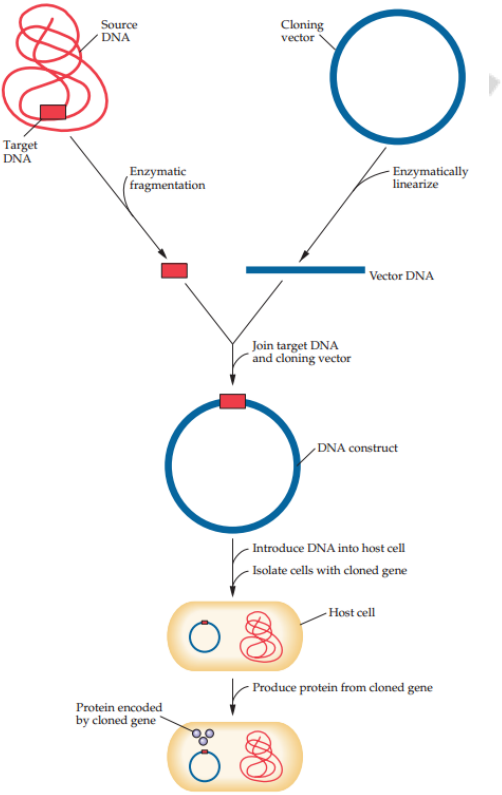

Fig. 11. Recombinant DNA-cloning procedure [2]

DNA ISOLATION AND PURIFICATION

To obtain rDNA researchers need to discover a method to isolate DNA from different organisms. Isolating DNA from bacteria is the easiest procedure because bacterial cells have little structure beyond the cell wall and cell membrane. Bacteria, such as *E. coli*, are the preferred organisms for manipulating of any type of genes. *E. coli* maintain both genomic and plasmid DNA within the cell. Genomic DNA is much larger than plasmid DNA, allowing the two different forms to be separated by size.

To release the DNA from a cell, the cell membrane must be destroyed. To destroy the cell wall of gram-positive microorganism lysozyme is used. To destroy the cell wall of gram-negative microorganism lysozyme with detergent is used. *E. coli* is a gram-negative microorganism. For other organisms breaking of the cells depends on their structure. DNA can be isolated first by removing the cell wall and cell membrane components. Next, the proteins are removed by phenol, and finally, the RNA is removed by ribonuclease.

RESTRICTION ENDONUCLEASES

For molecular cloning both the source DNA and the cloning vector must be consistently cut into discrete fragments.

The type II restriction endonucleases are commonly called restriction endonucleases or simply restriction enzymes. Type II restriction enzymes can either cut both strands of the double helix of DNA at the same point, leaving blunt ends, or they can cut at different sites on each strand leaving single stranded ends (sometimes called sticky ends).

One of the first type II restriction endonucleases was obtained from the bacterium *Escherichia coli* and was originally designated as *Eco*RI.

EcoRI is a homodimeric protein that binds a DNA region with a specific palindromic sequence (**recognition site, or binding site**).

The EcoRI recognition sequence consists of 6 base pairs (bp) and is cut between the guanine and adenine residues on each strand.

EcoRI specifically cleaves the internucleotide bond between the oxygen of the 3′ carbon of the sugar of one nucleotide and the phosphate group attached to the 5′ carbon of the sugar of the adjacent nucleotide.

The symmetrical staggered cleavage of DNA by EcoRI produces two single-stranded complementary cut ends, each with extensions of 4 nucleotides, known as sticky ends (fig. 12).

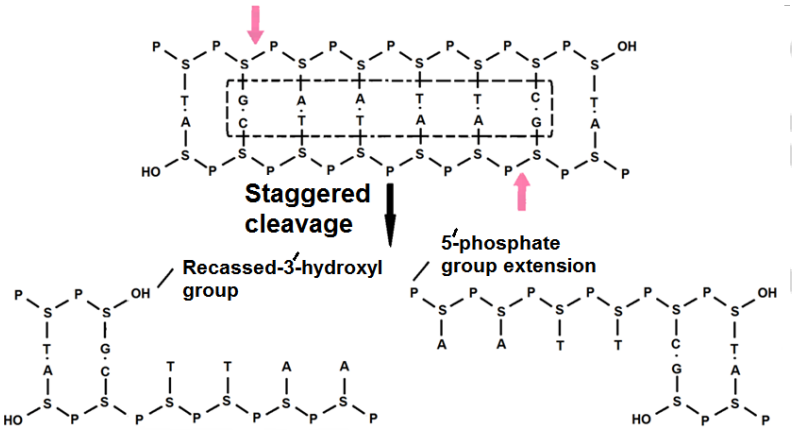

Fig. 12. Cleavage of DNA by EcoRI [2]
The EcoRI recognition sequence is highlighted by the dashed line.

Some restriction endonucleases digest (cleave) DNA, leaving 5′ phosphate extensions (protruding ends, or sticky ends) with recessed 3′ hydroxyl ends; some leave 3′ hydroxyl extensions with recessed 5′ phosphate ends; and some cut the backbones of both strands within a recognition site to produce blunt-ended (flush-ended) DNA molecules (fig. 13).

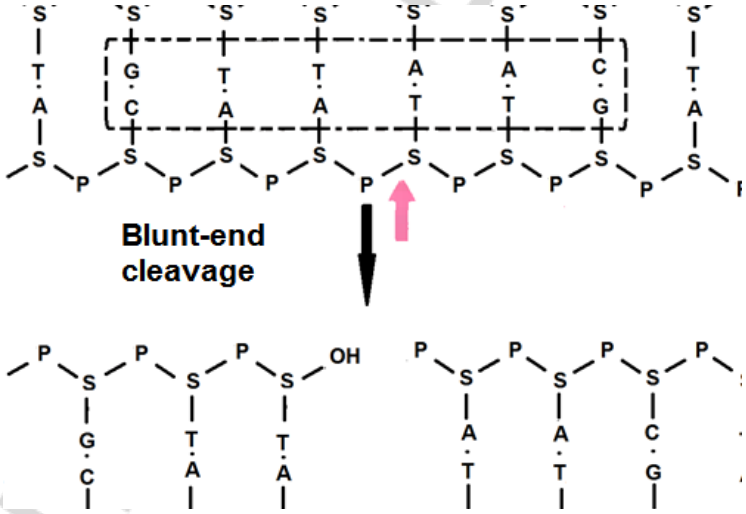

Fig. 13. Cleavage of DNA by HindII

The lengths of the recognition sites for different enzymes can be four, five, six, eight, or more nucleotide pairs (fig. 14) [2].

Enzyme	Recognition site	Type of cut end
EcoRI	G↓A–A–T–T–C C–T–T–A–A↑G	5′ phosphate extension
BamHI	G↓G–A–T–C–C C–C–T–A–G↑G	5′ phosphate extension
PstI	C–T–G–C–A↓G G↑A–C–G–T–C	3′ hydroxyl extension
Sau3AI	↓G–A–T–C C–T–A–G↑	5′ phosphate extension
PvuII	C–A–G↓C–T–G G–T–C↑G–A–C	Blunt end
HpaI	G–T–T↓A–A–C C–A–A↑T–T–G	Blunt end
HaeIII	G–G↓C–C C–C↑G–G	Blunt end
NotI	G↓C–G–G–C–C–G–C C–G–C–C–G–G–C↑G	5′ phosphate extension

Arrows denote cleavage sites.

Fig. 14. Restriction Endonucleases [2]

Restriction endonuclease cleavage is important in molecular cloning to insert target DNA into a cloning vector. However, restriction enzymes alone are not sufficient for molecular cloning.

DNA LIGASE OBTAINED FROM BACTERIOPHAGE T4

This enzyme catalyzes formation of phosphodiester bonds at the ends of DNA strands that are already held together by the base pairing of two extensions. DNA ligase also joins blunt ends that come in contact when they both bind to the enzyme (fig. 15).

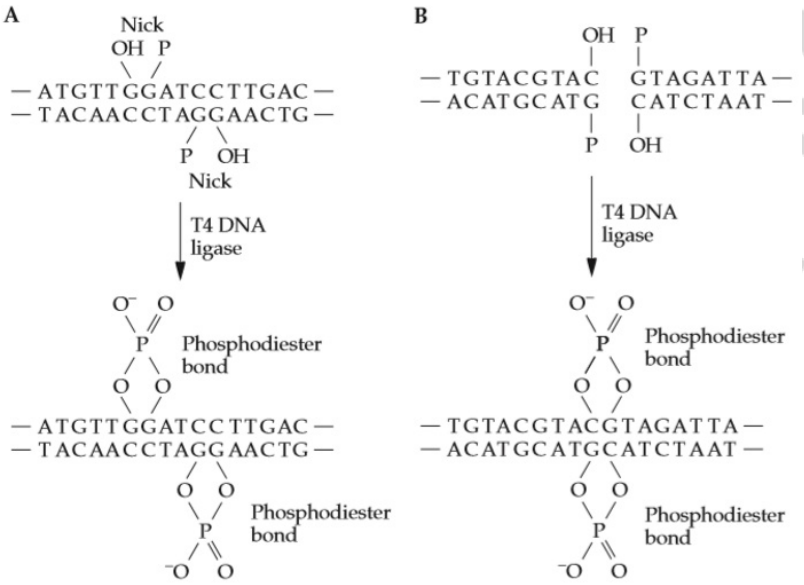

Fig. 15. Mode of action of T4 DNA ligase [2]

The enzyme T4 DNA ligase forms phosphodiester bonds by joining 5′ phosphate and 3′ hydroxyl groups at nicks in the backbone of double-stranded DNA. Ligation of sticky-ended DNA; (B) ligation of blunt-ended DNA. A, C, G, and T represent nucleotides

The ability to join different DNA molecules is not by itself useful unless the new DNA combination (i.e. recombinant DNA) can be perpetuated in a host cell. Cloning vectors overcome this problem.

Digestion of the source DNA containing the gene of interest with a restriction endonuclease produces a mixture of DNA molecules, and a number of different DNA constructs are formed after ligation with a cloning vector. Consequently, there has to be a way of identifying the DNA combination in a host cell that contains the target DNA sequence. Screening procedures have been devised to detect host cells carrying a specific cloning vector–DNA insert construct.

PLASMID CLONING VECTORS

Plasmids are self-replicating, double-stranded circular DNA molecules that are maintained in bacteria as independent extrachromosomal entities.

Each plasmid has a sequence that functions as an origin of DNA replication; without this site it cannot replicate in a host cell.

Some plasmids, because of the specificity of their replication origin, can replicate in only one species of host cell. Other plasmids have less specific origins of replication and can replicate in a number of bacterial species. These plasmids are called narrow- and broad-host-range plasmids, respectively. As autonomous self-replicating genetic elements, plasmids have the basic attributes to make them potential vectors for carrying cloned DNA.

However, naturally occurring (unmodified or nonengineered) plasmids often lack several important features required for a high-quality cloning vector.

The most important features are:

– a choice of unique restriction endonuclease recognition sites into which the insert DNA can be cloned and

– one or more selectable genetic markers for identifying recipient cell carrying the cloning vector–insert DNA construct.

In other words, plasmid cloning vectors have to be genetically engineered.

PLASMID CLONING VECTOR PBR322

In the 1980s, one of the best-studied and most often used «general-purpose» plasmid cloning vectors was pBR322 (fig. 16).

In general, plasmid cloning vectors are designated by a lowercase p, which stands for plasmid, and some abbreviation that may be descriptive or, as is the case with pBR322, anecdotal. The «BR» of pBR322 recognizes the work of the researchers F. Bolivar and R. Rodriguez, who created the plasmid, and 322 is a numerical designation that has relevance to these researches.

Plasmid pBR322 contains 4,361 bp. As shown in fig. 16, pBR322 carries two antibiotic resistance genes. One confers resistance to ampicillin (Ampr), and the other confers resistance to tetracycline (Tetr). This plasmid also has unique BamHI, HindIII, and SalI recognition sites within the Tetr gene; a unique PstI site in the Ampr gene; a unique EcoRI site that is not within any coding DNA; and an origin of DNA replication that functions only in *E. coli*, is maintained at a high copy number in *E. coli*, and cannot be readily transferred to other bacteria.

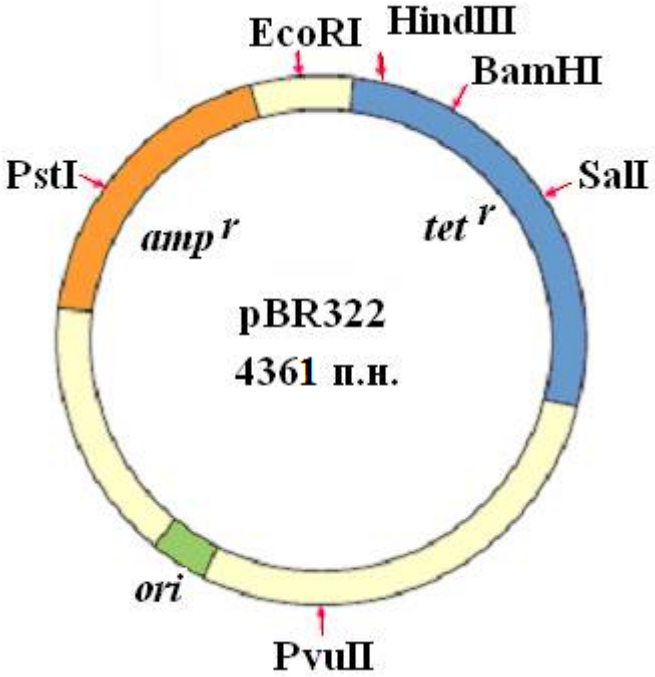

Fig. 16. Plasmid cloning vector pBR322 [2]

HOW DOES PBR322 WORK AS A CLONING VECTOR?

Purified, closed circular pBR322 molecules are cut with a restriction enzyme that lies within either of the antibiotic resistance genes and cleaves the plasmid DNA only once to create single, linear, sticky-ended DNA molecules. These linear molecules are combined with prepared target DNA from a source organism. This DNA has been cut with the same restriction enzyme, which generates the same sticky ends as those on the plasmid DNA.

The DNA mixture is then treated with T4 DNA ligase in the presence of ATP. Under these conditions a number of different ligated combinations are produced, including the original closed circular plasmid DNA. To reduce the amount of this particular unwanted ligation product the cleaved plasmid DNA preparation is treated with the enzyme alkaline phosphatase to remove the 5′ phosphate groups from the linearized plasmid DNA. As a consequence, T4 DNA ligase cannot join the ends of the dephosphorylated linear plasmid DNA (fig. 17).

However, the two phosphodiester bonds formed by T4 DNA ligase after ligation and circularization of alkaline phosphatase-treated plasmid DNA with restriction endonuclease-digested source DNA, which provides the phosphate groups, are sufficient to hold the two molecules together, despite presence of two nicks (fig. 17). After transformation these nicks are sealed by the host cell DNA ligase system. Digested fragments from the source DNA are also joined to each other by T4 DNA ligase. However, these unwanted ligation products do not contain an origin of replication and therefore will not replicate following introduction into a host cell.

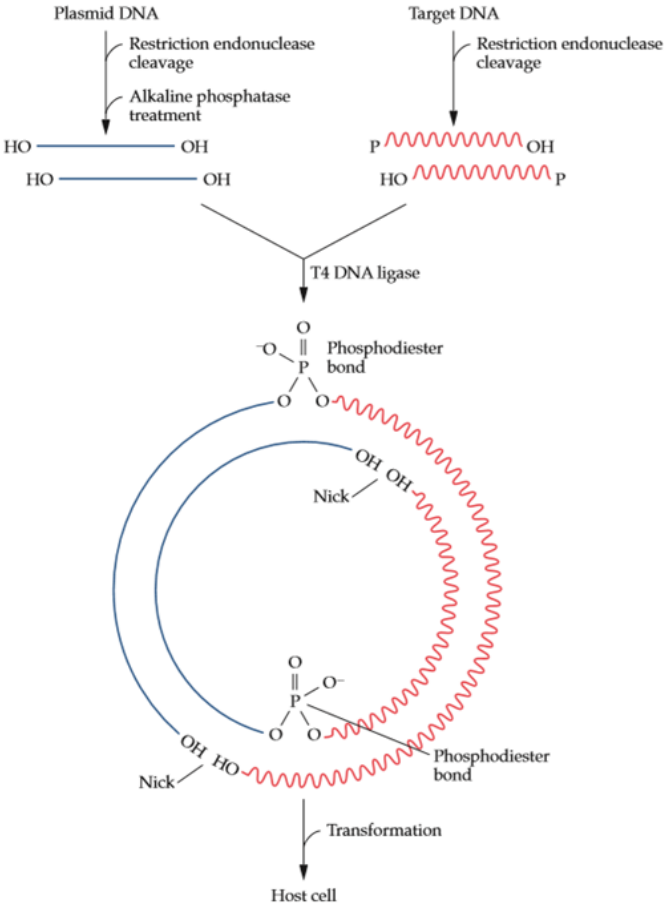

Fig. 17. Cloning foreign DNA into a plasmid vector [2].

After restriction endonuclease cleavage and alkaline phosphatase treatment, the plasmid DNA is ligated to the restriction endonuclease-digested target DNA, and two of the four nicks are sealed. This molecular configuration is stable, and the two DNA molecules are covalently joined. After introduction into a host cell, ensuing replication cycles produce new complete circular DNA molecules with no nicks.

TRANSFORMATION AND SELECTION

The next step in a recombinant DNA experiment requires the uptake of the cloned plasmid DNA by a bacterial cell, usually *E. coli*. The process of introducing purified DNA into a bacterial cell is called transformation, and a cell that is capable of taking up DNA is said to be competent.

If the introduced DNA is a plasmid, it is maintained in the cytoplasm after the second strand is synthesized. Competence and transformation are not intrinsic properties of *E. coli*.

However, competence can be induced in *E. coli* by various special treatments, such as cold calcium chloride, which in turn enhances the acquisition of DNA by the cell. A brief heat shock facilitates the uptake of exogenous DNA molecules.

After the transformation step, it is necessary to identify, applying the simplest method, those cells that contain plasmids with cloned DNA. In a pBR322 system in which the target DNA was inserted into the BamHI site, this specific identification is accomplished using the two antibiotic resistance markers carried on the plasmid. Following transformation the cells are incubated in medium without antibiotics to allow the antibiotic resistance genes to be expressed, and then the transformation mixture is plated onto medium containing antibiotic ampicillin. Cells that carry pBR322 with or without insert DNA can grow under these conditions because the Ampr gene on pBR322 is intact. The nontransformed cells are sensitive to ampicillin.

The BamHI site of pBR322 is within the Tetr gene (fig. 16), so the insertion of DNA into this gene disrupts the coding sequence and tetracycline resistance is lost. Therefore, cells with these plasmid–cloned DNA constructs are resistant to ampicillin and sensitive to tetracycline. Cells with recircularized pBR322 DNA, however, have an intact Tetr gene and are resistant to both ampicillin and tetracycline.

The second step in the selection scheme distinguishes between these two possibilities. Cells that grow on the ampicillin-containing medium are transferred to the tetracycline-containing medium. The relative positions of the cells transferred to the tetracycline–agar plate are the same as those of the colonies from which they were transferred on the original ampicillin–agar plate. Cells that form colonies on the tetracycline–agar plates carry recircularized pBR322 without insert DNA,

because, as noted above, these cells are resistant to both ampicillin and tetracycline. The cells that do not grow on the tetracycline–agar plates, however, are sensitive to tetracycline and carry pBR322–cloned DNA constructs (fig. 18).

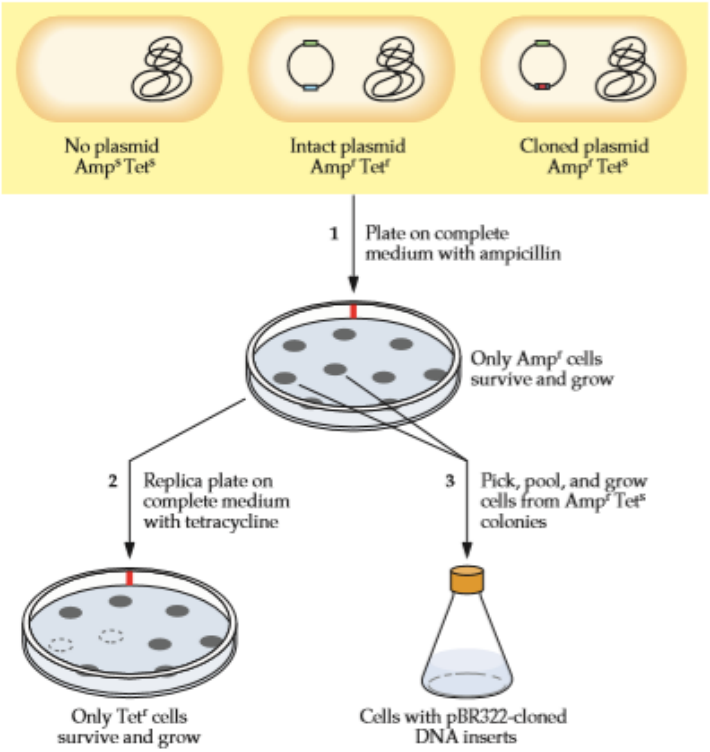

Fig. 18. Strategy for selecting host cells that have been transformed with pBR322 [2]

Individual cultures sensitive to tetracycline are established from each of the colonies on the ampicillin–agar plates. Later, additional screening procedures can be conducted to verify that these cells, called transformants, carry the desired pBR322–cloned DNA construct. The HindIII and SalI sites in the tetracycline resistance gene and the PstI site in the ampicillin resistance gene of pBR322 provide alternative potential cloning locations. When the PstI recognition site is used for cloning, the principle of the selection scheme is the same but the antibiotic sensitivities are reversed; thus, the first set of plates contains tetracycline

and the second set contains ampicillin. The pBR322 selection scheme to identify transformed cells with insert DNA–vector constructs relies on replica plating.

This technique, which can be used in many different ways for various purposes, was originally devised to isolate mutant bacterial colonies that require a supplement for growth, i.e., auxotrophic mutants (fig. 19).

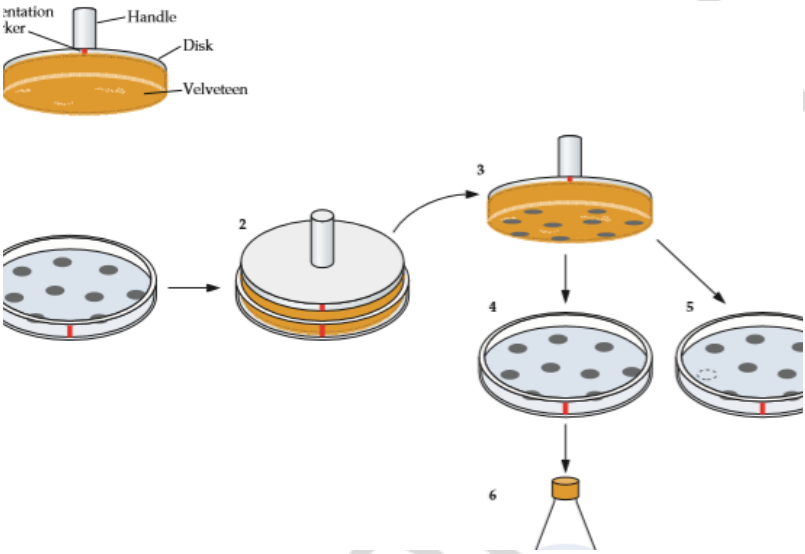

Fig. 19. Screening bacterial colonies for mutant strains by replica plating. (A) Replica-plating (colony transfer) device; (B) replica-plating technique [2]

Cells from each separated colony on a master plate (1) adhere to the velveteen of the replica plating device after it is gently pressed against the agar surface (2). The adhering cells are transferred (3) in succession to a petri plate with complete medium (4) and to one with selective medium (5).

The pattern of the colonies is consistent among the replicated plates because the orientation markers (red squares) are aligned for each transfer. In this example, minimal medium is the selective medium used to identify colonies that require a nutritional supplement for growth, i. e., auxotrophic mutants. The missing colony (dashed circle) on the minimal medium (5) denotes an auxotrophic mutation. The equivalent location on the plate with complete medium (4) has the colony with the auxotrophic mutation that can be picked and grown (6). Further analysis of the isolated strain is necessary to determine the nature of the auxotrophic mutation.

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REVIEW OF THE COVERED ISSUES

1. List the advantages of recombinant DNA technology.
2. Describe the DNA structure.
3. Describe the RNA structure.
4. Describe the packaging of DNA in prokaryotes.
5. Describe the packaging of DNA in eukaryotes.
6. What is splicing?
7. What is alternative splicing?
8. Describe recombinant DNA-cloning procedure.
9. What are restriction endonucleases?
10. How is DNA ligase obtained from bacteriophage T4 used?
11. Describe the plasmid cloning vectors.
12. Describe the plasmid cloning vector pBR322.
13. How does pBR322 work?
14. What is transformation?
15. Describe the strategy for selecting host cells that have been transformed with pBR322.
16. Describe the screening bacterial colonies obtained by replica plating.

FILL THE GAPS

1. Therapeutic proteins differ in many aspects from classical, small molecule drugs (size, … , production, … , contamination, side effects, stability, … , regulatory aspects).
2. … — RNA synthesis.
3. … — protein synthesis.
4. A protein consists of a precise sequence of …. essential for its activity.
5. The term «base» denotes any of the four bases found in DNA: Adenine (A), Guanine (G), Cytosine (C), … (…).
6. The sugar part of the nucleotides of RNA is … , which has hydroxyl groups on both 2′ and 3′ carbons of sugar.
7. Instead of thymine, the base … (…) is found in RNA.
8. In general, exons tend to be ... to … bases in length, and introns can vary from 40 to over … bases.
9. In prokaryotes translation and transcription in cytoplasm occurs … .
10. The vast majority of approved recombinant proteins have been produced in the bacterium ... , the yeast *S. cerevisiae* or in animal cell lines (most notably Chinese hamster ovary (CHO) cells or baby hamster kidney (BHK) cells.

11. To destroy the cell wall of gram- positive microorganism ... is used.

12. The type II restriction … are commonly called restriction endonucleases or simply restriction enzymes.

13. The EcoRI recognition sequence consists of 6 base pairs (bp) and is cut between the guanine and … residues on each strand.

14. The process of introducing purified DNA into a bacterial cell is called …

15. Screening bacterial colonies for mutant strains by … plating.

16. The symmetrical staggered cleavage of DNA by EcoRI produces two single-stranded complementary cut ends, each with extensions of 4 nucleotides, known as … ends.

17. Selected engineered biopharmaceutical products that have currently gained marketing approval.

Product	Alteration introduced	Results
Faster acting insulins	… amino acid sequence	Generation of … acting insulin
Slow acting insulins	… amino acid sequence	Generation of … acting insulin
Modified tissue plasminogen activator (tPA)	… of three of the five native domains of tPA	Generation of a … acting thrombolytic (clot degrading) agent
Modified blood factor VIII	….. of 1 domain of native factor VIII	Production of a … molecular mass product
Chimaeric/ humanized antibodies	Replacement of most/virtually all of the murine amino acid sequences with sequences found in human antibodies	Greatly reduced/eliminated immunogenicity. Ability to activate … functions

Is the statement True or False

1. The process of DNA synthesis is called replication. True/False.

2. The A · T base pairs are held together by three hydrogen bonds. True/False.

3. The G · C base pairs are held together by two hydrogen bonds. True/False.

4. In eukaryotes transcription and translation are spatially separated. True/False.

5. To destroy the cell wall of gram-negative microorganism lysozyme with detergent is used. True/False.

6. *E. coli* is a gram-negative microorganism. True/False.

7. Plasmid pBR322 contains 4,361 bp. True/False.

8. DNA ligase obtained from bacteriophage T4 catalyzes formation of phosphodiester bonds at the ends of DNA strands. True/False.

CONTENT

Учебное издание

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