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INTRODUCTION TO MEDICAL AND MOLECULAR BIOLOGY

Asklepios
Bratislava
2010

Introduction to medical and molecular biology

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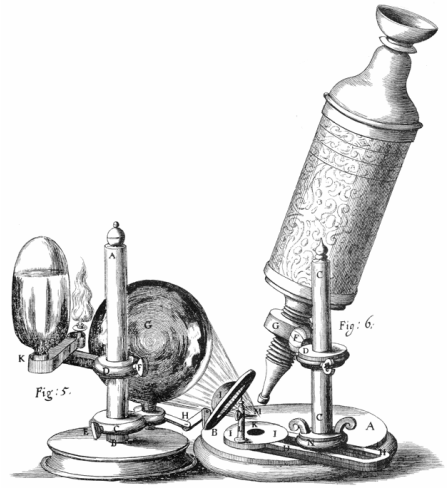
Asklepios, Bratislava, 2010

ISBN: 978-80-7167-151-0

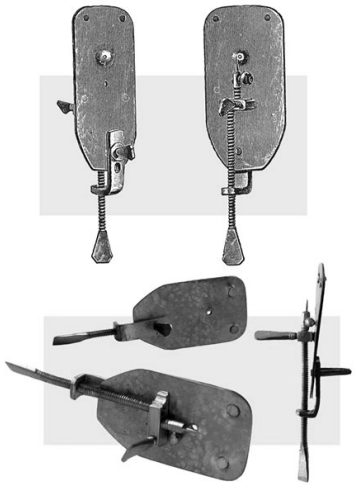
1. Introduction to microscopic techniques

Microscopes are optical devices which allow observation of objects of microscopic size (less than 70µm) and which are invisible for human eye. The main goal of this chapter is to provide basic information about types and design of microscopes, as well as about principles of work and the use of microscopic techniques in the biomedical disciplines.

The first microscope was probably constructed in 1590 by Dutch sunglasses manufacturer Zacharias Jansen. Another pioneer of microscopy was Robert Hook, who described the construction of microscope (Fig. 1) with separated objective, eyepiece and a source of light in his book *"Micrographia"* (1665). He observed and depicts several organisms – e.g. fungi, mosses and small insects. Moreover, he introduced a biological term – cell (*cellula*). A simple microscope (Fig. 2) was also designed and constructed in 1676 by Antonie van Leeuwenhoek. He was able to observe many micro-organisms, as well as blood cells, sperm and muscle fibres. During the next two centuries, microscopes and microscopic techniques improved. The mass production of microscopes was initiated by the German company Carl Zeiss in 1847. Another significant milestone of microscopy was construction of first electron microscope in 1933. Recently, microscopes belong to standard laboratory equipment in the biomedicine.



● **Figure 1.** A Hook microscope



● **Figure 2.** A van Leeuwenhoek microscope

1.2 Types of microscopes

According to type of used radiation the light and electron microscopes are distinguished.

Light microscopes use white or ultraviolet light. Sunlight, bulb or vapour lamp is used as source of light. Optical parts are made from cut glass. The resolving power (resolution) of this type of microscopes is 0.2 µm and maximal theoretical magnification is 2 000 times. In practice, the objects are usually observed at a magnification of up to 1 000 times. In the routine conditions, the slides (native or fixed) are observed in the passing light. The method of lighting from above slides is used mainly in fluorescence and inverted microscope.

Electron microscopes – radiation is a stream of electrons emitted by cathode. The function of optical parts occupy special electromagnetic lens. The resolving power is 0.2 nm and maximal useful magnification can be up to 1 000 000 times. Slides have to be prepared by special techniques (fixation, staining, contrasting, etc.). According to way of sample visualization the **transmission electron microscope** (TEM; electron beam passes through the preparation impregnated by electron-dense particles) and **scanning electron microscope** (SEM; electron beam proceeds on the surface of sample and bring information about surface details, shape as well as size of observed object) are distinguished.

1.2.1 Stereomicroscope

Stereomicroscopes, also called dissecting microscopes belong to two light microscopes which focus on the same point from slightly different angles. This allows the specimen to be observed in three dimensions. Stereomicroscopes are relatively low power compared with light microscopes – useful magnification is usually below 100 times. They can have a single fixed magnification, several discrete magnifications, or a zoom magnification system. Working distance is much longer than with a typical microscope as well. It allows work to be done on the specimen while it is being observed through the microscope. Stereomicroscopes are usually used in diagnostics (e.g. in gynaecology) and for various types of surgery (e.g. in neurosurgery, vascular surgery, ophthalmology and otolaryngology).

1.2.2 Inverted microscope

It is special type of light microscope with changed order of optical part and source of light. Optics is under the slide and light source is above. It is mainly used for monitoring cell cultures, when is necessary to visualize the growing of cells on the bottom of culture vessels (Petri dishes or cultivation flasks).

1.2.3 Fluorescence microscope

Fluorescence microscope belongs to light microscopes which use vapour lamp as a source of UV radiation. Specially prepared biological samples are illuminated with light of a specific wavelength which induces emission of light with longer wavelength – visible light. There are only few objects with natural ability of fluorescence. For that reason, the special fluorescent dyes (fluorochromes) have to be used. It allows not only observation of cell structures, but also it is very useful method for molecular cytogenetics (e.g. FISH).

1.2.4 Polarized microscope

It uses polarized light. The optical part contains special Nicol prisms to generate a beam of polarized light. They are used for observation of structures such as chitin, cellular fibres and crystalline cell inclusions.

1.3 Construction of light microscope

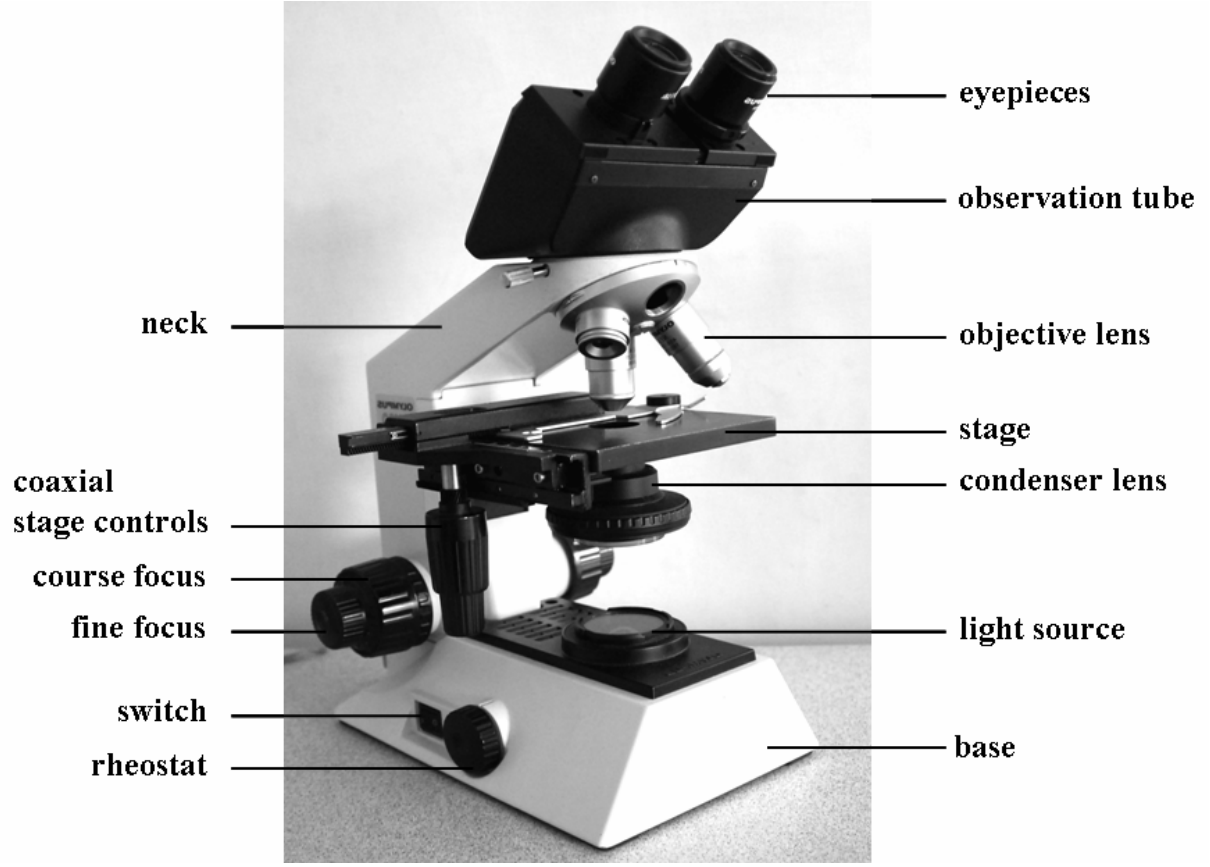
Generally, every light microscope consists of optical, lighting and mechanical parts. Figure 3 demonstrates construction of typical laboratory microscope.

Optical part consists of two types of lenses – objective and eyepiece. Objective lenses are oriented to the observed sample and eyepiece is turned to the observer eye. Both of them are involved to formation of resulting image which is real, inverted and magnified. Other additional lenses should be added to this basic system.

Lenses and eyepieces have several properties, which are based on fundamental laws of optics. Most important of them is magnification. The resulting magnification is calculated by multiplying of objective magnification and magnification of eyepiece (e.g. 40 x 10 = 400 times).

According to the number of eyepieces we distinguish monocular and binocular microscopes. Special microscopes – demonstration and operating ones may have multiple sets of eyepieces. If necessary, the obtained image should be visualized on the monitor screen or recorded by digital camera and projected.

Working distance (distance between slide and frontal lens of objective) is shortened by increasing of objective magnification. For the smallest objectives it is several centimetres, but in commonly used objectives with magnification of 40 times it is practically less than the sum of the thickness of microscopic slide, preparation and cover slide. Objective with magnification of 100 times in which frontal lens lies on the cover slide (or microscopic slide) is used with immersion oil.



● **Figure 3.** Construction of typical laboratory microscope

Lighting part of laboratory microscope consists of two systems. The first one represent source of light. The second one is formed by optical network components, filters and diaphragms designed to adequately illuminate the slide.

The halogen or vapour lamps (in fluorescence microscope) are most common source of light in modern microscopes. The main advantage of the halogen lamp is its long life and high intensity. They are powered by an external or built-in source.

The system of prisms, lenses, filters and diaphragms is used for adjusting of light beam. Condenser and iris diaphragm belongs to standard equipment of light microscopes. **Condenser** is concave lens and change of its position ensures the most appropriate lighting of preparation.

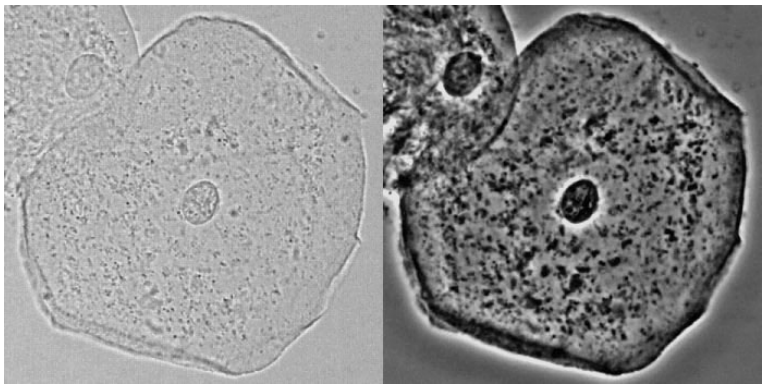
Special type of condenser is used for observation in "**dark field**". It is condenser which has deflector in its system of lenses which lead into releasing of sidelight. It allows observation of beams reflected from preparation. Mentioned is used in dermatology and venerology for the direct diagnostics of treponemas (Fig. 4).

For the observation of native slides, the **phase contrast** is used to highlight transparent objects with weak contrast interface (Fig. 5).

Filters allow adjusting the light intensity and colour of observed structures or their background. In practice, it must be remembered that utilization of filters which increases convenience of observation may change demands for photographic or digital image recording.

In conventional microscopes, between the condenser and preparation there is **iris diaphragm**. It is used for adjusting the light intensity of observed preparations.

● **Figure 4.** Observation in dark field (*Treponema pallidum*)



● **Figure 5.** Phase contrast microscopy – observation of epithelial cells

Mechanical part of the light microscope belongs to the most variable components. **Observation tube** represents the basic element of the light microscope. It holds the **eyepieces** in place above the **objective lens.** Eyepieces are changed mechanically. Objective lenses are mounted on a **rotating turret (nosepiece)** which assures a correct adjusting of the objective into the optical axis.

The microscope focusing can be achieved by vertical positioning of the observation tube or by stage moving from or to the objective lens. To focus the microscope, **coarse** and **fine focus knobs** are used. They are also **coaxial focus knobs** which are built on the same axis with the fine focus knob on the outside.

Stage is a platform with a central aperture which can be moved horizontally using two axial or one coaxial stage controls (in left-right and up-down position). Preparation (microscope slide) is hold either by clips or is inserted into the cartridge.

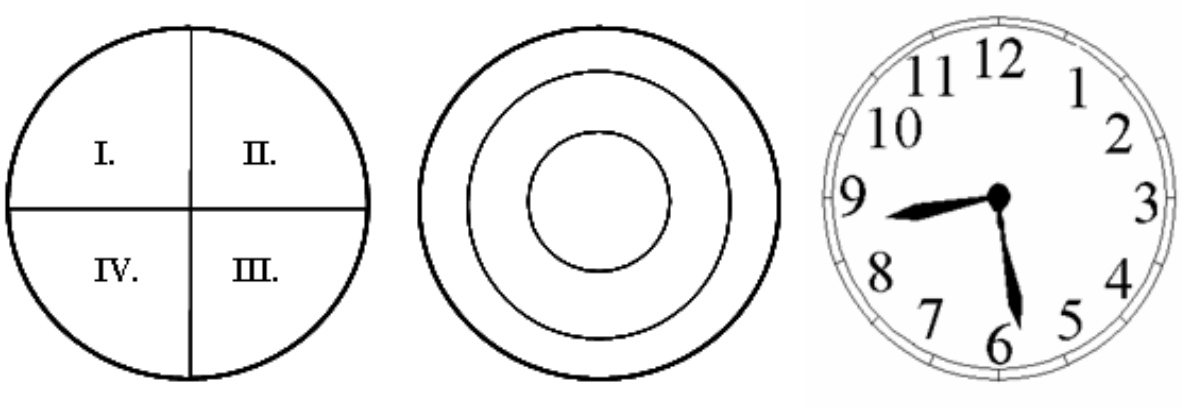
1.4 Recommended procedure for microscopic observation

Place the slide on the stage of the microscope so the specimen faces the objective lens. Secure the slide with the stage clips and move it to the optical axis. Set the light control (condenser is in a low position; iris is open, use filter if needed). Always start with the low power (shortest) objective. Move the stage to the upper position, under the objective. Look through the eyepieces, use the coarse focus knob to lower the stage until the image of a specimen is visible, and then fine focus knob for accurate focusing. Change the magnification by rotating the objectives on the rotating turret and adjust position of the condenser (move upper). For accurate focusing, use the fine knob.

1.4.1 Orientation in the microscopic view

When observing the specimen in the microscope, position of an object can be specified by three methods (Fig. 6):

- quadrants – the optical field is divided clockwise into four quadrants I. – IV.
- concentric circles – central, pericentric and peripheral circle
- according to clock face



● **Figure 6.** Orientation in a visual field

1.4.2 The most common errors and causes in microscopy

The most common errors in microscopy, their causes and possible eliminations are described in Table 1.

Error	**Possible cause**	**Elimination**
dark or weakly illuminated visual field	condenser is in a too low position	raise the condenser
	iris diaphragm is closed	open the iris diaphragm
	condenser is not located in the optical axis	centre the condenser
non-uniformly illuminated visual field	objective is not located in the optical axis	rotate the objective so it clicks into place
	optical components are dirty	clean optical components with alcohol
obscure or dirty visual field	slide is dirty	clean the slide
	optical components are dirty	clean optical components with alcohol
	condenser or illuminator is dirty	clean the condenser or illuminator
object cannot be focused	microscope slide is too thick	use a thinner microscope slide
	slide is inverted	turn the slide upside down
	coarse focus knob is full throttle	release the coarse focus knob
	frontal objective lens is dirty	clean the frontal objective lens with alcohol

● **Table 1.** The most common errors and causes in microscopy

1.5 Types of slide preparations

According to mode of preparation different types of microscopic slides are recognised:

- **impression preparations** – a new clean slide is slightly pressed on the surface of the examined tissue and attached cells are observed (e.g. cells of liver or brain);
- **smears** – e.g. a small drop of suspension containing cells is placed near an end of a slide and is spread across the slide by the edge of another slide (e.g. blood smear);
- **covered slides** containing cell suspension or processed histology tissue covered by cover slip.

Depending on methodical approaches of cells or tissue slides preparation native and permanent histological slides are distinguished. They have both positives and negatives so they must be used adequately in experiments and for diagnosis.

Native slides are used to observe physiological manifestations of cell (e.g. movement, cell division, particles ingestion etc.) or its typical shape. Because refractive index of organelles is very similar this technique does not allow observing intracellular structures. For this purpose phase contrast or dark field microscopy are used. Sometimes vital staining can be utilized e.g. to demonstrate phagocytosis of cells or to evaluate viability of cells using trypan blue dye.

Permanent slides allow detailed observation of cell morphology. The preparation of permanent slides consists from fixation and following staining of cells or tissue slices.

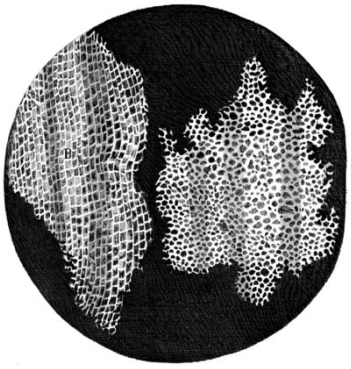
Fixation terminates any ongoing living and autolytic biochemical processes in cells. Chemical or physical fixation is possible. During a **physical fixation,** used mostly for smears and touch preparations, biological sample is heated and dried at laboratory temperature or above a burner flame. **Chemical fixation** requires a liquid chemical fixative (e.g. formol, methanol, ethanol etc.).

Fixation is followed by **staining** in different types of solutions which is based on affinity interactions between cell structures and stain components. They are informative (e.g. Nile Blue) or specific stains (e.g. Giemsa stain for chromatin visualization). Some dyes have both fixative and staining effect (e.g. Lugol's solution, orcein).

2. The cell

The cell (*cellula* – Latin, *kytos* – Greek) is the basic morphological, functional and reproductive unit of all unicellular and multicellular organisms. It is autonomous and dynamic system which is characterized by basic life manifestations (metabolism, growth, irritability, reproduction and development). Science which deals with the study of cells is called **cytology**.

The term "cell" was used for the first time in 1665 by Robert Hooke, who observed structure of cork (Fig. 7) with simple microscope. From 1715 to 1722 Leeuwenhoek observed some cellular structures (e. g. chloroplasts). During this period cytology started to form as a separate biological science. In 1838 Schleiden and Schwann formulated the **cell theory** in which they appointed plant and animal cells as elementary constituents of all living organisms. In 1855 Virchow revised cell theory and summarized it into three general points:



1) The cell is basic unit of all organisms;
2) Every cell consists of nucleus and cytoplasm;
3) Every cell originated from existing cell („*Omnis cellula e cellula*").

● **Figure 7.** Drawing of cork structure (R. Hook, *Micrographia*, 1665)

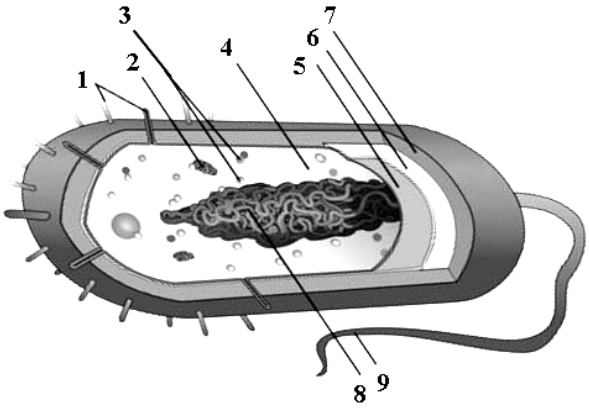
Improvement of light microscopy and introduction of electron microscopy helped to a more accurate understanding of the structure of cells. In general it can be claimed that all cells are composed of **nucleus, cytoplasm** and **cytoplasmic membrane**. In some specialized cells in their differentiation some component should be reduced or disappeared. Each cell contains ribosomes. The presence of other organelles is dependent on cell type.

According to the organization of nucleus and other structures, we distinguished prokaryotic and eukaryotic cells. **Prokaryotic cells** have a single circular DNA, which is not separated from the cytoplasm by membrane. They do not have any membrane organelles, cytoskeleton. Small ribosomes are present in its cytoplasm. They have a cell wall on the surface. **Eukaryotic cells** have nucleus which is separated from cytoplasm by the nuclear envelope. Cytoplasm contains various membrane organelles, cytoskeleton and ribosomes. The cells of plants and fungi have cell wall on the surface.

2.1 Prokaryotic cell

Prokaryotic cells have a simple structure (Fig. 8). They are composed of cytoplasm, nukleoid (meaning nucleus-like), cytoplasmic membrane, and on the surface they have a cell wall.

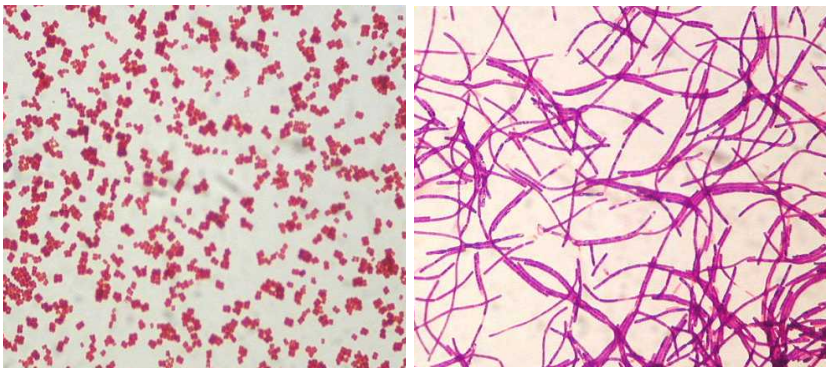
Nucleus of prokaryotic cell, assigned as **nukleoid**, is formed by a single circular DNA which is connected with internal part of cytoplasmic membrane. Cytoplasm contains prokaryotic ribosomes, which are smaller when compared to ribosomes in eukaryotic cell. They are composed of three types of rRNA and 52 proteins. Their sedimentation constant is 70S. The cytoplasm of prokaryotic cells may contain plasmids (episomes) – small circular molecules of DNA. Photosynthetic bacteria and cyanobacteria have simple vesicles from cytoplasmic membrane with enzymes responsible for photosynthesis (thylakoids). Some prokaryotic cells may also have locomotive organelles (e.g. flagellum, cilia etc.)



● **Figure 8.** Morphology of prokaryotic cell (1 – pili; 2 – plasmid; 3 – ribosomes; 4 – cytoplasm; 5 – plasma membrane; 6 – cell wall; 7 – capsule; 8 – nukleoid; 9 – flagellum)

Prokaryotic cells form only single-cell organisms (e. g. bacteria and cyanobacteria). Most important are **bacteria**. They are frequently reproduced by asexual reproduction – by **amitosis (**binary fission) which occurs immediately after DNA replication. Some bacteria should be reproduced by **conjugation**. It is based on the ability of transfer genetic material between bacteria through the conjugative plasmid. The genetic information transferred is often beneficial to the recipient bacteria. Benefits may include antibiotic resistance which has serious negative consequences for the possible treatment of bacteria related diseases.

Bacteria display a wide diversity of shapes (cocci, bacilli and others) and sizes (0.3 to 2 μm). Most bacterial species are either spherical, called **cocci** (Fig. 9a), which can be arranged in chain (streptococci) or clusters (staphylococci). Other bacteria are rod-shaped, called **bacilli** (Fig. 9b). They vary in length and thickness; some may have bizarre shapes (e.g. form of spirals such as *Treponema*) and special locomotive organelles.



a. **b.**

● **Figure 9.** Basic morphology of bacteria**: a.** cocci, **b.** bacilli (magnification 1 000 x)

Bacterial cell wall is composed of peptidoglycan murein, which allows only minimal staining identification. However, it has significant importance for the recognition by the host immune system.

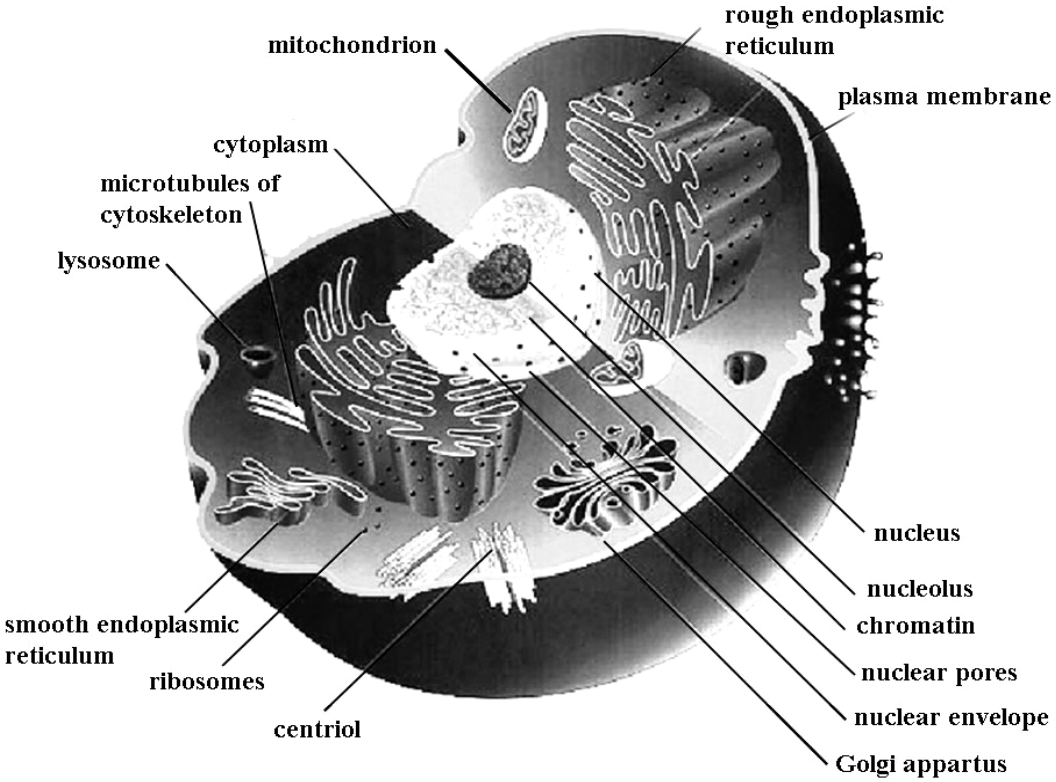
Microscopic diagnosis of bacteria usually does not provide enough opportunities for identification originator of inflammatory disease. The most commonly used Gram staining technique dividing bacteria to Gram-positive (purple) and Gram-negative (red), but crucial evidence has their cultivation. It is based on the type of soil (culture medium), in which bacteria grow, the appearance of the colonies and its effects on the environment. Cultivation also allows performing tests of bacterial sensitivity to antibiotics.

Bacteria can be aerobic and anaerobic. Some of them (especially anaerobic) are capable of forming **endospores** (anabiotic stages), able to overcome adverse environmental conditions. By this way they facilitate their spreading (e.g. in *Clostridium tetani* and *Clostridium botulinum*).

Some bacteria are essential for human being (e. g. *Escherichia coli*) and some of them are parasites which cause purulent inflammatory diseases.

2.2 Eukaryotic cell

Eukaryotic cells (Fig. 10) form unicellular and multicellular organisms. According to way of nutrition we distinguish autotrophic (protophyta) and heterotrophic (protozoa) organisms. Eukaryotic multicellular organisms are fungi, plants and animals. They undergo process of differentiation and specialization. They are composed from tissues, which are organizes to organs and organ system.



● **Figure 10.** Morphology of eukaryotic cell

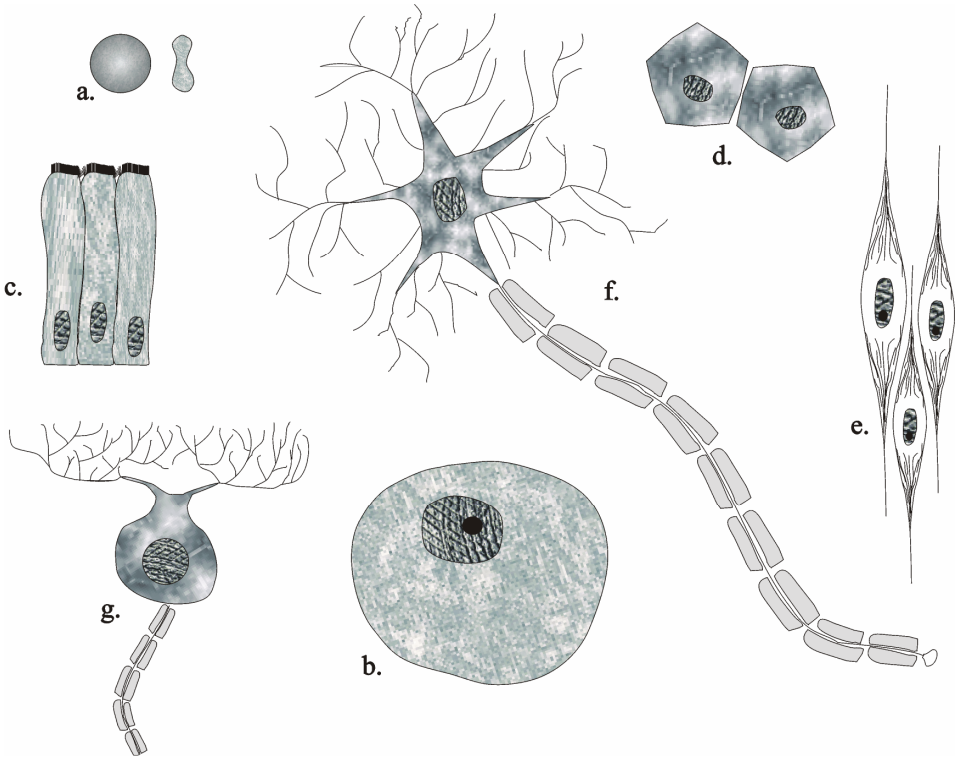
2.2.1 Shape and size of cells

The shape and size of cells is genetically determined and is related to their location and function in the body. The basic cell shape is spherical (e.g. human oocyte, leukocytes) and other shapes are derived from it (Fig. 11) – biconcave disc (e.g. human erythrocytes), squamous (e.g. epithelial cells of skin or oesophagus), cuboidal (e.g. germinal epithelium of ovary, epithelium of ducts of many glands), columnar (e.g. epithelial cells of small intestine), polygonal (hepatocytes), spindle-shaped (e.g. fibroblast, myocytes), multi-polar (e.g. neurons, astrocytes), pear-shaped (e.g. Purkinje cells), pyramidal cells (e.g. pyramidal neurons) etc.

Cells may have different projections. For example cells with fibrous projections (e.g. motor neurons, astrocytes); with irregular cytoplasmic projections (e.g. leukocytes, pericytes); with flattened projections (e.g. cells of tendons); with cilia and microvilli (e.g. cells of the small intestine, respiratory tract and uterus); with flagellum (e.g. sperm).

Cells according size are:

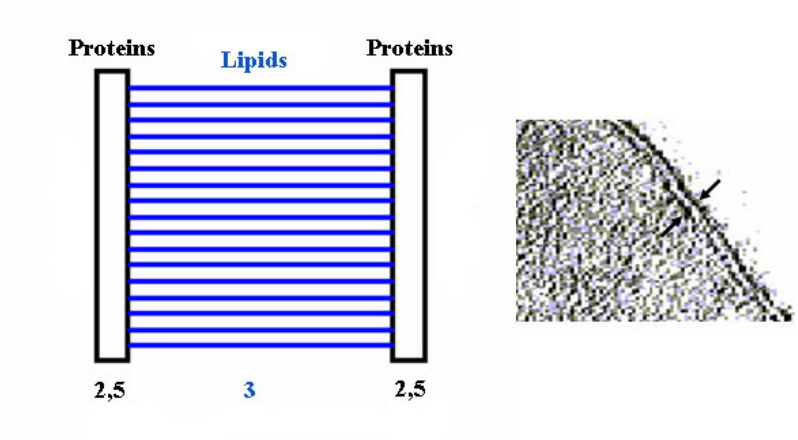
- **small,** which reach size to 10 µm (e.g. erythrocytes, lymphocytes). The smallest cells are occurred in the *stratum granulosum* of cerebellum;
- **middle sized**, their size varied from 10 to 30 µm (e.g. plasmatic cells, chondrocytes). Most of cells in human tissues are of this size;
- **big**, with size over 30 µm (human ova, megakaryocytes, motoric neurons).



● **Figure 11.** Morphology of cells: **a.** biconcave disc – human erythrocyte; **b.** spherical cell – oocyt; **c.** columnar – enterocytes; **d.** polygonal cells – hepatocytes; **e.** spindle-shaped cell – myocytes; **f.** multi-polar – neurons; **g.** pear-shaped cell – Purkinje cells

2.2.2 Molecular structure of cell membranes

Cell membranes (**biomembranes**) are important part of all cells. Their discovery is closely related to the upgrading of microscopic techniques, especially with the design of transmission electron microscopy, by which was observed typical trilaminar structure (Fig. 12). Further observations showed that biomembranes in the cell are similar in structure and slight differences in chemical composition are due to cell differentiation and specialization.

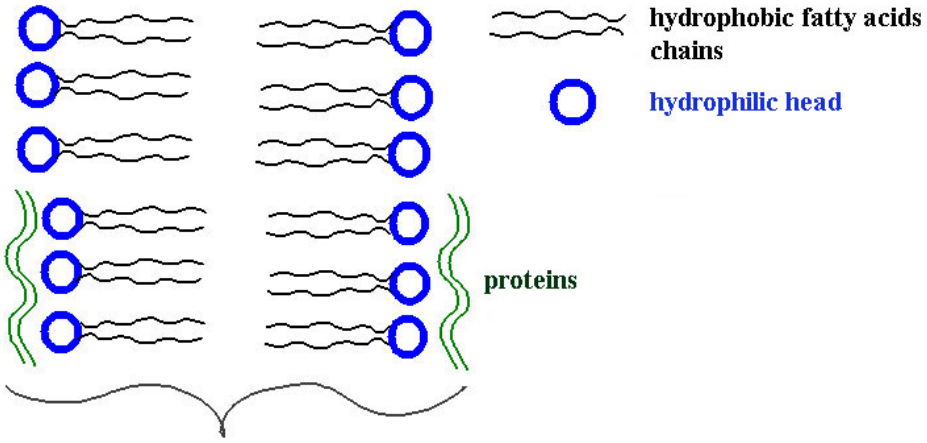


● **Figure 12.** Trilaminar structure of biomembranes – dark layer of proteins (2.5 nm) and light layer of lipids (3 nm)

Every cell is surrounded by the cytoplasmic membrane, which separates intracellular from extracellular space. Its average thickness is 60 – 10 nm. Cell membrane is selectively permeable boundary, which ensures the maintenance of dynamic equilibrium between cell and environment. It contains enzymes, receptors, transport proteins, signalling systems and antigens. It performs different functions, e.g. intake of substances, interactions, recognition of signals, etc. The biological membrane is a part of many important cellular organelles. It

makes their border and also it is involved in the execution of physiological processes (e.g. oxidative phosphorylation).

The main components of cell membranes are **phospholipids**. Most abundant are lecithin, sphingomyelins and amino phospholipids. The specialized cells also contain phosphatidylglycerol, phosphatidylinositol and cardiolipin. The molecule of phospholipids is composed of polar (hydrophilic) head and two non-polar (hydrophobic) fatty acids chains. In the aqueous environment the hydrophilic parts are oriented towards the water around them and fatty acids chains to each other, creating so-called **phospholipid bilayer** (Fig. 13). Given that phospholipids are not chemically bound to each other, their lateral movement is possible. Mentioned is in relation with their **fluidity**. It is affected by **cholesterol** (found only in animal cells), which increases the rigidity of biomembranes.



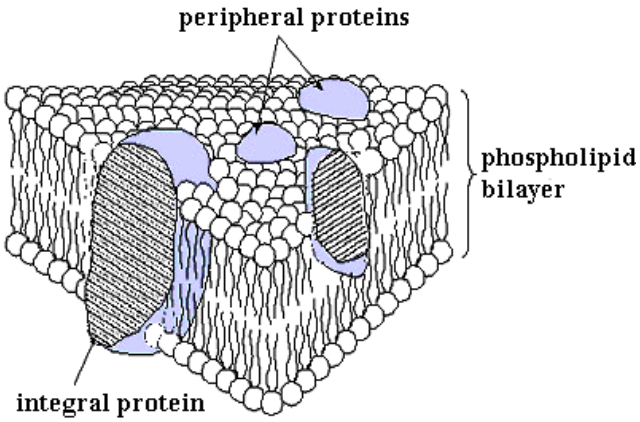
● **Figure 13.** Schematic representation of phospholipid bilayer

Other important components of biological membranes are proteins (Fig. 14). They may be:

- **integral**, which affect the hydrophobic parts of the phospholipid bilayer or transgress it. They are hardly separable from the biomembranes;
- **peripheral**, which lie outside the lipid bilayer. They are associated with electrostatic bonds and can be easily separated from biomembranes.

Types and number of proteins in biomembrane is variable. It is dependent on cell differentiation and cell cycle phase. Specific protein composition of biomembranes is regulated by cell.

Membrane proteins perform various functions. They are part of biomembrane structure (structural proteins). Some of them are involved in the transport of ions across the membrane (pump and ion channels) or in the transfer of substances along the electrochemical gradient by facilitated diffusion. Many of them are part of the receptors that are able to specifically bind hormones, neurotransmitters and other signal molecules. Some have the role of enzymes. Proteins and glycolipids are part of the antigens.

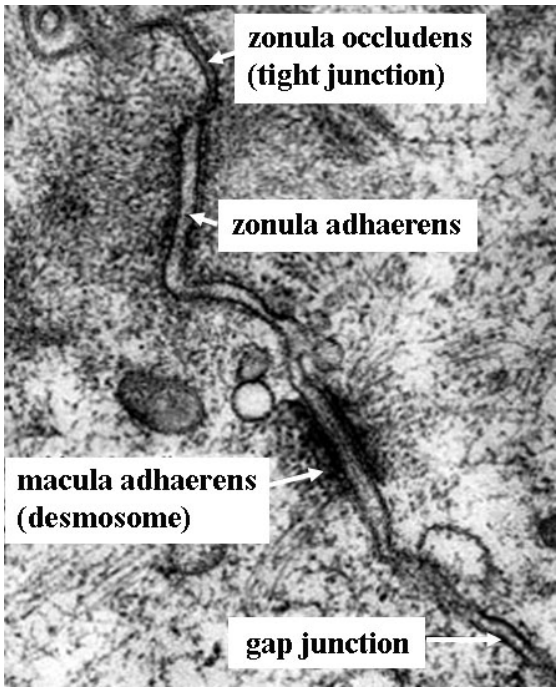


● **Figure 14.** Structure of cell membrane (fluid mosaic model)

2.2.2.1 Intercellular junctions

Cell membrane is actively involved in the creation of intercellular junctions. According to the number of layer contacts, thickness of intercellular space and its symmetry, intercellular junctions may be classified into three types (Fig. 15):

- ***zonula occludens* –** close connection, at which the cell membranes of neighbouring cells make contacts. If the distance between membranes is 2 – 3 nm, but they are not fully merged, it is **gap junction**. When the membranes fuse together it is **tight junction**. With this type of connection is carried intercellular communication (e.g. coordination of activities of neighbouring cells, synchronization of cilia oscillations etc.);
- ***zonula adhaerens*** arises as a continuation of the *zonula occludens* towards to basal part of cells. It surrounds cell by the perimeter and thus contributes to the cohesion of the tissue;
- ***macula adhaerens*** (desmosome) – strongest and most complex cell connection in shape of disk which arises at the base of cells. It develops by attachment of neighbor cell membranes which create cavities (~24 nm). Intercellular space is filled by electrondense grain mass (central lamella). From it arises out filaments, that are in contact (connection) with cell membrane. Cytoplasm in area of desmosome is modified and contains cytoplasmic plate which is associated with tonofilaments.



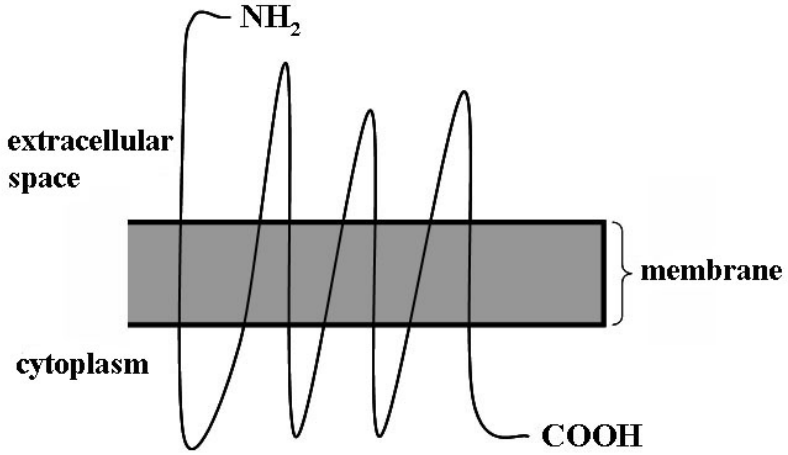
● **Figure 15.** Types of intercellular junctions

2.2.2.2 Membrane receptors

Membrane receptors are protein structures located in cell membrane, which are responsible for recognition and binding of signal molecules (e.g. hormones, neurotransmitters etc.). Through these receptors cell interacts with its surroundings. Membrane receptors may be classified into:

- **receptors which are part of the ion channels** – these are receptors for transport of cations (e.g. nicotine-acetylcholine receptor and receptor for excited amino acids) and anions (e.g. glycine receptor and receptor for gamma amino-butyric acid);
- **receptors with enzyme activity** are receptors with intracellular protein subunit which catalyzes certain chemical reactions. These include receptors with tyrosine kinase activity, insulin receptor etc.;

- **receptors coupled to G proteins** – the largest group of membrane receptors (five families are known). They are composed of polypeptide chains that pass through the membrane (Fig. 16). Extracellular part contains the N-end of the chain while in the cytoplasm there is C-end. Chain makes three string loops in the cell and three outside. Outer loop is used to bind signal molecules and the interior is essential for subsequent interaction with G proteins (activated after binding of guanosine triphosphate – GTP). Activated G protein stimulates the effector enzyme (e.g. adenylatcyclase, phospholipase, etc.), ensuring "transmission" of signal inside the cell. The result is known as second messenger (the first messenger is a signal molecule)



● **Figure 16.** Scheme of receptor coupled to G proteins

2.2.2.3 Transport of substances through the membrane

Transfer of substances into cells and outside of cells is realized by two basic mechanisms – passive and active transport.

The passive transport ensures transfer of substances in the direction of concentration gradient without consumption of energy (diffusion and osmosis). The speed of transition depends only on the size of the gradient (difference between concentrations in the cell and outside). Given the selective permeability of the cytoplasmic membrane only a few substances with low molecular weight (e.g. water, oxygen, carbon dioxide, urea, methanol and ethanol) can be transported by this way.

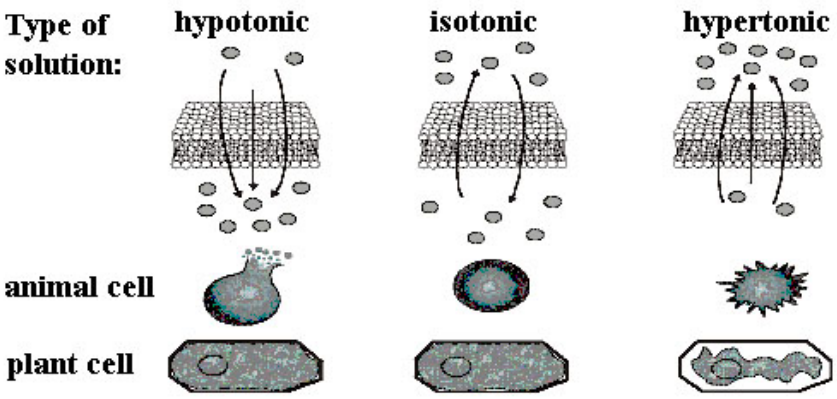
Diffusion is an unordered movement of molecules in solution. It results in the movement of dissolved substances from the higher concentration to places of lower concentration. This movement will stop as soon as the concentration of the substance on both sides membrane equalized.

Osmosis is a process in which water passes through the cytoplasmic membrane from the environment with a lower concentration in more concentrated environment. The process takes place until equalization of concentrations of both solutions. In case, that both solutions are **isotonic** to each other and cells that are in it perform no changes. For human cells the isotonic solutions are 0.9% NaCl saline and 5% glucose solution.

If the solution in the extracellular environment is more concentrated than inside the cell, it is **hypertonic** solution. The cells lose water and shrink. In plant cells occurs **plasmolysis** (separation of the plasma membrane from the cell wall).

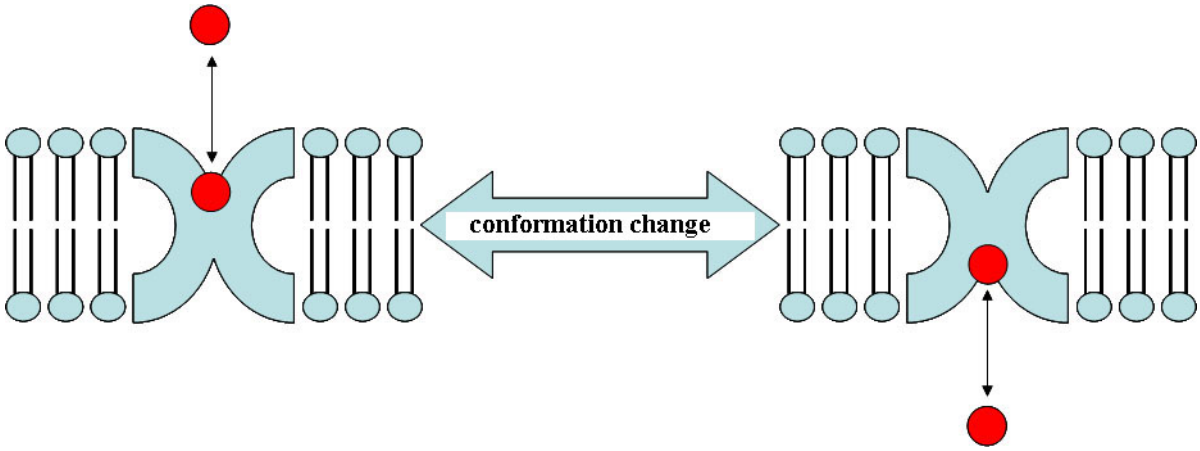
If the solution outside the cell is of lower concentration as in the cell, it is **hypotonic** solution. Water penetrates into the cell. Animal cell increases in size and burst (**cytolysis**); e.g. **haemolysis** of red blood cells. In plant cells only it increases their turgor – cell wall prevents them against **breaking**.

Phenomena of osmosis in plant and animal cells are presented by figure 17.



● **Figure 17.** Phenomena of osmosis in plant and animal cells

Facilitated diffusion (Fig. 18) represents the transport of substances mediated by plasma membrane proteins without consumption of energy in the direction of concentration gradient. This process occurs so that the substance is bound to transport protein on the cell surface. It changes its conformation and the substance is released into the cytoplasm (e.g. transport of glucose).



● **Figure 18.** Facilitated diffusion

Given that water diffuses across the cell membrane very slowly and in limited quantities, the transport is ensured through the special water channels – **aquaporins**. These are protein structures with a diameter less than 0.2 nm. This is to achieve high selectivity – release only water, but does not release her from of secondary ions (H^+, H_3O^+ a OH^-) or small polar molecules such as urea. It is expected that through the structure of aquaporin "leak" about 2 – 4.10^9 water molecules per second. Mechanism of regulation of these channels is currently unknown.

Active transport is the transfer of substances against concentration or electrochemical gradient without moving membrane. This process ensures due to consumption of energy obtained by dissociation of ATP (adenosine triphosphate) to ADP (adenosine diphosphate) or up to AMP (adenosine monophosphate). This process is provided by special transportation systems (channels and pumps), protein complexes, which pass through the membrane. It is a rigorous selective and managed process, often controlled by receptors. There are two basic types of active transport – primary and secondary.

Primary active transport is realized against concentration or electrochemical gradient with the energy consumption (obtained by hydrolysis of ATP). This process is performed by cyclic phosphorylation and dephosphorylation of transport proteins. This also changes the affinity to the substrate – alternately on the outside and inside the membrane. The whole process can be summarized as follows – transported substance (substrate) is attached to

phosphorylated transport protein; protein is dephosphorylated to open the binding site toward the cytoplasm and the substrate is released. The function of Na^+– K^+ pump (Na +-K +-ATP-ase) and H^+ pump (H^+–ATP–ase) is realized by this way.

In **secondary active transport**, the affinity of membrane transport protein is not changed by phosphorylation, but by the attachment of ions (e.g. Na^+). These proteins have two sites, first one for connection with ion and second one for transported substrate. In the case of the substrate and the ions are transported in the same direction, it is **cotransport**. In the case of transport in the opposite direction, it is **antiport**.

In addition to the basic types of transportation some substances may transfer through transportation (ion) channels:

- **channels activated by electrical changes** are opened and closed by changing membrane potential (e.g. sodium channel);
- **channels activated by receptors** – to change the channel passage is caused by attachment of hormone or neurotransmitter to receptor;
- **channels activated by mechanical stress** – channel opening is caused by a mechanical change in membrane tension. They are part of mechanoreceptors.

2.2.2.4 Endocytosis and exocytosis

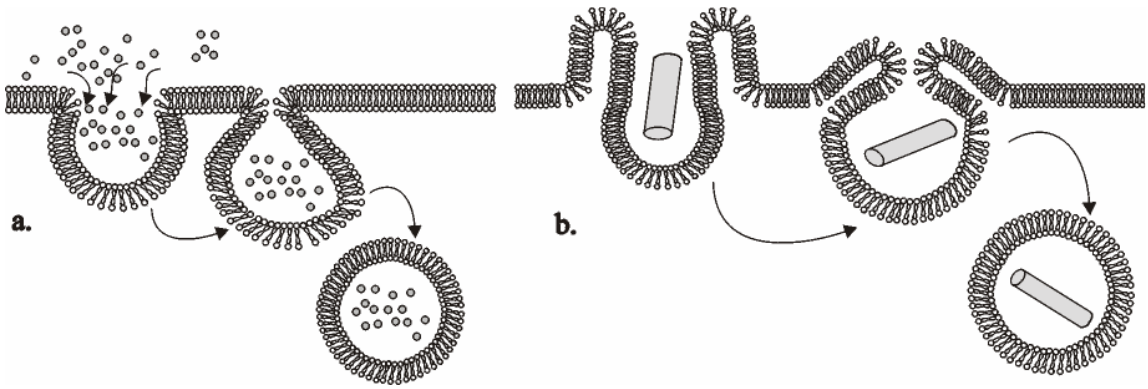
Transport of substances with high molecular weight is carried by endocytosis and exocytosis. Both processes are associated with active participation of the cytoplasmic membrane (changes in its structure or its movement).

Endocytosis is the process by which substances are transported into cells. According to transported substances we distinguished pinocytosis (especially the transport of soluble substances) and phagocytosis (transport of solids).

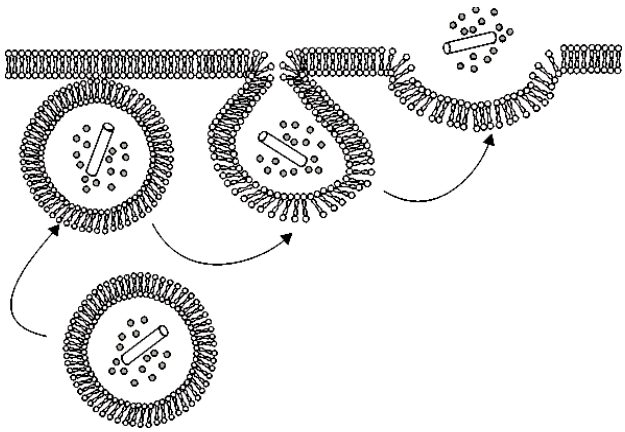
In **pinocytosis** (Fig. 19a) the transported substance is attached to receptor in the plasma membrane, which "creates" hollow inside the cytoplasmic membrane. The resulting cavity gradually increasing and surrounded transported substance. Finally, it is closed and creates a pinocytotic vesicle, which is released into cell and travels to the place of further processing. After that plasma membrane integrity is restored.

Phagocytosis (Fig. 19b) represents transport of solid particles, for example phagocytosis of bacteria. The cell generates plasma membrane processes (pseudopodia) which surround the transported material. It enters cells in vesicles and is processed.

In **exocytosis** (Fig. 20) the substances are transported from the cell into the extracellular environment. Secreted material is located in vesicles, which usually arise from endoplasmic reticulum and Golgi apparatus. Vesicle approaches the plasma membrane, touched her, and merging with it and the substances are released into the environment.



● **Figure 19. a.** Pinocytosis, **b.** Phagocytosis



● **Figure 20.** Exocytosis

2.2.3 Intracellular communication

Intercellular communication is carried out by transmission of information (signals) between cells. It is mostly based on the production, transport and recognition of specialty chemicals by cells. It influences the spatial structure of molecules that bear information (ligands) as well as the structure and location of receptors responsible for signal recognition. To maintain the accuracy of information transfer there is strong affinity between signal molecule and receptor.

There are two types of communication – nerve and humoral. In the **nerve regulation** the transmission of signal occurs by mediator directly transferred close to the target cell – by nerve cell projection. In the **humoral regulation** the information molecule (e.g. hormone) is excreted to body fluids by which it is transported to the cell with proper receptor.

In regard to the place of signal molecule production and overcome path to the receptor, we distinguish three main types of humoral communication:

- **endocrine** – signal molecules are hormones produced by glands of internal secretion. Hormones are transported to the target cells by blood and interstitial fluid;
- **paracrine** – signal molecules are excreted by cells of tissue and effect only cells in their neighborhood. They are spread by interstitial fluid to a short distance (only few millimeters). This regulation ensures management of activity and regeneration of tissues. Moreover, it has important role during embryonic development;
- **autocrine** – the cell manages its own activity. Proper timing of certain processes in the cell is essential (e.g. in cell division). The process can take place only when the cell synthesizes signals molecule which is attached to the receptor inside the cell, or effects other important protein.

2.3 Cell organelles

Cytoplasm represent basic inner environment of the cell. It is composed mainly of water (70 – 80%). It also includes a considerable number of ions, inorganic and organic compounds (e.g. fatty acids, amino acids, lipids, carbohydrates, nucleic acids, proteins). Cytoplasm is a semi-liquid mass, which should be in form of sol (liquid) or gel. Protein content and their ability to bind water influences its viscosity. The cytoplasm of eukaryotic cells contains a variety of cell organelles.

According to their composition cell organelles are distinguished into three basic types:

- **membrane**, which are composed from one membrane (endoplasmic reticulum, Golgi apparatus, lysosomes, peroxisomes, vacuoles and other vesicles) or from two membranes (nuclear envelope, mitochondria and chloroplasts);

- **composed of proteins –** cytoskeleton (microtubules, microfilaments, intermediate filaments, flagellas, cilia etc.);
- **composed of proteins and nucleic acids –** ribosomes, nucleolus.

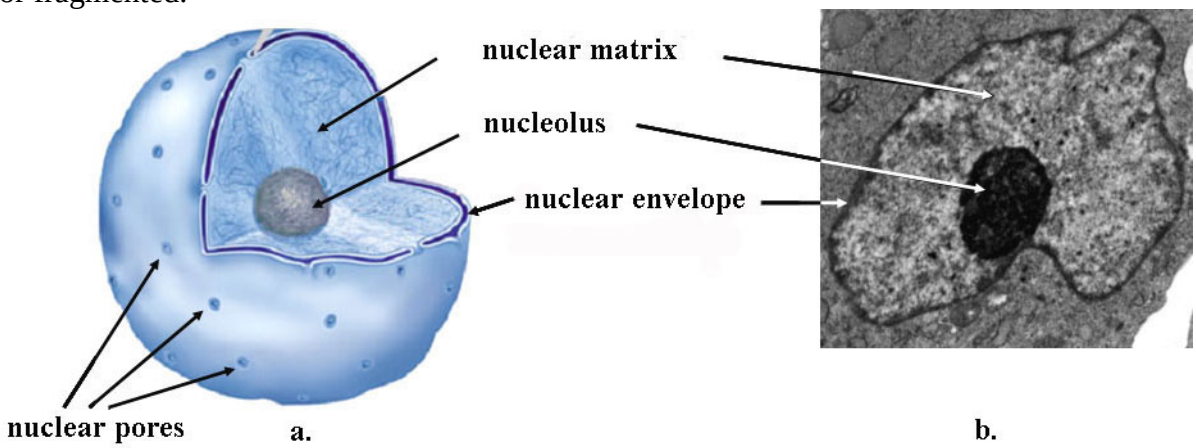
2.3.1 Nucleus

Nucleus is the coordinating and control center of cells. It contains the genetic information that is embedded in the structure of DNA. The inner structure of nucleus is very complex and is characterized by high dynamics of the processes that occurs there. During the cell cycle is periodically changed. **Interphase nucleus** is observed between two mitotic divisions. The second form is the **mitotic nucleus** which morphologically "disappears" during the indirect cell division.

Most of eukaryotic cells contain only one nucleus. Some cells lose nucleus in the process of differentiation (e.g. mammalian erythrocytes). On the other hand, some cells of plants or protists may have two or more nuclei.

Nucleus is composed of three basic parts – nuclear envelope, nuclear matrix and chromatin. Integral part of the interphase nucleus is the nucleolus (Fig. 21a, b).

The size of the nucleus varies from 4 to 30 μm, depending on cell type, DNA content and the "age" of cells (in the young cells is greater than in the older). Nucleus shape mostly depends on the shape of cells. Basic shape is spherical, but it may be oval, flattened, irregular or fragmented.



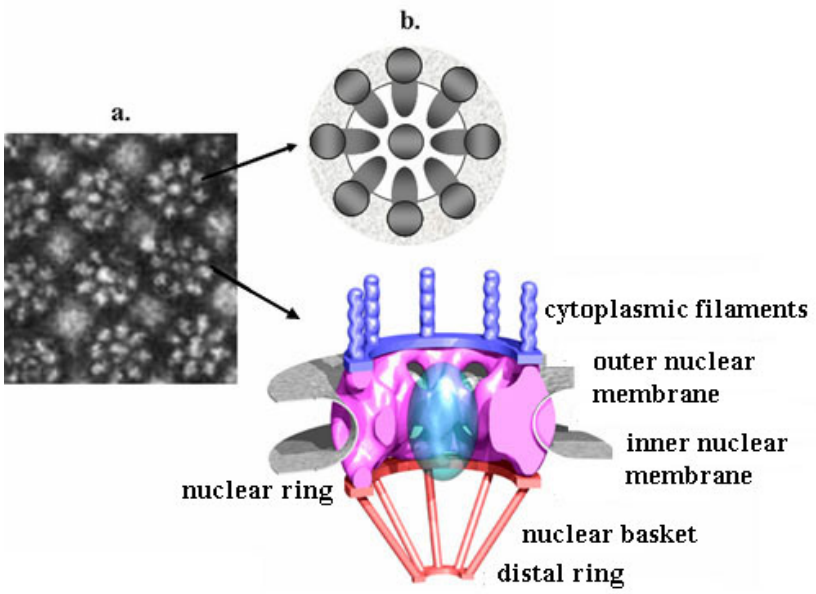
● **Figure 21. a.** Scheme of nucleus; **b.** electrongram of nucleus (TEM)

2.3.1.1 Nuclear envelope

Nuclear envelope of eukaryotic cells consists of two membranes. The inner membrane encloses the contents and the outer is in contact with the cytoplasm and endoplasmic retikulom. The space between these membranes is wide 20 – 80 nm and is called as perinuclear space. Both membrane in some places are merged and creates nuclear pores.

Nuclear pores represent complicated gaps in the nuclear membrane with a diameter of about 70 nm and occupy 5 – 25 % of nuclear envelope surface (e.g. in human cells there are 3 – 4 000 nuclear pores). They consists of protein fibers and granules, which together form the pore complex (Fig. 22a).

From upper view the nuclear pore has circular shape. In his perimeter there are 8 subunits (granules of nuclear origin) and in the middle it has central granule (Fig. 22b). It is associated with circumferential sub-units through protein fibers creating a diaphragm. From side view the nuclear pore is composed of two identical rings – external and internal (Fig. 22c).



● **Figure 22. a.** Pore complex; **b.** scheme of nuclear pore (upper view); **c.** scheme of nuclear pore (side view)

Nuclear pores provide active transport of substances from the nucleus to the cytoplasm (especially RNA subunits and ribosomes) and from the cytoplasm to the nucleus (e.g. transport of histones, nutrients and regulatory proteins).

2.3.1.2 Nuclear matrix

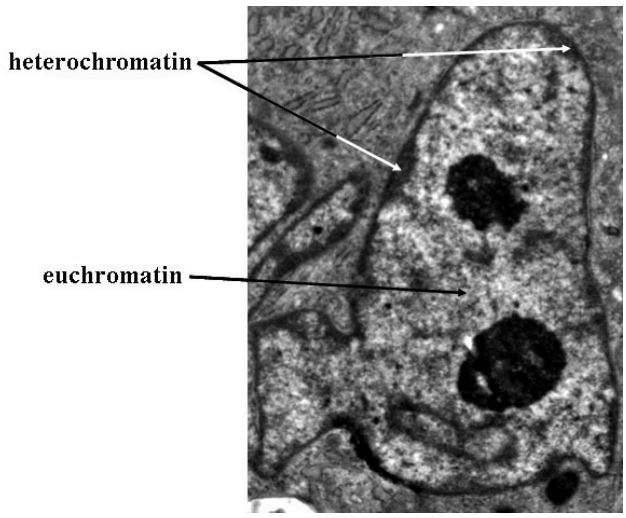
Nuclear matrix represents the most important extrachromatine component of nucleus. It consists of lamins (A, B and C) that form a protein layer on the inner side of the internal nuclear membrane envelope (i.e. lamina). Moreover it is formed by a complex network of fibers which consists of "Y" shaped 10 nm subunits from lamin A and is attached to the lamina. In this network, there are compressed spaces with enzymes and other proteins necessary for transcription (transcriptomes) and replication (replicosomes). Chromosomes are attached on lamin B and lamina through specific protein, which contributes to their spatial arrangement. Lamin B also participates in the attaching of other lamins on the inner nuclear membrane and is connected with intermediate filaments of cytoskeleton, which is crucial to the defragmentation of nuclear envelope in prometaphase and its reconstruction in telophase. The main components of the nuclear matrix are lamins A and C. They participate in the reconstruction of the inner organization of nucleus after cell division.

2.3.1.3 Chromatin

Interphase nucleus is filled by seemingly amorphous mass – **chromatin** which is composed of linear DNA associated with the histon or non-histon proteins. Histon proteins have structural and regulatory function. Non-histon proteins have mainly regulatory functions and manage the internal organization of the nucleus. Detailed information on the organization of the deposit and the use of genetic information is described in the Genetic aspects of the normal and pathological traits in humans (see chapter 1).

In eukaryotic cells, we distinguish two types of chromatin – heterochromatin and euchromatin (Fig. 23).

Heterochromatin consists of condensed chromosome segments and produces dense aggregations, which are mostly located near the nuclear envelope. It can be also irregularly distributed throughout the nucleus. Heterochromatin located by the nucleolus is called



perinucleolar chromatin. If euchromatin is in the nucleolus, it is intranucleolar chromatin. It is a transcriptional inactive chromatin. It may be facultative or constitutional.

Euchromatin has soft, foamy or almost fibrillar structure. It consists of decondensed segments of chromosomes. It is transcriptional active and is present in the cells with high protein synthetic activity.

● **Figure 23.** Heterochromatin and euchromatin **(**TEM)

2.3.1.4 Nucleolus

Integral part of eukaryotic cell nucleus is the **nucleolus**. This functional organelle is most abundant in the G1 phase of the cell cycle. It has spherical shape of about 1-5μm. From a chemical point of view it is formed of RNA molecules and proteins. It is a place of rRNA synthesis, post-transcriptional modification and completisation of ribosome subunits.

In electron microscope we distinguished three basic parts of nucleolus:

- *pars granulosa* – composed of ribonucleoprotein particles, which are precursors of ribosomes;
- *pars fibrosa* – composed of soft filaments, which are stored close to each other. Contains precursors of rRNA;
- nucleolar organizer region – the place of nucleolus restoration after cell division.

Classification of nucleolus according to arrangement of ribonucleoprotein granules:

- **nucleoli with nucleolonema** – the most common in animal cells. The active synthesis of rRNA take place there;
- **ring-shaped nucleoli –** they are characterized by lack of granules in central zone. They are present in resting cells;
- **compact nucleoli** – they posses less fibrous structures. Granules are present in whole nucleolus. They are typical for embryonic and cancer cells.

During the cell cycle, nucleolus overcomes **nucleolar cycle**. In prophase nucleolus disappears. Its structure is restored in the telophase at nucleolar organizer region (so-called NOR).

2.3.2 Mitochondria

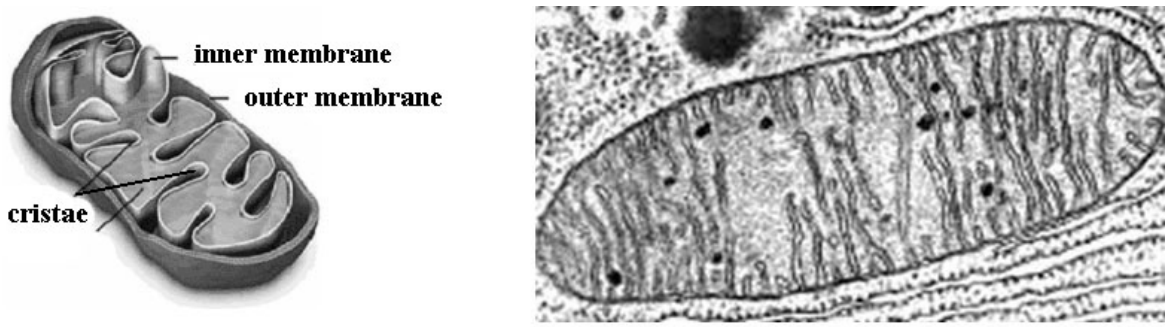
Mitochondria are organelles inevitable for the life of eukaryotic cells. They are involved in generating energy for cells by breaking down of saccharides, lipids and other energy-rich organic compounds.

Mitochondria can vary in number and shape according to the type of cell. Their number is in a direct proportion to the intensity of cell's energy metabolism. They consist of two biological membranes (Fig. 24). The outer membrane encloses it while the inner one is folded into the mitochondrial cristae expanding its surface. The enzyme system (H+ATP synthase) is localized in the inner membrane and is responsible for cellular respiration (oxidative phosphorylation). This is where the glucose is broken down with the freed energy

bounding into ATP molecules (ADP + E + P = ATP). It also contains other important enzymes, e.g. cytochromes, NADP dehydrogenases etc.

The space between the cristae is filled with mitochondrial matrix made up of phospholipoproteins and ions of calcium and magnesium. Moreover it also contains enzymes of the citric acid cycle (the Krebs cycle).

Mitochondria have their own circular DNA and ribosomes of the prokaryotic type. This enables their auto-reproduction and synthesis of their own proteins. Most of mitochondrial enzyme substance is however coded by nuclear genes. They are synthesized on endoplasmic reticulum, modified in the endoplasmic reticulum and completed in Golgi apparatus. Consequently, they are transported into mitochondria where they are enhanced with proteins synthesized in mitochondria and become functional.



● **Figure 24. a.** Scheme; **b.** electrongram of mitochondrion

2.3.3 Chloroplasts

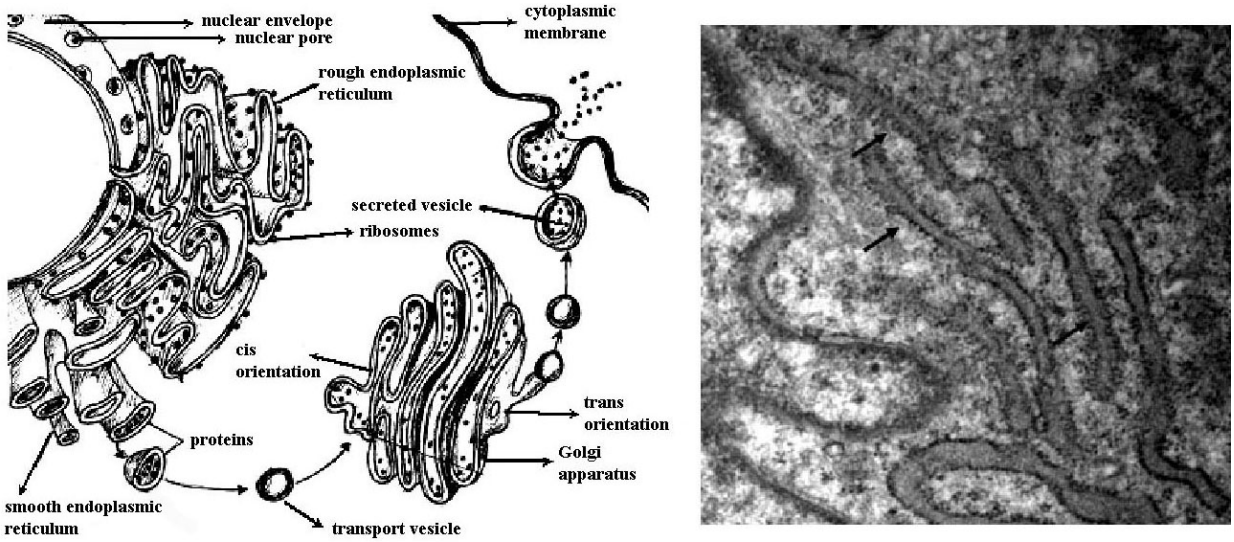
Chloroplasts belong to organelles of eukaryotic cells that are made up of two biological membranes. They are only present in green plants. They contain chlorophyll and are involved in photosynthesis (the transformation of the light energy into the energy of chemical bonds in the glucoses). Similarly as mitochondria, they contain their own genetic information and ribosomes – of prokaryotic type.

2.3.4 Endoplasmic reticulum

Endoplasmic reticulum (ER) is a system of tubules, cisterns and flat vesicles from the biological membrane closely communicating with the nucleus and via transport vesicles with the Golgi apparatus and cytoplasm (Fig. 25a). ER is responsible for synthetic processes. From the morphological and functional aspect, we distinguish between two types of endoplasmic reticulum. **Smooth ER** which is the place of synthesis of saccharides and lipids (including parts of the biomembranes), steroid hormones and cholesterol. The detoxication function of the smooth ER is also significant – excessive cell‘s intoxication leads into apoptosis. The muscle cells contain a special form of endoplasmic reticulum – **the sarcoplasmic reticulum.** It ensures the transport of Ca^{2+} cations that are inevitable for muscle contraction.

Rough endoplasmic reticulum (Fig. 25b) contains ribosomes on its surface and is the place of protein synthesis. Formed proteins penetrate its structure and are modified for the first time. For further processing they are transported in a vesicle from the membrane into the Golgi apparatus.

The ratio of the rough and smooth ER in a cell depends on its function and products it creates.



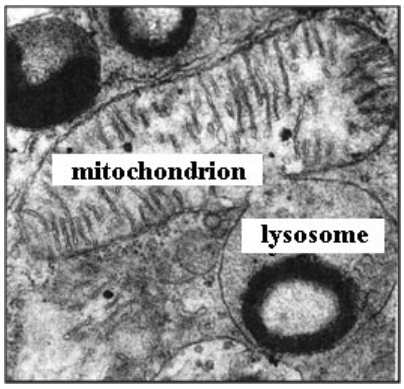
● **Figure 25. a.** Scheme of endoplasmic reticulum and Golgi apparatus; **b.** rough endoplasmic reticulum (TEM)

2.3.5 Golgi apparatus

It is made up of a system of flat cisterns (Fig. 25a) whose sides are from the functional aspect divided into cis and trans. Proteins synthesized on the endoplasmic reticulum, usually enclosed in a transport vesicle, come to the cis side. Their posttranslational modification continues inside of the Golgi apparatus. They leave Golgi apparatus from the trans side in the form of vesicles to cytoplasm. Some of them fulfill specific tasks in a cell, others join the cytoplasmic membrane and their content comes into the extracellular environment. Some vesicles only take part in "circulation" of cell membranes.

2.3.6 Lysosomes and other vesicles

Lysosomes are vesicles from single biological membrane (Fig. 26) created by being detached from the Golgi apparatus as a **primary lysosome**. They contain digestive enzymes (e.g. hydrolases for protein digestion). Following connection with an unneeded organelle or pinocytotic (fagocytotic) vesicle creates a **secondary lysosome** and lysosomal enzymes break down the content of these structures. In plant cells and some protists, the function of lysosomes and many other tasks is performed by **vacuoles**.



Peroxisomes belong to the large family of vesicles in cytoplasm, the role of which is to ensure activity of various enzymes. Peroxisomes contain catalase eliminating the aggressive hydrogen peroxide. Enzymes in the cytosolic vesicles are thus available for the cell; however, they are not directly contained in the cytoplasm, but used when the cell needs them.

● **Figure 26.** Mitochondrion digested by lysosome (TEM)

2.3.7 Cytoskeleton

Cytoskeleton (Fig. 27) is the internal functional and dynamic skeleton of eukaryotic cells. It participates in forming of the shape of cells, distribution of organelles and performing of some intracellular activities (e.g. movement, contraction etc.). Cytoskeleton consists of protein-based microtubules, microfilaments, intermediate filaments and microtrabecules, while each of these plays a different role.

Microtubules are the same in all eukaryotes. They are long, firm and hollow cylinders about 25 nm in diameter comprising the molecules of tubulin (dimers consist of α and β monomers). Microtubules are made longer or shorter through polymerization and de-polymerization (adding or removing of tubulin dimers). They participate in the transport of vesicles between endoplasmic reticulum, the Golgi apparatus and cytoplasmic membrane. They play an important role as part of mitotic spindle fibers in the movement of chromosomes during cell division. They also make up the structure of centrioles, tails and cillia.

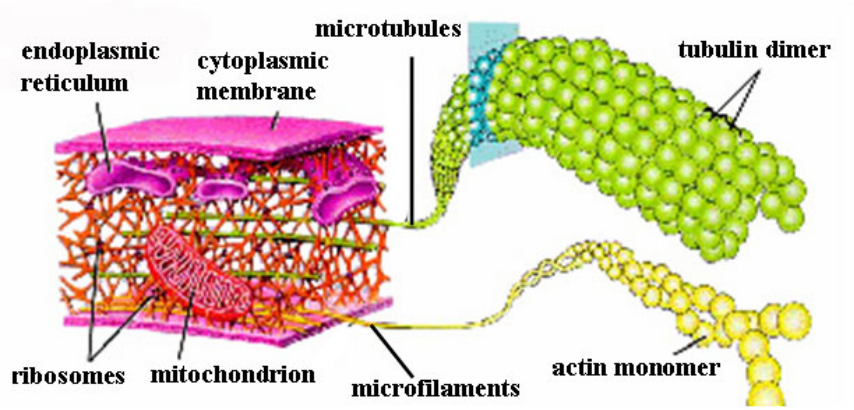
Microfilaments are thin (7 nm in diameter) filaments of helical structure made up of globular protein called actin to which the proteins of myosin are bound together creating (via clutching) a contractile system of muscle cells.

Intermediate filaments are think fibers (10 nm in diameter) that are not able of contraction. These include, for example, lamins of the nuclear matrix, epithelial keratins, vimentins of the intercellular substance (EDM) of connective tissues – networks of proteins connecting cells and neurofilaments (the transport system of nerve cells). They provide for cell resistance to pulling and pressing. They also participate in the distribution of organelles and inclusion within a cell.

Microtrabeculae are considered to be the skeleton of the cell itself.

From the cytoskeleton, the movement organelles of some eukaryotic cells, e.g. flagella and cilia, are also derived.

Kinetochore, a structure important for cell division, is also made up of proteins. It is the protein complex attaching to the centromere of chromosomes (chromatids) and enabling in the course of cell division free attachment of tubulin fibers of the mitotic spindle.



● **Figure 27.** Cytoskelton

2.3.8 Centrosome

The centrosome is a structure of eukaryotic cells located near the nucleus. It is of vital importance for the process of cell division. It consists of two complexes of three couples of orthogonally arranged tubulin fibers (centrioles). The centrosome doubles prior to cell division.

2.3.9 Ribosomes

These might be one of the smallest cell organelles, but they are crucial, because of protein synthesis. They are made up of a large and small sub-unit that is connected only during translation (the protein synthesis). The sub-units are made up of proteins and ribosomal RNA.

Prokaryotic ribosomes are smaller, but their gravitation density if 70S (Svendberg units, determining the sedimentation speed at ultracentrifugation). They are present only freely in the cytoplasm of prokaryotic cells. They are made up of three types of rRNA and 52 proteins.

The **ribosomes of eukaryotic cells** are larger and their gravitation density is 80S. They consist of four types of rRNA and 82 proteins. They are present freely in cytoplasm, but their prevailing majority participates in the creation of rough ER. This enables the eukaryotic cell to effectively manage the course of protein synthesis.

In the eukaryotic cells there are also ribosomes of the prokaryotic type, specifically in mitochondria and chloroplasts. These organelles synthesize their own proteins needed in order to activate their enzymes and auto-reproduction.

3. Reproduction of cells

Multiplication (division) of the cell belongs to its primary functions. Cell division is a part of subsequent processes, known as **cell cycle.** In multicellular organisms it is not only way how to increase number of cells, but include also structural and functional specialization of cells – via differentiation. If particular cell will continue in cell cycle toward its division, depends on many factors – extracellular and intracellular, stimulating or inhibiting.

In regard to course of division and its result, we recognize generally three types of cell division – amitosis, mitosis and meiosis.

Amitosis (direct division) happens immediately after replication of DNA. In form of "binary fission" is typical for bacterial cells. Multiplication of intracellular endosymbiont organelles (mitochondria and chloroplast) termed as "endoreduplication" or fission.

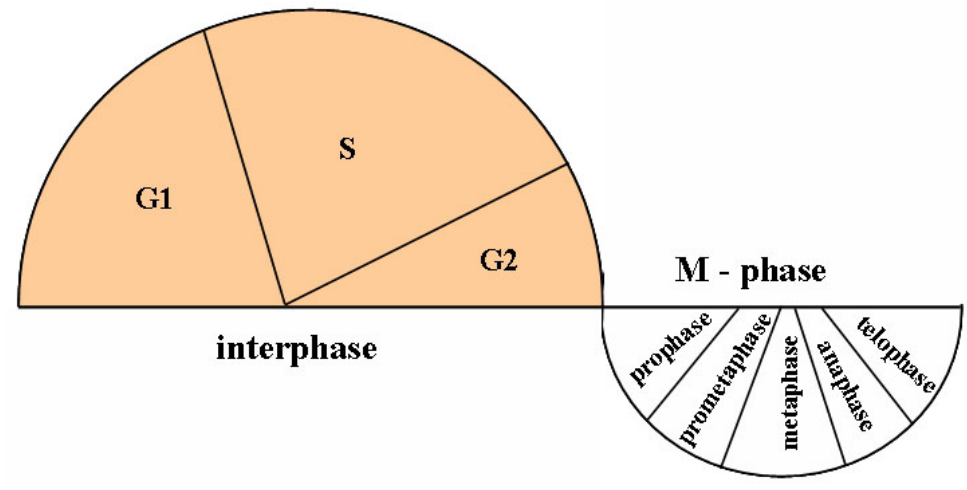
Mitosis is indirect division, because between replication of DNA (S-phase of cell cycle) is intermission (gap) – G2 phase of cell cycle. Mitosis is for multicellular organisms standard mode of cell division, because it guarantees genetic identicalness (concordance) of daughter cells.

Meiosis (reductive division) is essential precondition to gametes origin – e.g. haploid cells having single chromosome of each type. Fertilization of gametes recreates original – species-specific (diploid) number o chromosomes.

In the world of protozoa, especially parasitic ones, does exist many extraordinary ways of cell division, but these out of range of this text.

3.1 Cell cycle of eukaryotic cells

The cell cycle consists of two main phases, which are **interphase** and **M-phase** (mitosis phase). The individual phases of the cell cycle proceed after each other (Fig. 28). The process is regulated by a complex of regulatory proteins, which are coded by tumor suppressor genes (they have a control function) and protooncogenes (stimulating division). A failure of their normal function can cause deregulation of the cell cycle and a consecutive malign transformation of the cell, meaning a change to a cancer cell.



● **Figure 28.** Scheme of the cell cycle

The time duration of the cell cycle is genetically determined and is connected with the telomeres of chromosomes, but is also effected by different signal molecules from the environment and by the cell itself (look up part 2 – chapter 10). It differs, depending on the cell type of various tissues.

Interphase is a time between two divisions and is made up of G_1, S a G_2 phase. The duration of the phases differs and depends on the type of the cell and the life period of the individual.

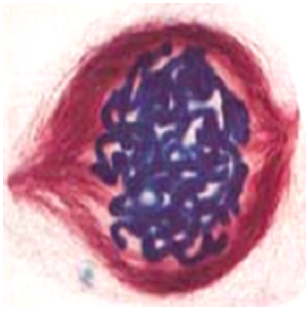
G_1 phase begins after the end of the previous mitosis. It is characterized by an intensive synthesis of proteins, and usually also growth and of new cell. During this process, in the so called **G_0 phase**, the cell differentiates, to fulfill specialized functions for organism. Time duration of G_0 phase is the most variable component of the interphase. In embryonic cells it is short, while with older individuals it is longer. In case that the cell will not divide (e. q. a mature human erythrocyte) this phase is the final one. At the end of the G1 phase is the so called **main checkpoint**, in which it is decided weather the cycle will continue or not. If it will, it is necessary to find and repair mutations in DNA. If the number of mutations is higher then can be repaired in a given time limit, the protection mechanisms evoke a „silent"cell death (**apoptosis**). This step is an important protection of the organism against the accumulation of mutations and the consecutive formation of cancer cells.

S phase (synthetic) is a time during which the duplication (semiconservative replication) of the nuclear DNA occurs. Considering the length of the DNA in the nucleus (in women around 2 m) and the processes of their repeating control and the repair of defects, it is the longest phase of the cell cycle, even tough the replication takes place at numerous places simultaneously. At its end each chromosome is doubled, meaning it consists of two chromatids connected by Scc1 and Scc3 proteins – cohensins.

G_2 phase is a relatively short period of preparation for mitosis and it contains another checkpoint of the cell cycle. After replication it is important for the cell to check the DNA and repair the potential mistakes. It is also important to prepare the necessary proteins, mainly tubulin, as well as sufficient sources of energy. During this phase the duplication of the centrosome occurs (made up of centrioles). At the same time, on the second centrosome, a so called astral complex and the basis of non-kinetochore microtubules are formed.

M phase - mitosis is a part of the cell cycle, during which the division of the nucleus occurs (karyokinesis) and consecutively the division of the whole cell (cytokinesis) happens. It was first described and named by Walther Flemming (1887 – 1880).

Mitosis is divided into five phases – prophase, prometaphase, metaphase, anaphase and telophase.

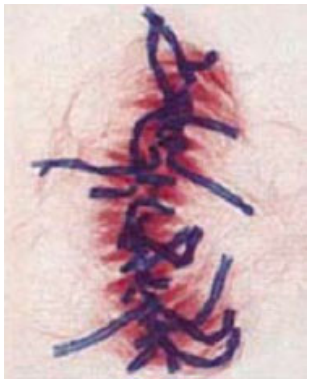


During **prophase** (Fig. 29) the condensation of chromatin (chromosomes) begins. Continue elongation of the non-kinetochore microtubules, from each centrosome toward another one. These fibrils slide on each other, which starts the movement of centrosomes towards the opposite cell sides (poles). An early division spindle is formed. Starts the process of nuclear envelope disorganization and when it "disappears" finishes prophase.

● **Figure 29.** Prophase in plant cell

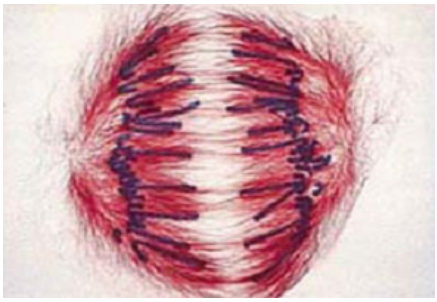
During **prometaphase** the movement of the centrosomes towards the poles continues. The condensation of the chromosomes goes ahead and they can be observed as stick-like formations. The process of chromatid separation from the end part of the chromosomes (telomeres) begins. On the outer side of each chromatid centromere functional kinetochores are formed. At the same time, kinetochore microtubules (KMT) "grow out" from each centrosome (elongated by polymerization of tubulin dimers) and enter the area of the former nucleus – "searching" for connection to kinetochores. When kinetochore microtubules connect to both kinetochores of particular doubled chromosome, they begin to elongate and shorten (by depolymerization), to transport the chromosome to the central (equatorial) plain of the cell. This takes a certain amount of time, making prometaphase the longest period of mitosis.

Metaphase (Fig. 30) is a relatively short period during which the duplicated chromosomes are located in the equatorial plain of the cell. The centrosomes are pushed to the opposite sites of the cell – spindle body is finished. All the kinetochores are occupied by kinetochore microtubules. Cohesins, except for the parts between centromeres of sister chromatids, are destroyed. This is why the metaphase chromosomes have the shape of the letter X. By this, all the conditions for the activation of the so called anaphase promotion complex (**APC**) are fulfilled and mitosis can continue to anaphase – chromatids are separated and transfer of daughter chromosomes can start. The mechanism is described in detail in chapter 10 of the second part of the text-book.



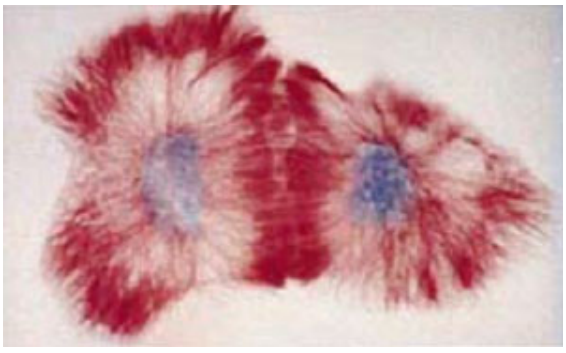
● **Figure 30.** Metaphase in a plant cell

During **anaphase** (Fig. 31) two parallel processes take place. Anaphase A is characterized by the shortening of the KMT, which is responsible for transporting ("pulling") of the individual daughter chromosome, to the centrosomes. In anaphase B the elongation of the non-kinetochore microtubules continues which elongates the whole cell and creates the space for cytokinesis. Both processes are supported by the activity of the so called motor proteins – dyneins and kinesins.



● **Figure 31.** Anaphase in a plant cell

During **telophase** (Fig. 32) the nucleus is reformed close to each centrosome. The formation of two new nuclei in the cell is called **karyokinesis**. Chromosomes decondense and the functional organization of the nuclei is renewed. Parallelly – the cell divides (**cytokinesis**) and two new identical daughter cells are formed – in animal cell by "cleavage" and plant cell by building of septum "from inside". Important is, that each of the two daughter cells retains one centrosome near the nucleus with the base of the non-kinetochore microtubules – new cell keeps the essential components necessary for the next division. If the cytokinesis doesn't take place, a so called syncytium is formed.



● **Figure 32.** Telophase (cytokinesis) in a plant cell

Mitosis makes up only about 5 – 10 % of the cell cycle duration. This is the reason why – even within very intensively proliferating tissues – it is difficult to find cells undergoing a certain phase of mitosis. Because of this, phenomena connected with the division of somatic cells are studied mainly on tissue cultures during *in vitro* cell cultivations (see chapter 4).

4. Cell and tissue cultures

Cell and tissue cultures represent a complex of methods, which enable maintaining basic physiological processes ***in vitro*** (out of living organism) longer than 24 hours. **Cell culture** is a complex of cells, which proliferates *in vitro* and is not organised as tissue. Tissue or organ culture represents any tissue or organ cultivated which preserve its structure and several functions.

The aim of this chapter is basic resume of latest discoveries about cell and tissue cultures and its utilization in a biomedical practice.

4.1 Brief history of cell and tissue cultures

First experiments of maintaining the live cells or tissues isolated from animals were performed at the beginning of 19^{th} century. These experiments were based on rinsing tissues or organs in different culture media (nutrition solutions), which should substitute *in vivo* system conditions. First successful cultivation had been described in 1907 by Harrison, who maintained isolated nerve tissue of a tadpole on coagulated frog plasma for several weeks.

Carrel and Burrows established methods of cultivation tissues and cells isolated from adult mammals. They experimentally proved growth stimulating effect of embryonal extract for several cell types. On the basis of achievements gained from these experiments they prepared culture medium consisted from chicken coagulated plasma and serum.

In the 1920s and 1940s epithelial cells isolated from tissue of various adult animals has been successfully cultivated. At the time it had been intensely worked on the development of cartilage and bone cells cultivation methods.

Important goal was management of cultivation fibroblasts isolated from mouse tissues, by which a malignant transformation *in vitro* was succeeded. Dr. Earle with coworkers established a cell line from these cells. Later from this cell line Sandford with the work team isolated first cell clone. In 1952 Dr. Gey established permanent HeLa cell line from the malignant human tissue, which is still used in biomedical praxis.

In 1960s and 1970s special cultivation techniques begun to implement which enabled long-term *in vitro* cultivation of malignant cells isolated from bioptic material in a patients with malignant diseases. At the time great amount of permanent human cells lines has been maintained. During this period many methods used in cytogenetics were also developed.

In the last 10 years cell culture techniques are orientated mostly on *in vitro* preparation of artificial tissues or parts of the human organs in the new biomedical field – **tissue engineering**. In this context it is necessary to master the cultivation methods of different cell types. Thus prepared artificial tissue could be used not only in the substitutive and reconstructive medicine, but also in toxicological experiments as three-dimensional models, which can be utilized for potential toxicity testing of various chemicals.

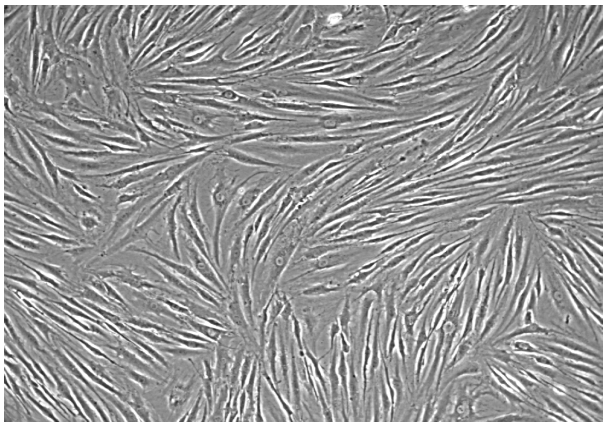
Recently the majority of scientific interest took place in cultivation of embryonic and adult stem cells. These are undifferentiated cells capable of long term self-renewing which might differentiate in other cell types. Stem cells are found in living organism during the whole life. They have an important role in embryogenesis and the tissue regeneration.

4.2 Biology of cultivated cells

Cell biology during the *in vitro* condition is not comparable with the conditions *in vivo* (in organism). Cultivated cells have usually different phenotype; they differentiate from cells in living organism morphologically as well as biochemically. Contact between cells and also cell to extracellular matrix is minimized, due to the low heterogeneity and absence of three dimensionality of tissue. Moreover, they miss many regular nutritions and hormonal stimuli. The effect of culture environment on cultivated cells can be summarized:

- characteristics of the substrate the cells grow on (surface of cultivation flask, semisolid gel, solution in suspension cultures etc.);
- extent of contact with other cells;
- composition of culture medium (inorganic salts, aminoacids, hormones, growth and differentiation factors etc.);
- gas content in the culture system (CO_2, O_2, etc.);
- incubation temperature.

For the initiation of proliferation under *in vitro* conditions, most of the cells isolated from the solid tissues must **adhere to the cultivation substrate** (Fig. 33). Originally for this purpose, glass culture flasks with partial negative surface charge has been used. Currently, various plastic flasks (e.g. polystyrene, polypropylene etc,) are used. Attachment of the cells to the substrate is ensured by the specific surface receptors for molecules that are found in the extracelullar matrix. Therefore in many cases a surface of the cultivation flasks is coated by several extracellular matrix components such as collagen and fibronectin.



● **Figure 33.** Example of adherent culture (human fibroblasts)

There are three main transmembrane proteins that provide adhesion cells to the substrate or to the other cells. Contact cell to cell provides a Ca^{2+} independent molecules (CAMs) and cadherins (Ca^{2+} dependent molecules). Interaction cell to substrate is provided by integrin, which is the receptor for collagen, fibronectin, enactine and laminine. The last group consists of transmembrane proteoglycans; which also provides the interaction between cells and intercellular matrix.

The disaggregating of the tissue and releasing of the cells from the substrate by proteases result in interruption of the interactions. Final concentration of the proteases and the exposure period is depended on the type of tissue or cultured cells. On the other hand while readhesion on the substrate after sub-passaging, the proteins must be synthesized by the cultured cells itself or it is necessary use the coated cultivation flask, with implemented synthetic proteins.

By camera observation of the cultivated cells, it has been proved that adhered cells are able to move on the substrate. The fastest cells are connective tissue cells – fibroblasts cultivated in low density. At the specific moment when they reach another cell, they begin to migrate to the opposite direction. If grown to confluence, the cells stop to migrate and a **contact inhibition** occurs, which leads to the termination of cell division. Similar migration is seen at the myoblasts and epithelial cells, whereas after the confluence the cells might result to differentiation depended on the microenvironment. In case of epithelial cell the migration is stopped immediately after the contact with another cell.

In vitro cultured cells are capable of differentiation. However, the following conditions must be performed – high cell density, cells interactions and the presence of differentiation factors. For this reason various three dimensional culture systems have been

developed. Most commonly used cultures are macroagregate cultures prepared by centrifugation. In addition, cells may be cultivated on natural or synthetic biomaterials. Major impact for *in vitro* differentiation has the composition of culture medium (e.g. hormones, growth and differentiation factors content).

Another unique phenomenon in the cultured cells biology is dedifferentiation process. Many cells in *in vitro* conditions lose its original biological and biochemical properties. This is due to microenvironment changes in which they are located (e.g. loss of three-dimensionality, the lack of intercellular interactions, the absence of hormones etc.). This process can be fully or partially reduced by using a suitable cultivation system.

Proliferation, migration, differentiation and apoptosis are *in vivo* regulated with reciprocal cells interactions and extracellular matrix, through various signal molecules (e.g. endocrine regulation, paracrine regulation, autocrine regulation). Under *in vitro* conditions previous processes are regulated only by the autocrinne and paracrine regulation, which limits the use of cell and tissue cultures. For this reason a special culture media are used, which are enriched by various exogenous factors (e.g. hormones, differentiation factors etc.).

The last important factor in the biology of cultured cells is their energetic metabolism. In most cultivation systems main source of energy is anaerobic glycolysis, which can operate without the presence of atmospheric oxygen. Main source of carbon is glucose; which is a part of almost every culture media. Not only glycolysis but also Krebs cycle remains active. Source of carbon is amino acids, mostly glutamine, dipeptides glutamyl–alanine and glutamyl–glycine.

4.2.1 Conditions for cultivation of cells *in vitro*

Successful cultivation requires **correct sampling** off the tissue or cells (biopsy or necropsy) and its adequate transport to the laboratory.

Precondition for eukaryotic cells of multicellular organisms cultivation is creating conditions that imitate the environment in the organism. The important factor is ensurance of appropriate culture conditions:

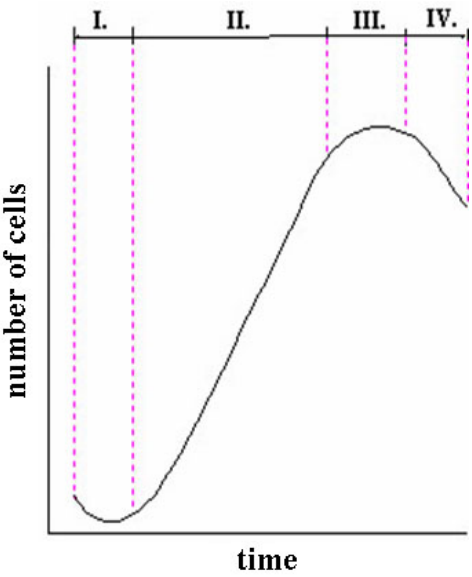
- **sterility** – prevents contamination of cultures by viruses, bacteria or fungi. Different antibiotics (e.g. penicillin, streptomycin, gentamicin) or antifungals (e.g. amphotericin B). Sterilization of the working place, materials, tools and solutions is necessary (hot air sterilization, autoclave, filtration, ionizing radiation, etc.);
- **temperature** – for most animal cells the optimum temperature is around 37 °C. The temperature is ensured by using special culture boxes – incubators;
- **pH** – the optimum concentration of hydrogen ions in the range 7.1 to 7.4;
- **osmotic pressure** – roughly 7.6 atmospheres (cells tolerate a change of ± 10%);
- **culture medium** – it is necessary to provide trophic of cultured cells. Composed primarily of water, ions (Na^+, K^+, Ca^{2+}, Mg^{4+}, Cl^-, $(PO4)^{3-}$ etc.), carbohydrates, amino acids, vitamins, proteins;
- **serum** – contains amino acids and proteins (e.g. growth factors, hormones, etc.);
- **substrate** – in the case of adherent cultures – bottom of the dish to which the cells are able to adhere. In some cases, the substrate itself is also the culture medium (suspension cultures).

4.2.2 Periods of cell cultures

The course of cultivation can be illustrated by the growth curve (Fig. 34), which has four phases and is valid for all types of cells:

- **lag phase** – cells adapt to *in vitro* conditions, they are mostly spherical in a shape and some of them adhere to the substrate (in the case adherent cultures);

- **logarithmic growth phase** (log phase) – cells are proliferating vigorously (depending on the type of cells it may take several hours to days), their shape is changing – mostly to the spindle, polygonal or radial one;
- **stationary phase** – cells are living in culture, but as a result of contact inhibition does not proliferate any more;
- **degradation phase** – cells degenerate (vacuolization of the cells, changes in the morphology of the cytoplasm and nuclei can be observed) and begin to release from the culture substrate.



● **Figure 34.** Growth curve (I. – lag phase; II. log phase; III. – stationary phase; IV. – degradation phase)

During the cultivation, regular exchanges of the cultivation medium are needed, depending on the amount and types of the cultured cells. Culture must be continually observed and evaluated in an inverted microscope. If necessary it can be photographically recorded. When cultured cells reach to approximately 80% of confluence (confluent layer), a passage is necessary – harvesting of the cell population and its transfer to the new cultivation dishes. The number of cell passages depends on the type of cultured cells. Passaging runs in four steps – releasing the cells from the cultivation surface (substrate), mechanically by scraping or enzymatically by trypsin; inactivation of trypsin by adding serum containing antitrypsin; re-suspension in fresh culture medium and finally platting cell suspension into new culture vessels.

4.3 Types of cell cultures

Primary culture is a cell population directly isolated from the tissue or organ that has not been passaged. In regard to the duration of cultivation short and long term primary cultures are recognized. **Short term cultures** – duration is up to 24 hours or maximum several days. Cultivated cells may have either high spontaneous mitotic activity (e. g. bone marrow cells, trophoblast cells) or the mitotic activity have to be stimulated by mitogenic activator (e.g. peripheral blood lymphocytes activated by phytohaemagglutinin). **Long term cultures** are maintained for more than 10 days (e. g. placenta, amnion and chorion cells).

The population of cells derived from the primary culture by passaging is called **cell line**. It has a limited life (life span) – number of division (e.g. human fibroblasts can be passaged from 25 to 30 times).

After reaching 50 passages, if the culture has still the ability for further cultivation (other passages) it is called stabilized (immortalized) cell line (e.g. human HeLa cells isolated from cervical carcinoma). These cultures can be also obtained by the transformation of cells by various viruses (e.g. Epstein Bar virus – EBV, SV40, etc.).

4.4 Utilization of cell and tissue cultures

Cell and tissue cultures are used in various biological and medical branches.

In **virology** the cell culture are used as a culture environment for virus multiplication, titration and identification. It is also used in the preparation of vaccines and monitoring of cytopathic effect depending on the type and concentration of the virions (titer) of virus.

Pharmacotoxicology utilize the cell cultures as biological models for *in vitro* testing of cytotoxic effect of various chemicals and pharmaceuticals remedies. Primary cultures of human cells (e.g. peripheral blood lymphocytes) as well as animal cell lines (e.g. V79 lung fibroblasts of Chinese hamster) are used.

In **human genetics**, the cultured cells (e.g. peripheral blood lymphocytes, amniotic fluid cells – amniocytes, fibroblasts) are used for cytogenentic analysis, DNA analysis as well as for biochemical testing.

In **experimental oncology** *in vitro* cell lines are commonly prepared from biopsies of patients who suffer from cancer for testing sensitivity or resistance of cancer cells to cytostatics.

In **gynecology and obstetrics**, *in vitro* cultivation is used for ova for *in vitro* fertilization.

In the last few years, cell cultivation is applied also for the therapeutic purposes. A new bio-medical branch – **tissue engineering** has been developed, which widely uses the techniques of cell and tissue cultivation. The aim of the tissue engineering is to create a functional anatomical unit (graft) suitable for the application in **regenerative and reconstructive medicine**. Nowadays tissue engineering is being successful not only in preparation of functional skin substitutes (used for the burned skin) but also cartilage (e.g. external ear fully prepared from *in vitro* cultured chondrocytes within collagen based extracellular matrix).

5. Relationship between microorganism and macroorganism

Single-cellular and multi-cellular organisms relate to each other in very complicated relationships which can seriously influence their vital manifestations.

According to mutual influence the following types of relationships between microorganisms (viruses, bacterias, and protozoa) and macroorganisms (human) are distinguished**:**

- **indifferent** – most of these organisms live outside of the human body, which is not their host. For example phytotropic viruses (multiplicated in plants) don‘t parasite in animal and human bodies;
- **symbiotes and commensals** do live on the surface and cavities of the human body. They are necessary for a proper functioning of the human organism, forming biocoenoses and are named as “human flora”. They mainly involve bacteria as *Staphylococcus epidermidis* on the skin, *Streptococcus salivarius* in the mouth cavity, *Escherichia coli* in the intestine and *Lactobacillus doederleini* in the vagina. In biocoenosis – under normal condition – proportional ratio of microorganism phyla and species is balanced and dominant species (symbiont or commensal) makes conditions, that are suitable for it and our organism (sc. **eumicrobiotia** appears). When their living conditions are disturbed, for example by antibiotics, dominant microbe is weakened – the **dysmicrobiotia** can develop. It means the overgrowth of other (ordinarily suppressed by dominant symbiotic bacteria) pathogenic microorganisms with the pathological side effects. The commonly dymicrobiotias are digestion problems (obstipations or diarrhea) or the development of candidosis in the vagina, potentially in the mouth cavity (soor);
- **pathogenic microorganisms** cause inflammatory diseases. They use the macroorganism as a source of nutrition and an environment where they are relatively safe. The ability to cause a disease is called **pathogenic** and it is a characteristic of the species or phyla of microorganisms. The level of pathogenecity of a specific phyla for certain a host is called **virulence**. Besides the virulence, the development of a disease and its course also depends on the immunity (protection mechanism) of the infected host, for example a human.

Pathogenic microorganisms are divided into obligatory and opportune.

Obligatory pathogens cause a disease after the first infection (primo-infection) almost in all people (e.g. *Staphylococcus aureus, Vibrio cholerae, Salmonella typhi,* plasmodia, trypanosomes etc.). This is enabled by their genetic predisposition and the unreadiness of the immunity system of the host.

Opportune pathogens cause a disease if they get to a place in the organism where the body isn’t able to tolerate them (for example *E. coli* in the urinary system) or during defects of the immunity system. The failure of the immunity causes that a big proportion of the people with AIDS die after the outbreak of diseases present in the body for a long period of time (for example *Toxoplasma gondii*).

From a epidemiological point of view, to understand the spread of pathogens (mainly parasitic elements) it is important to recognize the term **infestation**. It informs about the proportion of the population (in %) which has specific antibodies against certain pathogen (parasite).

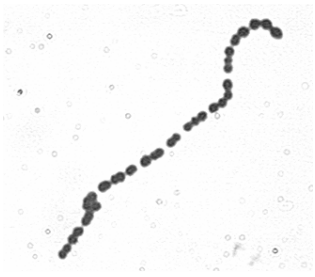
5.1 Bacteria

Bacterias belong to single-cellular prokaryotic microorganisms (see chapter 2.1). They are the cause of many diseases. Their average size is around 0.3 – 2.0 μm. Only some of them reach the size of several or a hundred micrometres (for example *Thiomargarita namibiensis*). They are present in two main shapes – globular, spherical to ovoid (coccus) and rod to thread-like (bacillus).

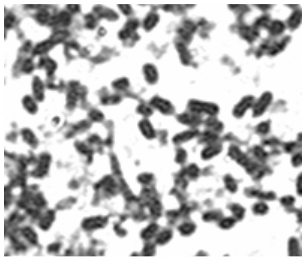
Cocci can be arranged randomly. If they are in pairs we label them as diplococcus, if in four tetracoccus, if they are more then eight they assemble as **sarcina**. In the microscope the bigger clumps (clusters) is **staphylococcus** (Fig. 35). The **streptococcus** assembles into a chain (the layout of the cocci depends on the number of plains where division takes place) (Fig. 36).

The second major form of bacteria is the shape of a simple rod named as bacillus (Fig. 37). The rods can be straight, slightly bended, spiral or fibrous. They can also contain locomotion organelles. Some of them form anabiotic phases – endospores.

● **Figure 35.** Staphylococcus



● **Figure 36.** Streptococcus



● **Figure 37.** *E. coli sticks*

Bacteria usually reproduce asexually (amitosis) and form clones. Sometimes they reproduce parasexually (conjugation), or by other forms of recombination of genomes.

At present there are several methods for the diagnostics of bacteria. The most commonly used, is the staining according to Gram, where positive (G+) bacteria are dyed blue and negative (G-) are red. The staining is an important distinguishing mark and so is the further microscopic examination, which has an important role in determining the exact inflictor of the disease and its properties.

But more important factor in diagnostics of infectious diseases is the isolation and indentifying of bacteria is the cultivation evidence. Bacteria reproduce well only if the conditions for their cultivation is adequate (temperature, gases, composition of cultivation soils etc.). They are diagnosed according to the soil in which they grow, the appearance of the colonies, and the effect which they have on their surroundings. Cultivation enables selective reproduction of specific species and the testing of their sensitivity or resistance on the effects of antibiotics and disinfection substances.

5.2 Parasitic protozoa

Are single-cell eukaryotic organisms, which are categorized amongst the **protozoa** (unicellular organisms), because they need nutritients from their surroundings (they are heterotrophic). They are aerobic organisms, meaning that they need oxygen for their reproduction.

The body of the protozoa is made up of only one eukaryotic cell, which is specialized to a parasitic form of life. Many of them have a complicated life cycle; they change hosts and the type of reproduction.

Some of them (eq. amoebas) are capable of **encystation**, or the formation of cysts with a thick coat layer and minimal metabolism. This enables them to survive in an outer environment for a very long period of time (years), until they manage to enter a new host. Here they excyst (they loose the coat layer) and change to vegetative forms capable of reproduction.

The size and the shape of protozoas, and the presence of accessory organelles (flagellum or cilia) are very diverse and specific for species (it will be mentioned during the description of different parasites).

The reproduction of parasitic protozoa is mainly asexual and takes place in the form of mitosis and its similar processes (eg. endomitosis). Gamogenesis is rarer and takes place by different, often bizarre methods (e.g. in plasmodias).

According to the **place of parasiting**, the protozoa are divided into such of them, that can parasite in **cavities**, **intestine** and **blood and tissue.** The localisation of their effect is connected with their demands on the optimal life conditions and the way of their transmition.

The knowledge about the place of parasiting has a major importance for their **practical diagnostics**. They relate to the sampling of material and the right method of examination. For the majority of parasitic protozoas, **microscopic diagnostics** is used:

- **native sample**, eq. slides of smear from the vagina (during the diagnostics of *Trichomonas vaginalis*), from the faecal discharge (cysts *Entamoeba histolytica*) or from the duodenal juice (trophozoites of *Giardia intestinalis*);
- **fixed and stained** (permanent) **sample**, most commonly from blood (blood smear or a thick drop) are used during the examination of blood and tissue parazytes (e.g. plasmodias and trypanosomas).

Other methods of diagnostics are:

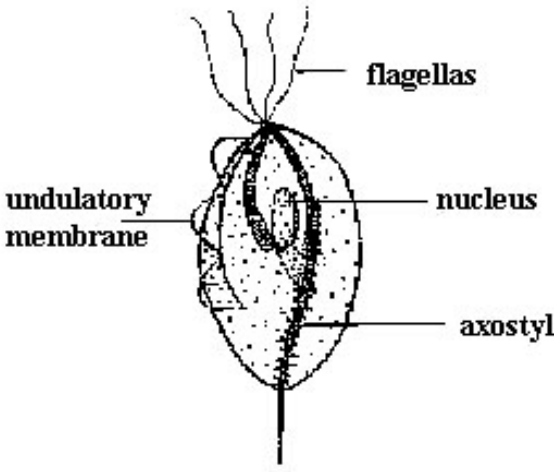
- **cultivation evidence** doesn't have such a huge importance in protozoas as in bacteria. Cultivation on living soils (for instance amoebas or trichonomas) or tissue cultures (toxoplasmas) is possible, but is only done in specialized laboratories. The motive is often to determine the strains resistant to commonly used drugs and finding the right form of therapy *in vitro;*
- **immunological evidence** (the examination of specific antibodies against parasite antigens), it is useful not only when a microscopic examination fails. Besides the evidence of the infection, it is also useful in the determination of the stage and dynamics of the disease;
- **molecular-genetic examinations** are at the present time gaining importance. They involve, for example, the prove of a DNA parasite by the polymerase chain reaction method, which is very specific and requires only a small amount of isolated material (sample).

5.2.1 Protozoa parasiting in cavities

They colonize the mucosa of the body cavities, especially of the mouth and vagina. A typical example is ***Trichonomas vaginalis***.

A common, sexually transmitted disease (STD), especially amongst women, is urogenital trichomoniasis, which is caused by the flagellate *Trichomonas vaginalis*, parasiting inside the urogenital tract of the men and women. It is most commonly transmitted by sexual intercourse. After a 10 day incubation period a vaginal effluence appears containing many trichomonas. In men the disease usually has a more latent course; only in a few per cents of cases it induces non-specific urethritis. Trichonomas is the largest flagellate which parasites in the body of the human (Fig. 38).

Trichomonas vaginalis has a pear like shape with a size of 15 – 30 µm. On its front part are four flagellas, the fifth one is connected to the body and forms an undulatory membrane. Axostyl spreads throughout the whole body and slightly overlaps its lower part. The nucleus is clearly visible. Living parasites have a typically circular motion, by which they can be easily detected in a native sample from a vaginal effluence.



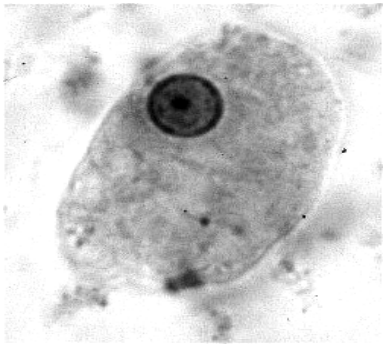
● **Figure 48.** Illustration of *Trichomonas vaginalis*

5.2.2 Protozoa parasiting in intestine

An example of **intestinal parasitic protozoa** is ***Entamoeba histolytica***. It is the only pathogenic amoeba, which lives in the alimentary tract of humans. It causes **amoebiasis** (tropical disentery). Amongst typical clinical manifestations are disenteric diarrheas with pain and blood in stool. Life threatening complications may arise in the case the amoebas infiltrate the different organs of the body.

In the human body *Entamoeba histolytica* appears in two forms (minuta and magna). It forms round cysts with 1 – 4 nuclei (Fig. 39). The cysts are resistant with the excrements they enter the external environment, foodstuff, and drinking water etc. The infection of a human therefore takes place by the so called **fecal-oral way** ("dirty hands disease"). The cysts resist the digestion in the stomach and excyst first in the small intestine. The vacant amoebas move around and gain food with the help of the pseudopodia (Fig. 40). They live in the large intestine, either as forma minuta or they infiltrate the mucosa of the large intestine and change into a dysenteric magna form. Magna form causes amoebic abscesses. This disease is infrequent in temperate climates; however in the tropical area amoebiasis is very common. In Central Europe it is only known as an "imported infection".

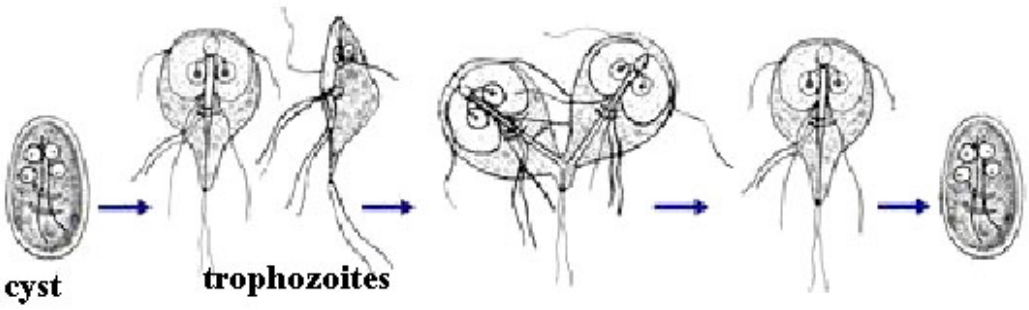
● **Figure 39.** Cyst *E. histolytica*



● **Figure 40.** Vegetative form *E. histolytica*

Giardia intestinalis is a flagellate which is present in the temperate climate mostly with young children with low level of hygiene standards, even tough can occur also amongst adult individuals. The parasite has a relatively simple life cycle (Fig. 41). The disease is spread with the help of cysts, which leave the body with the stool. Cysts have an average size of 12 x 6 µm and a typical structure; however they are not suitable for diagnostics, since they are excreted in small amounts. In the duodenum excystation and reproduction of the parasite occurs. Vegetative forms (**trophozoites**) of a parasite have a typical shape simulating

a double sided symmetry with dimensions 10 x 7 µm. On their ventral side they have a sucking disc, by which they attach to the mucosa of the intestine.



● **Figure 41.** Life cycle of Giardia intestinalis

The diagnostics of giardiasis is most commonly done by a microscopic examination of the duodenal juice, in which trophozoites can be found. This is because the parasites, as a film, lay out the surface of the mucosa of the small intestine, especially the duodenum. The change of chemism in this environment prevents the effects of bile and consequently cause the malabsorbtion syndrome – patient is hungry, uneasy and have bigger amount of fatty stool.

5.2.3 Blood and tissue parasitic protozoa

They are the cause of serious and most commonly present diseases of humans on earth.

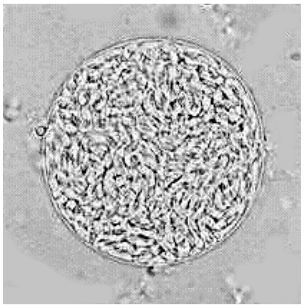
A representative of these parasites in temperate climate conditions is ***Toxoplasma gondii***. Infestation in population of central Europe is around 20 to 80% - in regard to nutrient habits, so it is a relatively frequent infection. In most cases of infected individuals *Toxoplasma gondii* doesn't clinically manifest itself, since they are avirulent phyla. Only seldom, a virulent phylum, which causes an inflammation in the organ which was settled down by the parasite appears. In the past, acute taxoplasmosis accounted for less then one per mille of diseases. At present it is gaining an increasing importance as an opportunistic infection in patients with immunodeficiency. It is estimated that about 30% of patients with AIDS die because of encephalitis, pneumonia, or hepatitis, as a "flare-up" of a toxoplasma infection present in an organism for a long time. It is a consequence of fact, that lymphocytes "arresting" parasite are destroyed by HIV multiplication.

Trophozoites of *Toxoplasma gondii* are present in two forms. The first form of trophozoites are the so called **tachyzoites** (Fig. 42), a virulent form, horn shaped with a curved end of a size 4 – 6 x 2 – 3 µm. They are pathogenic and adaptive to such a degree, that they are able to multiply also in cells of macrophages and neutrophile leukocytes, which phagocyte them.

The second form are avirulent **bradyzoites,** which assemble to several groups and form huge, so called **tissue cysts,** of sizes to 300 µm (Fig. 43), especially in the brain, liver and skeletal muscles. If a damage of the immunocompetence of the organism occurs, the bradyzoites change the virulence, and they become tachyzoites and cause an acute inflammation.

Even tough the parasite was discovered in 1908, until 1969 it wasn't its life cycle described (Fig. 44). On the beginning of the biological cycle is the cat (and other *Felidae*). Cat is classified as the definitive host, since the **sexual reproduction** of the parasite takes place in its intestine. The result of this is the formation of **oocysts**, which leave the body with the stool. In the outer environment they mature (sporulate) within 24 hours, and become very resistant.

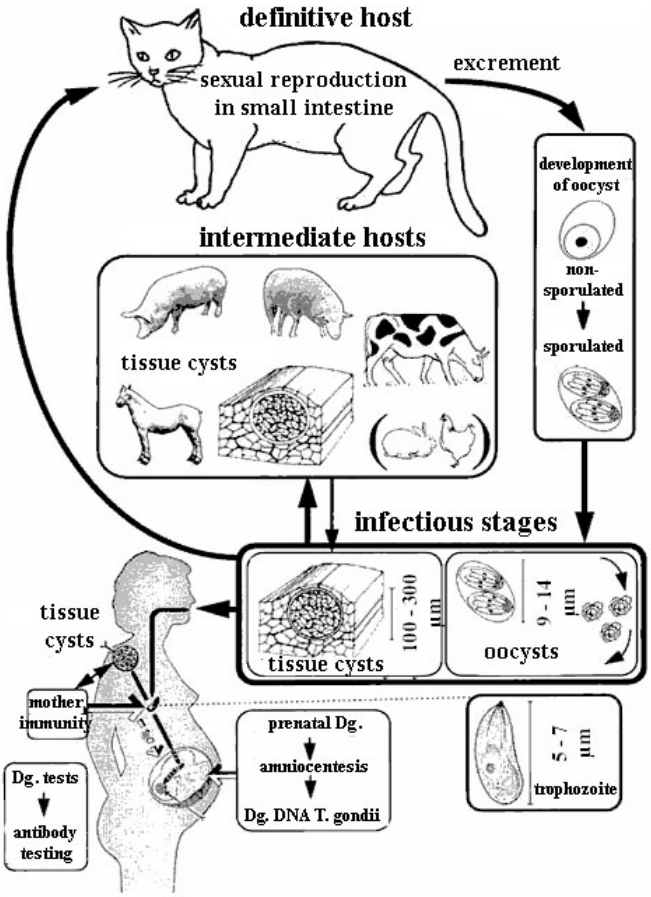
● **Figure 42.** Tachyzoites *Toxoplasma gondii*



● **Figure 43.** Tissue cyst *Toxoplasma gondii*

If any kind of warm-blooded animal swallows the oocyst, it excysts in its intestine and releases 8 tachyzoites. They enter the target organs (brain, skeletal muscles, liver, lungs etc.), where they slowly asexually reproduce and form tissue cysts. These organisms (including humans) are named as **intermediate hosts**. It was experimentally proven, that small animals, for example rodents, have a slower reaction time after the infection by toxoplasmas. For cats this makes their hunting easier, but also allows the infection of the cat by toxoplasmas. This finishes the life cycle of the parasite. The particularity of *Toxoplasma gondii* is that it can be **passed from human to human**, to be more exact the transplacental transfer – from the mother to the fetus etc. However this is a very rare occurrence. The condition for this is the primo-infection of the mother by a sufficiently virulent phylus. It was found out that the probability of infection of the fetus increases with the length of gravidity. Typical **congenital toxoplasmosis** is rare and manifests itself especially by brain damage (hydrocephalus) caused by the infection. More common are late side-effects of prenatal infection by toxomplasmas, as the typical affliction of the retina (coloboma).

In general, the etiological connection of the disease with abortion or defect of the fetus requires a direct evidence of the parasite (or its DNA) from a biological material.



● **Figure 44.** Scheme of the life cycle of *Toxoplasma gondii*

Plasmodia are globally one of the most spread blood and tissue parasites. It is estimated that malaria, the diseases they cause, effects about 20% of the world population. Every year more then 300 million new cases are reported, from which more then a million are fatal. In Sub-Saharan Africa, 3000 children younger then 5 years of age die everyday as a consequence of malaria. There are four types of parasiting plasmodia among humans — ***Plasmodium malariae, ovale, falciparum and vivax***. Each one of them causes a different type of malaria, their diagnostics is based on the recognition of the specific morphological sings of schizonts, merozoites and gemeotocytes during the examination of a microscopic sample from blood.

The **life cycle** of plasmodia is complicated. The definite host, in which the sexual reproduction of the parasite occurs is the **vector** (carrier of the parasites) – the female mosquito of the *Anopheles* species. Parasites asexually reproduce in the erythrocytes of the intermediate hosts, in this case, infected people. Some of them differentiate into germ cells, so called gametocytes.

First phase of the cycle takes place in the stomach of the female mosquito, that sucked the blood of the sick person. The gametocytes merge and a movable zygote (ookinet) is formed. This one enters the mucosa and its development continues, until the parasites, in the so called oocyste, does not multiply to a level that it snaps and the individual cells of the parasite (sporozoites) detach. By the lymphatic system the sporozoites enter the salivary glands of the female mosquito.

The second phase begins by the female mosquito releasing (before sucking of blood) saliva with the sporozoites into the blood of the human. These are firs stored for a while in the Kupffer cells of the liver – to adapt on antigene system of human cells. Plasmodium ovale and vivax can remain there for the entire life and cause constant relapses of the disease. Afterwards the parasites start to reproduce asexually. Schizonts enter, they burst, and merozoites enter the blood. This is the end of the so called **extraerythrocyte phase**. Merozoites attack the erythrocytes and reproduce inside them (**erythrocyte phase**). In regular intervals, blood cells burst and release merozoids, which causes a malaric seizure. The freed merozoites attack other blood cells etc.

6. Nucleic Acids

Nucleic acids and proteins are the basic macromolecules of living organisms. The linkage between nucleic acids and proteins is very close. Proteins are in structure extremely variable molecules – so like characteristics of cells and organisms, they perform. Deoxyribonucleic acid (DNA) is long molecule, but its structure is featureless – four types of monomers in various combinations. Researchers could not believe that DNA is molecule keeping genetic information. In 1952 an experiment was conducted by Hershey and Chase involving the bacteriophage T2 whose DNA was labeled by a radionuclide ^{32}P, and the protein part by a ^{35}S radionuclide. During the infection by a bacteriophage, only the DNA part of the virus entered the cell. This supported the evidence, that DNA is the carrier of the genetic information.

6.1 The Function of the Nucleic Acids

Each cell carries with its genetic information. It is stored in DNA, in almost all organisms – except RNA viruses. Nucleic acids also carry the genetic information to the offspring. Replication ensures the exact copying of the DNA molecule to two new identical DNA molecules. Before each cell division, the DNA is checked and the possible errors (mutations) are either eliminated or apoptosis of the cell is induced, which is a protective mechanism of the organism against origin of malignant cells.

The information that is stored in the DNA is not used directly, but in the form of copies (transcripts) – RNA molecules, with a limited life-time and function. Therefore nucleic acids carry and code the genetic information, which then leads to the synthesis of proteins. We call this processes transcription and translation. The properties of the proteins are dependent only on their primary structure, which means the sequence and types of the amino-acids they are made of. Information stored in the DNA, which determines this sequence, manages to ensure all the structural and functional properties of the cells and organisms. If turned around, errors in the genetic code lead to the synthesis of defective proteins, or to stop the synthesis of proteins overall.

6.2 Primary Structure of Nucleic Acids

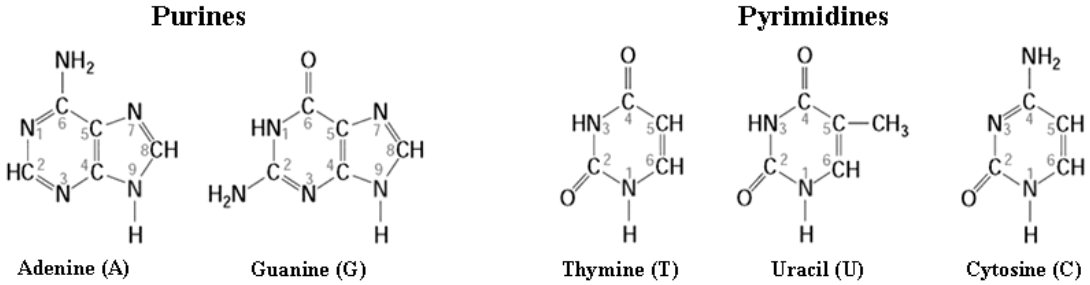
Nucleic acids were discovered as “nuclein” by a Swiss physician and biologist Miescher in the year 1869, and their name was created by Altmann (1889).

For the synthesis of nucleic acids nucleosides are used, which are made of a nitrogenous base and pentose (five carbon atoms sugar), connected by a N-glycosidic bond to the first carbon being a nitrogenous base. In DNA the saccharide is deoxyribose (2-deoxy-β-D-ribose), while in RNA it is ribose (β-D-ribose) (Fig. 45). They differ from each other because ribose contains on its second carbon an –OH group while deoxyribose only has a –H group.

β-D-ribose 2-deoxy-β-D-ribose

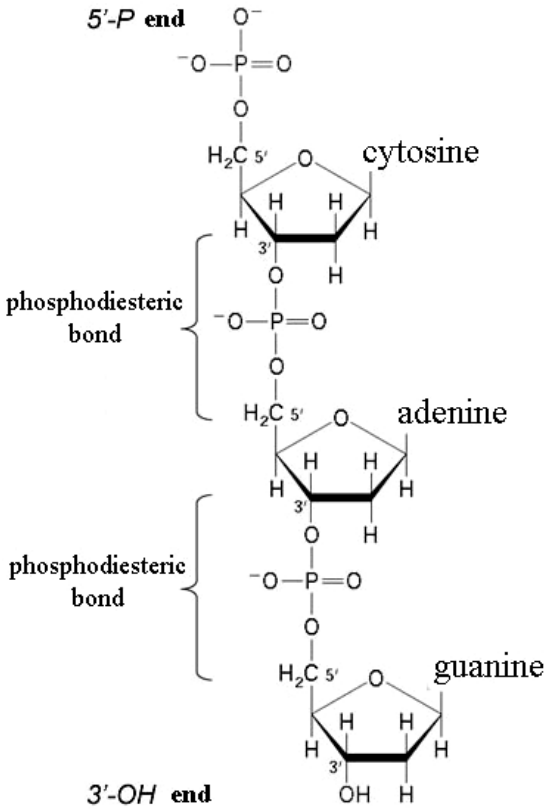
● **Figure 45.** Chemical structure of ribose and deoxyribose

The genetic information in the nucleic acids is carried by the nitrogenous bases (Fig. 46). From a chemical point of view, we divide the nitrogenous bases to **purines (adenine** and **guanine)** and **pyrimidines (cytosine** and **thymine** – in DNA, and **uracil** in RNA.



● **Figure 46.** Chemical structure of nitrogenous bases

A nucleotide is a monomer of a nucleic acid polymer and is made of a nitrogenous base, pentose and phosphate (the rest of H_3PO_4). The individual nucleotides in a nucleic acid differ only by their nitrogenous base. In the DNA chain there are four types of deoxyribonucleotides, which we can call the „letters of the genetic code".



During the synthesis of nucleic acids the corresponding polymerase (DNA polymerase during replication or RNA polymerase during transcription) binds the pentoses molecules of nucleosides. This bond is carried out by the remainder of H_3PO_4 (phosphate) between the third carbon (3') of the preceding pentose and the fifth carbon (5') of the following pentose. Name of this bond is the **phosphodiesteric bond**. There arises a continual chain of nucleic acid, which is called a **polynucleotide chain**. At the same time, by the bonding, the **chain's orientation** is achieved. The 3' end of the chain contains on its third carbon free –OH group and on its opposite end, on the fifth carbon (5'), a phosphate group $–PO_4$. The adjoining of the individual nucleotides to the 3' end of the chain is the direction it grows (Fig. 47).

● **Figure 47.** Scheme of the polynucleotide chain

6.3 Secondary and Tertiary Structure of the Nucleic Acids

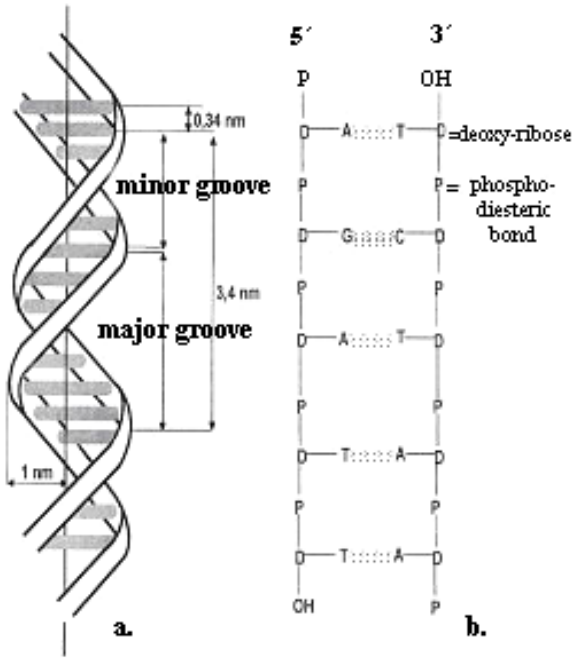
6.3.1 Secondary and Tertiary Structure of DNA

DNA is formed by two mutually connected strands – **double stranded DNA**, also referred to as the duplex. In 1953, Watson and Crick explained the spatial structure of the DNA molecule, the so called **secondary structure of DNA**.

The strands are in an **antiparallel** arrangement and differ from each other in the orientation of the phosphodiester bonds. Opposite to the 5' end of one strand is the 3' end of the second strand. The chain with the 3'-5' orientation is referred to as the **template strand**.

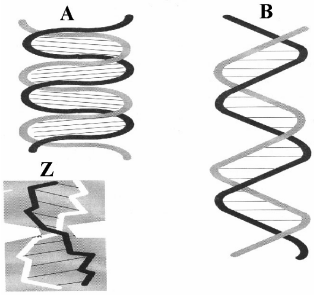
The complementary strand (with the 5'-3' orientation) is called a „sense“or **coding strand** (it has the same sequence of bases as will have mRNA transcribed complementary to template strand, but instead of uracil contains thymine). Both DNA strands are mutually **complementary** and in most cases forms a righthanded double strand, called α-**helix** (Fig. 48). The mutual bond of the strands is carried out through the **nitrogenous bonds** between the bases. Two nitrogen bonds are formed between adenine and thymine and three between guanine and cytosine (the more stable pair). That's why we call them the **complementary bases,** while chains of DNA molecules we refer to as **complementary chains**. Between RNA and during the interaction of DNA and RNA, adenine is complementary to uracil. No other base pairing combination does exist. The more C-G pairs a molecule contains, the stronger it is. The genetic information is, to a large extends, protected against damage, because the base-pairs are positioned towards the interior of the molecule. The bases are located in a plane, where each base pair is turned by 36°, compared to the previous pair. Phosphates and deoxyriboses form the outer side of the double strand, called the **pentophosphate skeleton**. This configuration of the DNA molecule is energetically the most stable. The presence of the H_3PO_4 rests gives the DNA a negative electric charge. The length of the nucleic acid chain is given by the number of base pairs (**bp**), or kilobases (thousand base pairs), in abbreviation "**kb**".

DNA can exist in different conformations, from which the most common are three: A and B-DNA (righhanded) and Z-DNA (lefthanded) (Fig. 49). The most common conformation is the B-DNA (α-helix**)** which has an average width of 2.37 nm. The distance between the bases („height of one stair“) is 0.34 nm and per one helical turn correspond 10 base pairs, and therefore the height of one helical turn is 3.4 nm. A-T pairs are „wider“(1.11 nm) and C-G pairs are „narrower“(1.08 nm) and therefore uneven helical turns arise. This means that **minor** (0.6 nm) and **major** (1.2 nm) **grooves** are formed. The major groove is wider than the minor and has an important function, because here bind regulatory proteins.



● **Figure 48**. **a.** Scheme of α-helix, **b.** the orientation of the DNA chains

When the content of water is lowered to 75% (a large concentration of salts and a low level of hydratation is done), the B form changes to the A conformational form. A-DNA is a righthanded double helix with and average width of 2.55 nm, in which each helical turn has a height of 2.46 nm and contains 11 nucleotides. Z-DNA is a lefthanded double helix with an average width of 1.84 nm and height of helical turn 4.56 nm, which contains 12 pairs of bases.



● **Figure 49.** Conformation types of DNA

6.3.2 Types of DNA

According to its location inside the cell we can divide DNA into more types. The most important is the chromosomal DNA. The mitochondrial DNA and plasmid DNA is extrachromosomal.

6.3.2.1 Chromosomal DNA

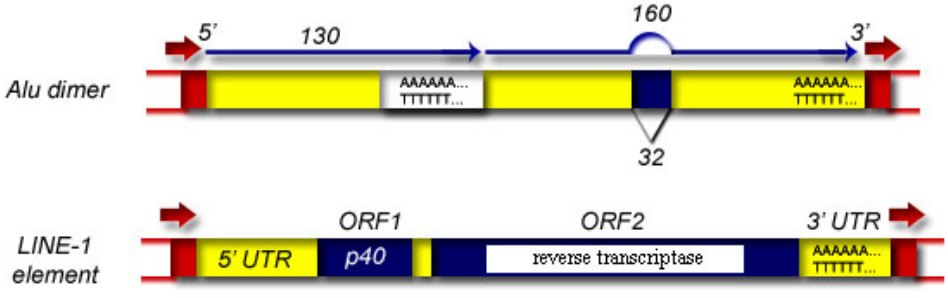
Chromosomal DNA can be divided into the certain groups according to more factors. According to its function, we can divide DNA into **coding** and **non-coding**. The coding DNA determines the sequence of amino in the polypeptide chain (structural genes), or nucleotides order in certain RNA (middle-repetitive sequences, e.g. for rRNA). There are more types of non-coding DNA – for example the DNA which has a control and regulatory function (for instance promoters). Some types of DNA have a specific function inside the chromosomes, for instance the repetitive sequences in the region of the centromeres or telomeres.

To non-coding DNA belong sequences of the DNA which still hasn't been explored, or the DNA whose function is unknown (for example pseudogenes).

Another approach is to classify DNA by the number of motifs or sequences copies in it, that are identical. In eukaryotes approximately 60% of their DNA is made of **unique – single copies** (or seldomly repeating) of sequences. To this group belong the **structural genes** (coding polypeptides) and **pseudogenes**. Only about 1.5 - 2% of the human DNA sequences code proteins. Pseudogenes are not functional genes. They appear in two forms – „cut", which are formed by reverse transcription of mRNA and don't contain introns, and „uncut" pseudogenes, which contain introns, but are not functional since they have a mutation on their promoter site.

Some parts of the DNA nucleotide chain are in more copies. We call them **repeating sequences** or repetitions. 30% of our nuclear DNA is made of **slightly (medium) repeating** sequences (the number of their copies in a genome is 10^2 – 10^5). Here belong the genes for rRNA (in a human being on the short arms of the acrocentric chromosomes), tRNA, signal sequences, and the genes which code the proteins of the histones.

The remaining 10% of DNA is made of **highly repeating** sequences (the number of copies in a genome is 10^6). They can be randomly spread – **interspersed**, or can be located directly behind each other, in blocks – **tandem repeats.** If the **interspersed** sequences are long, they are called LINE (long interspersed repetitive elements) (Fig. 49). **Even tough** they code for reverse transcriptase, they do not contain LTR or ENV genes (in comparison to proviruses from retroviruses). Their size is up to 6 kb. As un-truncated, they appear in the genome about 3500 times and as truncated 50 - 100 000 times. If they are short (80 – 400 bp), they are called as SINE (short interspersed repetitive elements). Most SINE is derived from the genes used for tRNA. For human beings are typical the **Alu** sequences – their name is derived from the restriction enzyme Alu1 and their length is 300 bp. They make up 3% of the genome and almost each 4 kb part of the human DNA contains this sequence.



● **Figure 49.** The comparison between LINE and Alu sequences

The example of tandem arranged repetitive sequences are the **satellite DNA**. According to the length of its motif (repeating sequence) we recognize:

- macrosatellite DNA with repeats of 100 – 6500 bp long sequences;
- minisatellite DNA with repeats of 6 – 24 bp long sequences;
- microsatellite DNA with repeats of 1 – 5 bp long sequences.

Microsatellite DNA is located in the centromeres of chromosomes. From the **minisatellite** DNA are important the VNTR sequences (Variable Number of Tandem Repeats). The high variability of microsatellite DNA – the so called CpG islands (C-cytosine, p – base, G – Guanine) and STR polymorphism (Short Tandem Repeats) – is used in human as a marker in certain methods of DNA diagnostics.

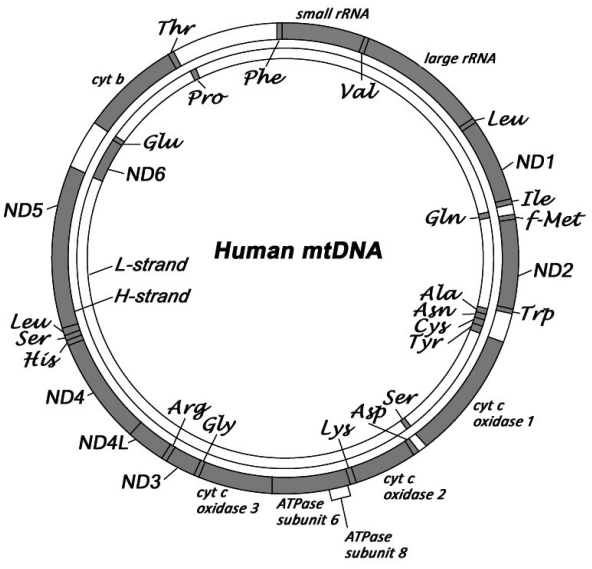
The DNA contains more types of other repetitions, as for instance the **inverted repetitions** or **long terminal repetitions**. The presence of inverted sequences in the DNA chain leads to the formation of **hairpin** and **cross structures**.

6.3.2.2 Mitochondrial DNA (mtDNA)

In humans, the non-chromosomal DNA is located in the mitochondria. These organelles belong to the group of semi autonomous cell organelles – they have their own genetic equipage.

The configuration of the mitochondrial genome is different from the nuclear genome. The mitochondria of a living cell contain one relatively small double stranded circular DNA, which is **three stranded** in the area of the D loop (Fig. 50). The strand rich on G is called **heavy strand** and is labeled **H** (heavy) and the strand rich on C is referred to as the **light strand - L** (light). The size of the mitochondrial genome of a human is 16569bp. The inheritance is matrocline, which means, that the father (sperm) doesn't carry his mtDNA to the offspring (zygote). The genome of the mitochondria contains 37 genes: 13 coding for proteins (structural genes), 2 for rRNA (23S, 16S) and 22 for tRNA. From these the H chain contains the information about the structure of 12 proteins, 2rRNA and 14tRNA, the rest of the information contains the L strand. The D loop doesn't contain coding sequences, in has two promoter sites, LSP (light strand promoter) for the light strand and HSP (heavy strand promoter) for the heavy strand. Through transcription, two long primary transcripts are formed.

Compact mitochondrial DNA doesn't contain repetitive sequences, 93% of mtDNA is made of genes, which do not contain introns. The genes are separated from each other only by a few nucleotides. The mitochondrial genetic code is slightly different from the nuclear one. The most important difference is, that nuclear stop-codon TGA is in mitochondria a codon for amino acid tryptophan.



● **Figure 50.** Diagram of the mitochondrial DNA

The mitochondria have the complete proteosynthetic apparatus, which synthesizes only certain mitochondrial proteins and is similar to the apparatus of the prokaryotic cell. Similarly as it is in bacteria, they contain 70S ribosomes (30S + 50S subunits). More than

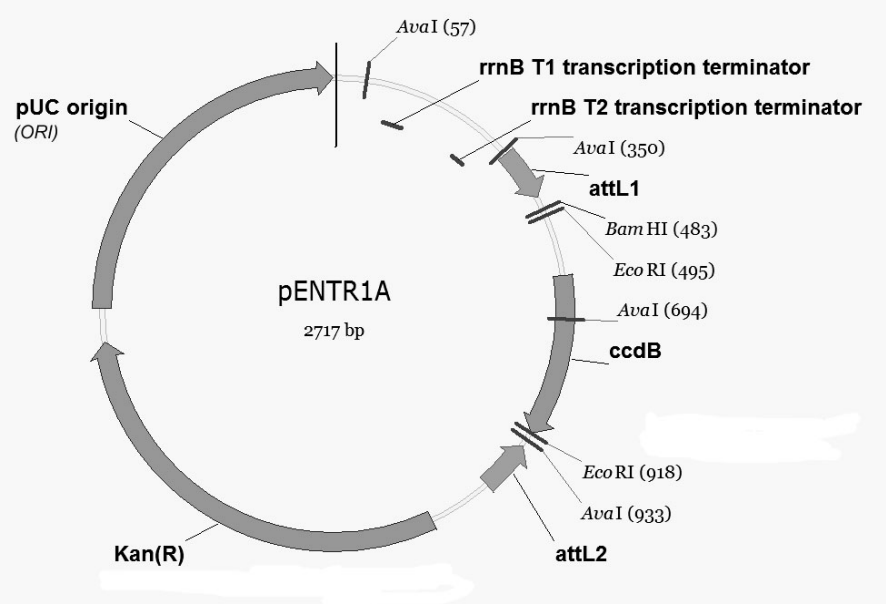
90% of all proteins necessary for the biogenesis of mitochondria are coded by the nuclear genes. Proteins are synthesized in the cytoplasmic ribosomes and are transported to the different structures of the mitochondria. Inside the mitochondria the replication of the mtDNA takes place, as well as the synthesis of all forms of RNA and the synthesis of proteins.

The first group of mitochondrial genes codes proteins that take part in the process of oxidative phosphorylation: apocytochrome b (cytb), subunits of cytochromoxidases (cox), subunits NADH dehydrogenases (nad), subunits of succinate dehydrogenase (sdh) and subunits of ATP synthetase (atp). The second group is made of genes necessary for the formation of the mitochondrial translation apparatus. They are the genes for tRNA, rRNA of the big (LSU, rRNA, rrn26) and small (SSU, rRNA, rrn18) subunits of the mitochondrial ribosome.

6.3.2.3 Plasmids

Besides the nucleoid (nuclear chromosome), the bacterial cells may contain in cytosol also other structures composed of DNA. They are called **plasmids**. They are extrachromosomal genetical elements (sc. episomes), which are made of a two-stranded circular DNA with a length of 2-15 kb. They replicate independently from the bacterial chromosome and often have ability for conjugation ("self" transport from one cell to another). Each plasmid contains an origin of replication (ORI) and genes which ensure its own reproduction. They can also contain other sequences, as:

- genes for resistance against antibiotics and for synthesis of certain enzymes – for example hyaluronidases etc., that give to the host bacteria selection advantages.
- restriction sites and selective markers, which allow the use of plasmids as vectors of genetic information (Fig. 51).



● **Figure 51.** Diagram of a plasmid with marked restriction sites

Plasmids which contain the required genes, and are able to replicate in different host cells, are called „shuttle" vectors.

Plasmids were indentified also in some eukaryotic cells. A cryptic plasmid was found in yeast, which was made of a double stranded circular DNA, while in certain fungi and algae linear DNA plasmids were found.

6.4.1 Secondary and tertiary structure of RNA

Ribonucleic acids (RNA) are relatively short linear **single-strand molecules**. There are more types, there. In prokaryotic cells three main RNA types are present – messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). Eukaryotic cells, besides these three main types, also contain small RNA molecules bond with proteins into ribonucleoprotein particles. RNA of viruses is their genetic information (genome), and therefore have a specific structure and function.

6.4.1.1 Messenger RNA

Messenger RNA (mRNA) is created by transcription from **structural genes** (in human cells there are more then 20 500). The result of transcription of a structural gene in the nucleus of an eukaryotic cell, is a precursor RNA (pre-mRNA), which is called also heterogeneous nuclear RNA (hnRNA). This primary transcript must be modified (**maturated**), in order to become a mature mRNA. Details of maturation are described in chapter Protein synthesis. Matured mRNA is a linear single-strand RNA chain, which has on its 5' end a guanine nucleotide bonded by an atypical 5' - 5' triphosphate bond, the so called "cap". These structure is not only the protection of the mRNA against the destructive action of RNases and site, where the regulation proteins bind to it, and „protect" the mRNA during its passage to the cytoplasm. The cap is recognized by sc. preinitiation complex, which allows the mRNA to connect to the small subunit of the ribosome, as essential proposal to intialization of translation (formation of active ribosome). On the 3' end of the mRNA is present long poly-A chain, containing about 100-250 nucleotides with adenyl, that not only protects the molecule from this end, but has a significant effect on the number of times, an mRNA can be used for translation. The task of the mRNA is to transfer the information from the genes in the nucleus to the ribosomes connected to the endoplasmatic reticulum (in eukaryotic cells). After its departure from the ribosome, each mRNA is disassembled by different enzymes (RNases), in mammals within 6 hours, so the cell doesn't undergo an information chaos.

In prokaryotic cells one mRNA carries the information for more polypeptide chains and we call it polycistronic. Eukaryotic mRNA is monocistronic, it contains the coding information only for one protein.

6.4.1.2 Ribosomal RNA

Ribosomal RNA (rRNA) is together with specific proteins a part of the ribosomes and makes up 80 % of the RNA of an eukaryotic cell. In a prokaryotic cell there are three types of rRNA there, while in an eukaryotic cell there are 4 types. Ribosomes are made up of two subunits (Fig. 52). An eukaryotic cell contains 40S and 60S subunits („S"means the Svedberg sedimentation constant, which is the scale of the gravitational density of the cell particles). The 40S subunit of a ribosome consists of an 18S rRNA and 30 different proteins; the 60S subunit is made up of 28S rRNA, 5.8S rRNA, 5S rRNA, and 45 different proteins.

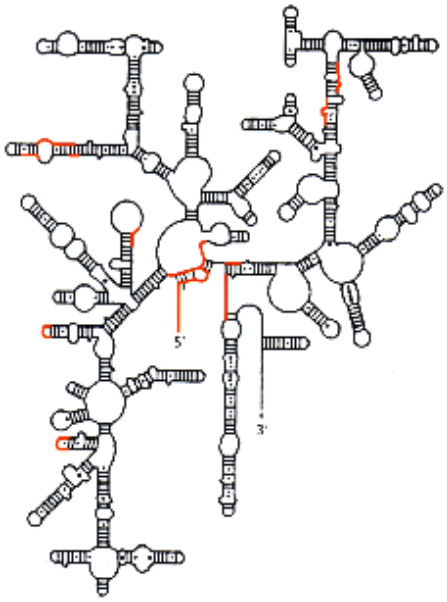
The rRNA synthesizes in the nucleus (in the so called NOR – nucleolus organizing region). rRNA is formed by the transcription from the genes for RNA, which are, except for the gene for 5S rRNA, located in the acrocentric chromosomes (D and G group, in the area of the satellites). In the DNA chain, they are close one to another in the form of transcription units. Each of the units is transcribed by RNA polymerase I into **45S precursor ribosomal RNA**. The transcription unit contains a promotor (180 bp long), base and regulatory part. Product of the 45S RNA transcription is in the next processing, with the help of snoRNA (small nucleolar RNA), modified directly in the nucleolus by a specific Rnase into 5.8S, 18S and 28S rRNA molecules. The posttranscription modifications start with the binding of

a substantial amount of methyl groups (in a human being more than 100) to nitrogenous base atoms, especially in the purine nucleotides. The resulting rRNA pairs together with itself creating a complicated 3D structure (Fig. 53). Molecules of rRNA together with the 5S rRNA bind to specific proteins and form subunits of ribosomes, that are then, with the help of specific transport proteins, transported through the pores of the nuclear membrane into the cytoplasm (5.8S rRNA is transported in 5 minutes, meanwhile 28S rRNA in 30 min.).

Genes for ribosomal RNA are, in prokaryotes, saved in one transcription unit. In the genome of a prokaryotic cell there are about 7 transcription units, which in the case of quickly growing cells, enables a sufficient production of ribosomal RNA. The transcription unit is transcribed into the molecule of the precursor RNA, from which 5S rRNA, 16S rRNA and 23S rRNA are generated.

Type	rRNA	Proteins
Prokaryotic 50 S 30 S → 70 S	23 S *(2904 b)* 5 S *(120 b)* 16 S *(1542 b)*	31 21
Eukaryotic 60 S 40 S → 80 S	28 S *(4718 b)* 5,8 S *(160 b)* 5 S *(120 b)* 18 S *(1874 b)*	49 33

● **Figure 52.** rRNA in a prokaryotic and eukaryotic cell



● **Figure 53.** Prokaryotic 16S rRNA

6.4.1.3 Transfer RNA

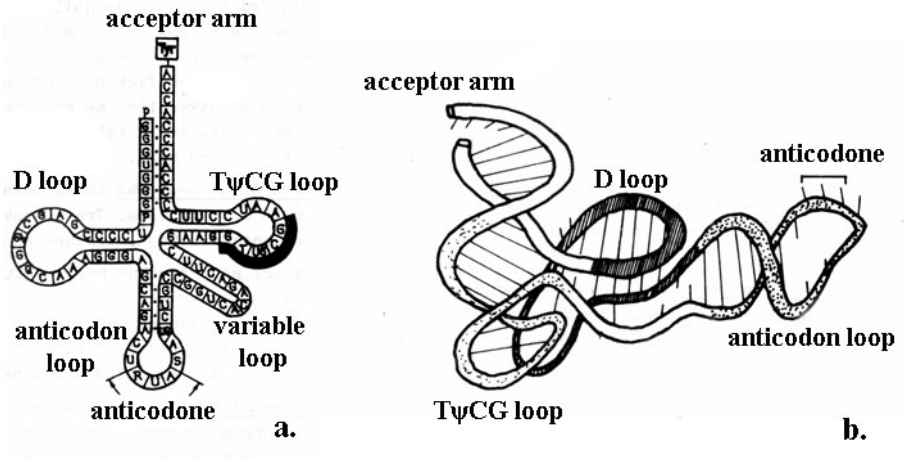
Transfer RNA (tRNA) has an important role in the synthesis of proteins in a cell. It binds to a specific amino acid and carries it to a ribosome, where – according to the code in the mRNA, it is inserted to the growing polypeptide chain. Each tRNA is made of 74 – 95 nucleotides. The primary structure of tRNA is made up of special bases, called **minority bases**. They are dihydrouracil (UH_2), methylguanine (GMe), inosine (I), methylinosine (IMe), thymine (T), pseudouridine (Ψ).

These bases are not added into the tRNA during transcription, but are formed later – by the enzymatic modification of the already existing chain. Because the genetic code is degenerated (particular amino acid could be coded by more variants of triplets), there may exist more than one tRNA – for the same amino acid. The structure of the tRNA (in 2D) is similar to a quaterfoil (Fig. 54a). The selfpairing of certain complementary sequences of nitrogen bases which are distant from each other, form double stranded segments, while the unpaired areas form **loops (arms)**. Inside unpaired parts of loops are located minority bases. On the 3' ends of the strand are the CCA sequences and on the terminal nucleotide with adenine connects the transported amino acid. On the 5' end of tRNA is usually a nucleotide with guanine. In the tRNA molecule two main parts are recognized. The first part is the so called **anticodon loop** (the middle loop), which contains the anticodon and perform tRNA function during decoding of the genetic information written in the structure of mRNA. The second major part of the tRNA is the **acceptor arm**, on whose 3' end a predestined amino acid is bound. Bases of the anticodon are complementary to the codon inside the mRNA, to which they are paired antiparallely. We read the anticodon sequence of bases from the 3' end of the tRNA molecule to 5' end. The anticodon enables the binding of a specific tRNA to an exactly defined spot on the mRNA, through the codon-anticodon bond, for example:

Codon in the mRNA: 5' – GUA - 3'
Anticodon in the tRNA: 3' – CAU - 5'

D and T arms help the assorting of the amino acids. Except these two major loops, the tRNA can also contain a **variable loop** (Tab. 2).

In space (3D) the tRNA has a shape similar to the letter L, and its angle formed by the two arms, is specific for bonding with a certain amino acid (Fig. 54b). The bonding of the amino acids to the tRNA is therefore given by the spatial structure of these molecules, they don't need any other helping apparatus, and it is conducted with a precision almost 100 %.



● **Figure 54.** Scheme of the tRNA (a) and the spatial model of tRNA (b)

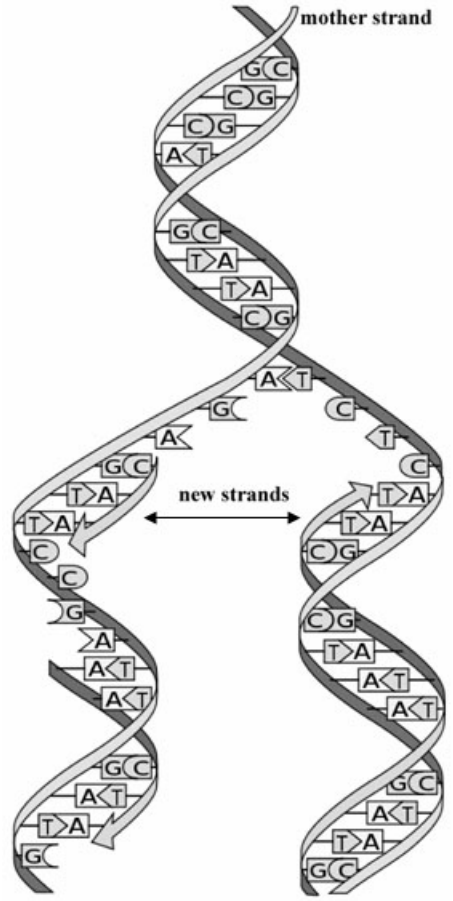
	Function
Acceptor arm	to its end an aminoacid binds
Anticodon arm with an anticodon loop	with this end the tRNA recognises the genetic code written inside the mRNA
Pseudouridine arm with S-loop	contains the modified uracil – dihydrouracil
Variable arm	can be variously long, it doesn't influence the biological function of the tRNA

● **Table 2.** Overview of the variable arms of tRNA

The tRNA is formed by the transcription of genes **for RNA** (in the cell a few dozens are present). Genes for transfer RNA are, in prokaryotes, located in the common transcription units.

Precursor pre-tRNA are formed in the nucleolus by the effects of RNA polymerase III, which catalyzes the synthesis of 5S rRNA and small RNA molecules. First the bases in the tRNA loops are alkylated and metylated. The pre-tRNA contains copies of introns, which are cut by other enzymes, the same way as in hnRNA maturation. The endonuclease splits the bonds between the exon and intron. By the effect of ligase the exons connect 3'-5' by the phosphodiester bond, and to its 3' end on each tRNA the CCA nucleotides are added.

7. Replication



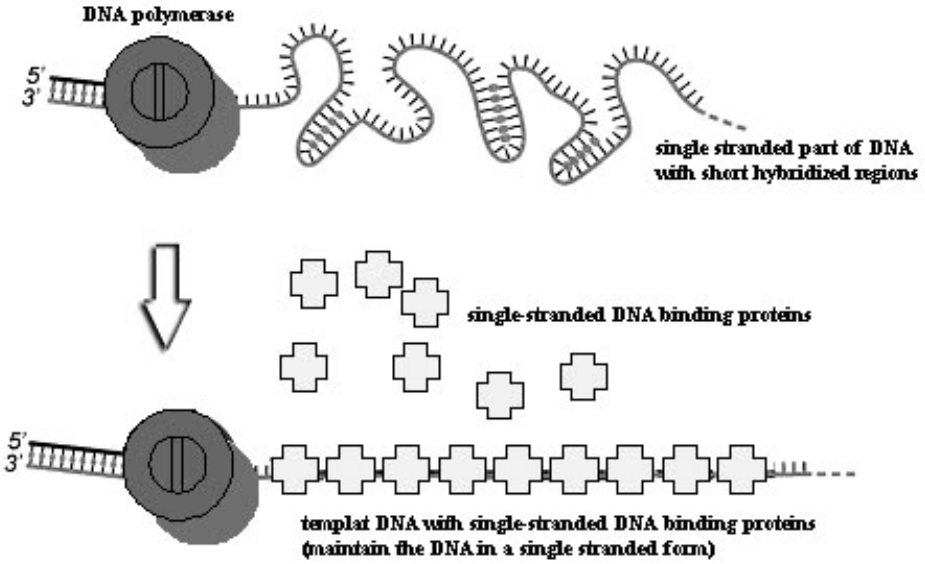
Replication is a process which is essential for the passing identical copies of genetic information from the mother cell to the daughter cells. Replication of DNA occurs between two cell divisions – in the S-phase of the cell cycle interphase. In eukaryotic cells it lasts about 12 hours and runs at approximately 400 places at the same time. The DNA of the mother cell is copied, and two identical DNA molecules are formed. The mechanism of replication is **semiconservative**, because to each of the former DNA strands a new complementary strand is synthesized. The former double strand DNA strands are separated and serve as a template for the synthesis of the two new DNA strands. To each former strand, a new DNA strand is synthesized. By this, two complete double helixes are formed, which contain a former strand = **mother strand** and the second newly synthesized strand = **daughter strand** (Fig. 55). Bases are bonded according to complementarity. For example, if the mother strand contains adenine, in the daughter strand it cans only nucleotide with thymine. By complementarity of nitrogen bases of nucleotides is preserved the identity of the genetic information for daughter cells.

● **Figure 55.** Scheme of semiconservative replication of DNA

Replication of DNA cannot start at a random position on the strand, but only on a specific point of the beginning of replication, which in a prokaryotic DNA is called **ORI** (ORIgin of replication). Bacteria *E. coli* contains only one DNA molecule and only one ORI, which has the size of 245 bp. The nucleus of eukaryotic cells contains many starts of replication and each of them is recognized and covered by **ORC** (Origin Recognition Complex) proteins. When starts replication, each ORC is released and specific proteins (sc. **DNA proteins)** bind to ORI sequences**.** The first that binds is the **dnaA** protein, which forms the complex dnaA-DNA. This enables the binding of the **dnaB** protein, which is a hexamer made of 6 peptide chains. The last that binds on these complex is the **dnaC** protein. Protein dnaB has the activity of **helicase** –interrupts the hydrogen bonds between complementary nitrogen bases. This causes separation of the DNA strands and formation of **replication bubble** in the middle of the strand, which spreads to both sides (Fig. 56). On the ends of chromosomes (telomeres) so called **replication forks** are formed by the separation of DNA strands, which spread in only one direction – towards centromera of chromosome.

● **Figure 56.** Replication bubble in a prokaryotic chromosome. Replication starts at ORI and spreads in both directions (labeled by arrows)

After the denaturation of DNA – separation of the DNA strands by helicases, to each strand of DNA immediately bind **ssDNAb proteins** (single-stranded DNA binding proteins). Their task is not only stabilize the denatured part of the DNA (maintain the DNA in a single stranded form) but also prevent formation of loops called "hairpins" (Fig. 57).



● **Figure 57.** ssDNAb proteins stabilize the denatured part of the DNA

When the replication bubble is enough wide, the enzyme **primase** (has the activity of **RNA-polymerase**) attaches to the complex of the DNA proteins, which forms a **primozome**. Primase generates and locate on proper positions on DNA short oligonucleotide strands of RNA (11-20 nucleotides) – **RNA primers**, that serve as the anchoring sites for of the DNA polymerase. In leading strand (with orientation 3´→ 5´) primer are applied at telomere sequence TTAGGG and on each ORI. In case of the lagging strand (with 5´→ 3´ orientation) apply primase primers in relatively equal distances. The polymerase must always firstly attach to the RNA primer, and after this has the ability to start synthesis of complementary parallel (daughter) DNA strand.

The most important enzymes in DNA replication are **DNA polymerases**. They ensure the prolongation (growth) of the DNA strand by assigning of new deoxyribonucleotides. Neighboring deoxyribonucleotides are connected by a phosphodiesteric bond. First phosphoesteric bond (PeB) is between phosphate ("rest of H_3PO_4") and third carbon (3´) of deoxyribose in previous deoxyribonucleotide. Second one connects the same phosphate to fifth carbon (5´) of deoxyribose in following deoxyribonucleotide. Process starts so, that polymerase "grabs" certain nucleoside (nitrogen base + deoxyribose) and make first PeB between latest phosphate of ATP and -OH group on fifths carbon (5´) of nucleoside pentose. Result is creation of nucleoside-triphosphate (N-3P). Then polymerase uses N-3P and inserts it opposite to complementary base in template strand. Finally – polymerase "cuts-out" ADP and makes second PeB with -OH group on the third carbon (3´) of previous nucleotide pentose. This is the reason, why synthesis of DNA (and all nucleic acids) occurs only in the 5´→ 3´ direction – to be template and newly synthesized strand complementary and antiparallel.

The direction of new strand synthesis and the necessity for the strands of the nucleic acids during its synthesis to be antiparallel, cause that the each DNA strands is replicated by different mechanisms.

In prokaryotic cells 5 types of DNA polymerase were so far identified. The most know are DNA polymerase I, II and III (Tab. 3). DNA polymerases IV a V, participate in the repair of DNA.

DNA polymerase	**I.**	**II.**	**III.**
Mr (kDa)	103	88	900
Number of subunits	1	4	10
Processing speed (nucleotides/sec)	16 – 20	7	250 – 1000
exonuclease activity 5´→ 3´	+	–	–

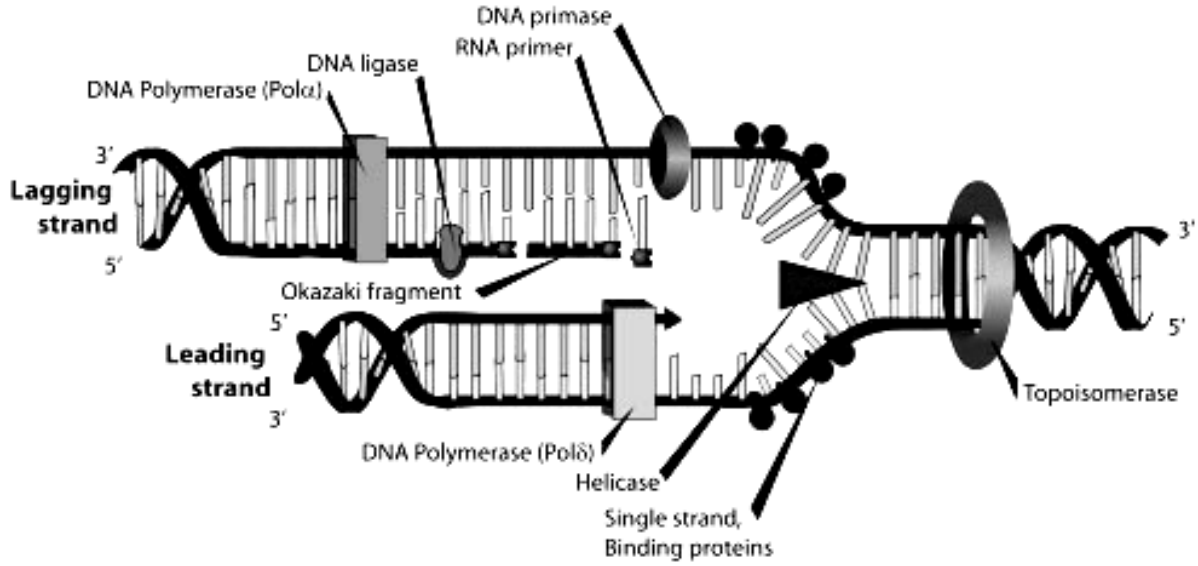
● **Table 3.** *E. coli* DNA polymerase

DNA polymerase I is formed by one polypeptide chain with the molecular weight of 103 000 Da. Inside the cell of *E. coli* there are around 400 molecules of this enzyme. The enzyme is responsible for repairing damaged DNA segments, and participates in the assembling of the lagging chain during DNA replication. It works at the speed of 20 nucleotides/sec and has a polymerase activity in the 5´→ 3´ direction, and an exonuclease activity (detaches the end nucleotide) in the 3´→ 5´ and 5´→ 3´ directions.

DNA polymerase II is also formed by one polypeptide chain with a molecular weight of 88 000 Da. Its *in vivo* function is not exactly defined, but it participated during the repair of DNA. It works with the speed of 7 nucleotides/sec., has a polymerase activity in the 5´→ 3´ direction and an exonuclease activity in the 3´→ 5´ direction.

DNA polymerase III catalyzes the synthesis of the DNA strands, while in the cell of *E. coli* there are only around 20 complete enzyme molecules. It works with the speed of 250 – 1000 nucleotides/sec. The enzyme has a complicated quartiery structure, it consists of many monomers (900 000 Da). **The catalytic center** of polymerase is made of monomers **α + ε + θ**. The important one is the **β-grip**, which moves towards the catalytic center and holds the mother and daughter strands together (it doesn't connect). The **γ complex** has an ATP-ase activity, it loads the β grips on to the DNA on the place where are the RNA primers.

Polymerases have their synthetic activity only in the 5´→ 3´ direction, and therefore the replication can only take place in this direction. The mother DNA strand with the 3´→ 5´ orientation is called as the **leading strand** while the one with the 5´→ 3´ direction as a **lagging strand**. The complementary strand to leading strand is synthesized continually and rapidly (approximately 1500 nucleotides/sec). The lagging (delayed) strand is replicated slower, discontinually, and with the help of small fragments (sc. **Okazaki fragments**), which are also synthesized in 5´→ 3´ direction (Fig. 58).



● **Figure 58.** Scheme of DNA replication

After the adding of a primer to the beginning of the leading strand, the primase disconnects. DNA Polymerase III starts the **continual** adding of the particular deoxyribonucleotides to the 3´ end nucleotide according to the rules of complementarity. If an error occurs, then during its 3´→ 5´ exonuclease activity it removes the fault deoxyribonucleotide off, and adds the right deoxyribonucleotide (sc. "self-proof" reading). The frequency of incorporation of a wrong deoxyribonucleotide is 1/10000. By this selfcontrolled corrective system, the occurrence of wrong deoxyribonucleotides is lowered by about a 1000 times.

The antiparallel strand is replicated **discontinually**, therefore by parts. The primase creates the first RNA primer that connects to lagging strand, then primase detaches and moves forwardd, where it forms second RNA primer. Polymerase III connects to the second primer and synthesizes the complementary DNA strand from to the proceeding RNA primer. This process repeats, forming complementary strand to lagging strand, in which DNA and RNA regions alter. The fragment of DNA in this hybrid we call the **Okazaki fragment,** according to the author who founded this kind of discontinual synthesis. The Okazaki fragments, in *E. coli*, contain around 2 000 deoxyribonucleotides. RNA primers are then removed from the chain by DNA polymerase I, which at the same time fills the gap (after the primer) by adding it up with deoxyribonucleotides. Except the polymerase activity, this enzyme also has a 5´→ 3´ exonuclease activity, by which it can gradually detach nucleotides from the 5´end of the DNA. Polymerase I has a sufficient amount of time, and therefore it works much slower then polymerase III. Alternating fragments are formed, which were made both by DNA Polymerase III and I. This short DNA fragments are finally connected together by **DNA ligase,** which forms phosphodiester bonds between the individual fragments of the DNA strand.

Replication of DNA in eukaryotic cells takes place according to similar mechanisms. The first important difference is in the polymerases. So far, 15 kinds of polymerases were isolated from the eukaryotic cells. Amongst the most important are:

- **DNA polymerase** δ catalyzes the synthesis of leading strand and finishes the synthesis of lagging strand;
- **DNA polymerase** α (DNA primase) catalyzes the synthesis of Okazaki fragments;
- **DNA polymerase** β catalyzes the synthesis of short fragments during DNA reparation;
- **DNA polymerase** γ catalyzes the formation of mitochondrial DNA in the mitochondria.

One of the units of the replication apparatus is the **PCNA protein** (proliferating cell nuclear antigen), which fulfills the function of the β-grip and the **RFC protein** (replication factor C), acting as an γ-complex in prokaryotes.

In eukaryotic cells, compared to the prokaryotes, there is a different method of the assembling of the delaying lagging strand. The splitting of the primer in eukaryotes is catalysed by an special exonuclease, also called **RNA-ase H**, which has a 5´→ 3´ exonuclease activity. The finishing of the synthesis of the Okazaki fragment is catalyzed by DNA polymerase δ (In prokaryotes it is RNA polymerase I). The connecting of DNA fragments, as in prokaryotes, is catalyzed by the enzyme DNA ligase.

Eukaryotic DNA is linear, and therefore a problem arises during the end part of the synthesis of the lagging strand. The end part cannot finish its replication on the 3´ end. Every eukaryotic chromosome, besides the centromere also contains a **telomere**, a structure with an overhanging (leading) DNA strand**.** On the 3´ end, the telomere is characterized by tandem repeats, which are type specific and rich on G. In humans it is the sequence **TTAGGG**. The telomeres shorten during each replication. Unless the main strand overhangs, the replication can not start. That is why this sequence must be recognized by the enzyme **telomerase** (specific DNA polymerase), which synthesizes, through the primer, on the 3´ end of the mother DNA, a short DNA strand, with the help of which the DNA polymerase finishes the synthesis of the end of the lagging strand.

The synthesis of eukaryotic DNA is 10-times slower then the synthesis of prokaryotic DNA. Human DNA polymerase connects approximately 50 nucleotides per second. Despite of this, new DNA is formed quickly even in eukaryotic cells. It is so, because a substantial amount of replicons are formed at the eukaryotic DNA, therefore synthesis occurs simultaneously on many places. Also the size of the DNA within the Okazaki fragments is diverse. In prokaryotes it is about 1 000 to 2 000 nucleotides, in eukaryotes the Okazaki fragments contain only about 150 nucleotides. In eukaryotes nucleosomes form immediately after replication. During replication the nucleosomes fall apart to form individual histones. Since for two new DNA strands a double amount of histones is required, intensive synthesis of these proteins occurs. Therefore the new nucleosomes randomly contain both new and old histone subunits.

8. Protein synthesis

Saccharides and lipids are, even within various organisms, almost identical, but proteins have the most important role in an organism. The properties of proteins are primarily determined by their primary structure, e.g. the sequence of amino acids that they are made of. The sequence of amino acids is coded inside the DNA (in structural genes).

The transfer of the genetic information occurs at three different levels: replication, transcription and translation.

By **transcription** we mean the rewriting of the genetic information from the DNA to the RNA.

Translation translates the genetic information from the RNA molecule into the primary protein structure – the sequence of amino acids in the polypeptide chain.

8.1 Gene

A gene is a basic functional and structural unit of genetic information. In meaning of molecular genetics gene is segment of DNA, that is transcribed and contain regulatory and coding parts. According to the genetic information that they carry, we can divide genes into three groups: structural genes, regulatory genes, and genes for the RNA molecules, except mRNA.

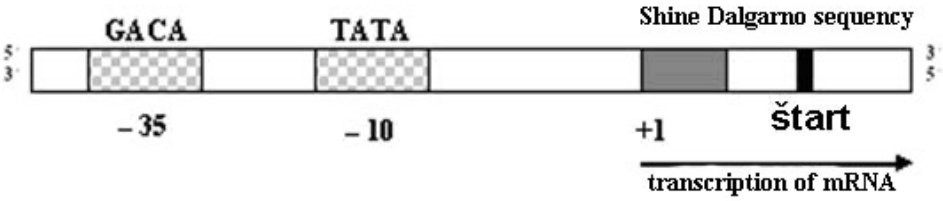
A Structural Gene is a part of the DNA chain (sometimes an RNA chain as in RNA viruses), which codes for the primary structure of the proteins. The size of the genes is expressed in the number of base pairs (bp) in the DNA, it contains. The larger units are called kilobases (kb) or megabases (Mb). The longest human gene (coding for dystrophine) is 2.4 Mb long and makes up 0.1 % of the entire human genome. The structural gene consists of two parts: **regulatory** and **coding** part. The **regulatory** part is called the **promoter**. It contains important sequences (parts of base sequences, the so called **boxes**), for example **TATA box**, **CAAT box** etc. Their task is to bind regulatory proteins, the so called **transcription factors**. These proteins must bind on the mentioned sequences of the nitrogenous bases in the right order for transcription (syntheses of mRNA) to begin, which is carried out by RNA polymerase.

The prokaryotic promoter (Fig. 59) contains two important functional parts. It is the sequence in the area of the nucleotide in the -35 location (before the start codon), which is called the **GACA box** and has the following primary structure:

5´– T T **G A C A** T – 3´

The second important sequence is in the area of the nucleotide -10, which is called the **TATA box** (Pribnow box) and has a sequence:

5´– **T A T A** A T – 3´



● **Figure 59.** Prokaryotic promoter

The Eukaryotic promot**e**r contains in the -34 to -26 area a **TATA box** (Hognes), to which the TFIID transcription factor binds.

5´– **T A T A** A A A – 3´

In the area between -75 and -80 is another important box called a **CAAT box**, to which the NF1 transcription factor binds, strengthening the promoter.

5´– G G C **C A A T** C T – 3´

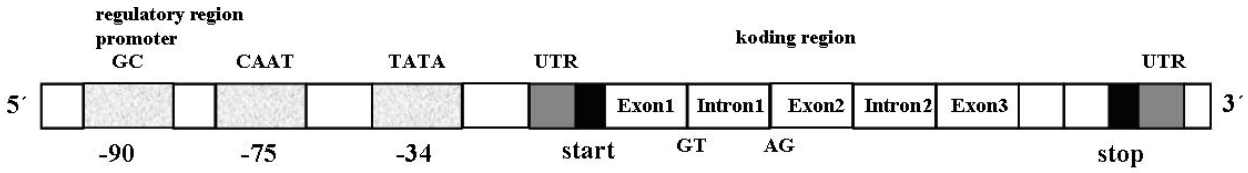
The third box is called the **GC,** it is in the area - 90 and is bonded by the transcription factor SP1, which also strengthens the promoter.

5´– G G **G C** G G – 3´

In **the coding areas** the eukaryotic genes (Fig. 60) often contain noncoding parts (**introns**) in between the coding parts (**exons**). The coding part of the gene starts with the untranslated region (**UTR**) on the 5´ end, which serves as a connection of the mRNA to a small ribosomal subunit. Immediately after it follows the first exon, which starts with the so called **start triplet** (ATG). The first exon is followed by the first intron. Then proceeds another exon etc. This area therefore contains alternating exons and introns. The last exon ends with the so called **stop triplet** (TAA, TAG or TGA). Transcription finishes on the 3´ UTR, that that continues. After it proceeds a sequence activating the **poly(A) polymerase** (forming a so called poly-A tail). In most cases the introns are longer then the exons and the length of the gene is measured from the start triplet to the stop triplet.

The regulatory gene is a part of the DNA which codes for the primary structure of the regulatory protein, which function is usually the induction or repression of the other genes expression.

The RNA coding genes are responsible for the primary structure of the ribosomal and transfer RNA and other types of smaller molecules of RNA.



● **Figure 60.** Structure of the Eukaryotic gene

8.2 Central Dogma of Molecular Biology

The transfer of genetic information by the process of replication, transcription and translation is in biology termed as the central dogma. The biosynthesis of proteins represents the process of the transfer of genetic information between three types of macromolecules: DNA, RNA and proteins. The direction of this transfer was postulated by Francis Crick in 1959 (Fig. 61).

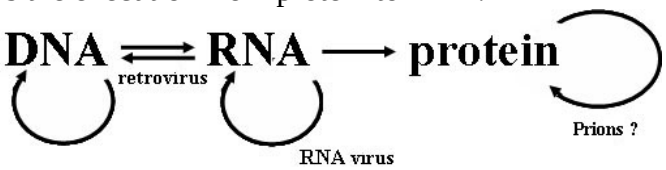
● **Figure 61.** Central biology dogma according to Crick

In the year 1970 Temin and Baltimore proved the existence of the transfer of genetic information from an RNA virus to a DNA of a host cell; with the help of the **reverse transcriptase** enzyme (it synthesizes DNA according to the RNA matrix). This altered the central dogma of molecular (Fig. 62).

● **Figure 62.** Central dogma of molecular biology according to Temin and Baltimore

Most recently certain abilities of glycoprotein's called **prions** were discovered, which are able to change similar types of proteins to their own (Fig. 63). Prions do not contain nucleic acids. The gene for a prion protein (**PrP**) is within humans located on the short leg of chromosome 20 and codes for a protein that is labelled $\mathbf{PrP^C}$. Prions are normally located in the neurons of a human. They can however change to a pathological form $\mathbf{PrP^{Sc}}$, which is resistant to proteolytic enzymes, is substantially stable, and is not able to physiologically degrade in the neurons. Because of this the protein accumulates, which then leads to the degeneration of neurons. We name such diseases as prionoses. It is a group of neurodegenerative diseases of humans and animals, scrapes of goats and sheep, with humans kuru, Creutzfeld-Jacob's disease, Gertsman's–Sträussler–Scheinker's syndrome, Bovine Spongiform Encephalopathy and Fatal Familiar Insomnia. These diseases are not caused by viruses, bacteria's or other microorganisms. Many experiments on mice lead to the hypothesis that infectious agens is of an entirely protein nature. The change of $\mathbf{PrP^C}$ into $\mathbf{PrP^{Sc}}$ is the basis of the pathogenesis of prionoses. The prion proteins synthesize as normal proteins and they become pathological as a consequence of postranslational editing, which shortens the sequence or changes the tertiary structure. Therefore only a conformational change occurs, which is not registered by the immunity system.

A pathway in which RNA or DNA is formed by a protein never occurs. The pathway therefore never follows the execution from protein to DNA.



● **Figure 63.** The central dogma of molecular biology involving prions

8.3 Transcription

In the process of transcription is the mRNA molecule formed according to the sequence of bases in gene. Later is mRNA transported from the nucleus into the cytoplasm – to the ribosomes. During transcription only a short part of one (sc. template) of the two DNA strands is transcribed. It is single gene or a group of genes, which are sequenced after each other in a DNA chain. Majority of genes are independent, however gene families do exist.

The transcription of the genetic information from the gene in the RNA molecule is catalyzed by the **RNA polymerase** enzyme. Sometimes a more complex name is used: **DNA dependent RNA polymerase.** It adds nucleotides according to the rules of complementarity. The nucleotides are connected in the 5´– 3´ direction by a phosphodiester bond. The synthesis of RNA takes place in the 5´→ 3´ direction for which a so called template DNA strand has a 3´→ 5´ orientation. By transcription in prokaryotes an mRNA is formed, while a so called **primary transcript** is formed in eukaryotes (sc. pre-mRNA or hn-RNA). It is a precursor

form, from which further processing makes functional mRNA molecules. The section of DNA which is transcribed into the primary transcript we call the **transcription unit**.

The transcription unit of prokaryotes, which has one or more structural genes, contains a so called **leading sequence**, located immediately after the promoter. A part of it is the **Shine - Dalgarno sequence**, which is transcribed into the mRNA as: 5´ – A G G A – 3´. By this sequence the mRNA binds to the 16S rRNA in the 30S subunit of the ribosome. This enables the formation of the mRNA + ribosome complex.

Prokaryotic mRNA is polycistronic, which means that it contains transcript of more structural genes following in each other.

In prokaryotic cells the production of all forms of RNA is catalyzed by only one type of RNA polymerase (465 kDa). The beginning of transcription is called **initiation**. At first the RNA polymerase connects to a promotor and after the double helix unwinds. A **transcription bubble** is formed. The second phase of transcription is the lengthening ("growth") of the mRNA chain and is called the **elongation phase**. This phase starts so, that **NusA protein** binds to the initiatory form of the RNA polymerase, forming an elongative form of RNA polymerase. In this form RNA polymerase "moves" on the DNA chain, denaturing it and then transcribing the template sequence onto the RNA chain with the speed of 60 nucleotides/s. At the same time, on the opposite end of the transcription bubble, the DNA renatures. In the transcription bubble there is an unwinded DNA sequence with an approximate length of 18 bp. The end of the transcription process is called **termination**. It occurs in the area of the "terminator", where a polyA polymerase activating sequence is located. The NusA protein recognizes this sequence and stops the movement of RNA polymerase on the DNA chain. In the area of the terminator the bond is weaker, since it is supported by only two hydrogen bonds between adenine and uracil. This kind of hybrid is easily destabilized and the mRNA is released from the complex. In certain genes a **ρ**-factor is needed for ending the transcription process. Transcription in prokaryotes is directly associated with translation, the lifespan of the mRNA is only a few minutes. The ribosomes start binding to the start codons in mRNA during its creation, this means that shortly after beginning of the mRNA synthesis, starts the production of proteins.

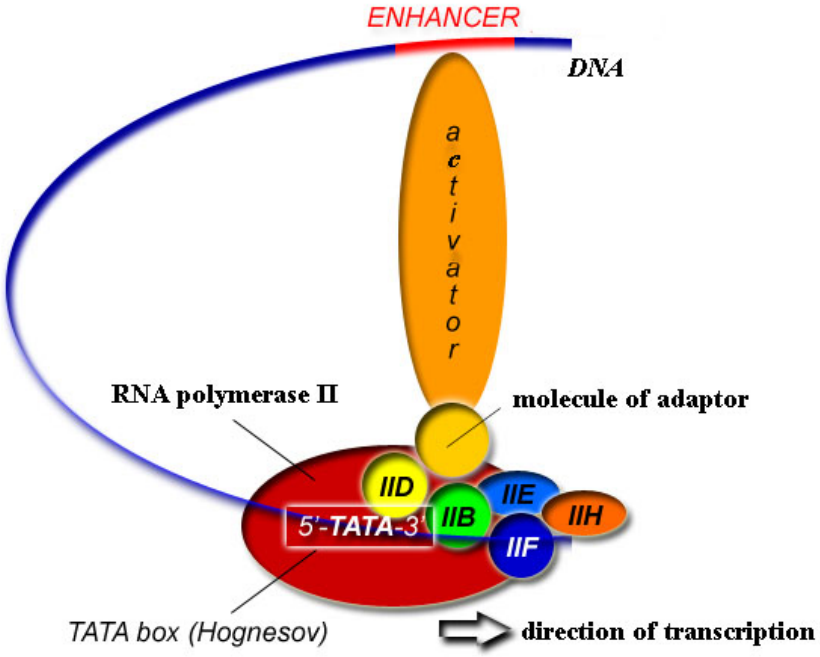
The synthesis of RNA in eukaryotic cells is carried out by three kinds of RNA polymerases: RNA polymerase I, II, and III (Tab. 4). **RNA polymerase I** catalyzes the transcription of genes for the rRNA. The product of this transcription is the **precursor rRNA (pre-rRNA).** By the post-transcription modifications it becomes 5,8S rRNA, 18S rRMA and 28S rRNA. **RNA polymerase II** catalyzes the transcription of structural genes with the creation of heterogeneous nuclear RNA (hnRNA or pre – mRNA). From this molecule, after certain modifications, a functional mRNA is formed. Besides hnRNA the RNA polymerase II catalyzes the production of small nuclear RNAs. RNA polymerase III transcribes genes for tRNA, and is creates a **precursor tRNA (pre-tRNA)**. From this molecule, after post-transcription modifications, particular tRNAs are created. RNA polymerase II also catalyzes the synthesis of 5S rRNA and small nuclear and cytosomal RNAs.

Enzyme	Location	Function
RNA polymerase I	Nucleolus	Transcribes genes for rRNA
RNA polymerase II	Nucleus	Transcribes structural genes and genes for certain small RNAs
RNA polymerase III	Nucleus	Transcribes genes for tRNA, 5S-rRNA and certain small RNAs

● **Table 4.** Overview of eukaryotic RNA polymerases

In eukaryotes, transcription requires the presence of many helping proteins, the so called **transcription factors**. They are regulatory proteins, which bind to the regulatory

positions of the promoter. During the initiation of transcription they carry out tasks that a prokaryotic RNA polymerase carries out by itself – the identification of boxes in the promoter, the binding of the RNA polymerase II to the promoter, the unwinding of the DNA double helix and the phosphorylation of RNA polymerase II (its activation to transcription). Regulators of transcription are called enhancers or silencers. They are bonded to complex transcription factors-RNA polymerase by collaborating molecules – the adaptor and activator (Fig. 64).



● **Figure 64.** Initiation of transcription

Initiation of transcription in eukaryotic cells is started by the binding of a regulatory protein to the big groove of the DNA in front of gene. The decomposition of nucleosomes and the linearization of DNA follows. The enzyme helicase separates the DNA strands, which are then stabilized by the helping proteins. Transcription factors (IID + IIB + RNA polymerase II + IIF +IIE +IIH) are then bonded to the main regulatory boxes (TATA, CAAT, GC). The TATA box has a high importance, since the **transcription factor TFIID** is bonded to it, by which the **preinitiaton complex** is formed. The basic component of the TFIID is the so called TBP protein (TATA box binding protein). It binds to the promotor, to other proteins but also with RNA polymerase II. The TBP protein is present in all eukaryotic cells.

The RNA polymerase and some other transcription factors bind to the preinitiation complex and form a so called **nonactive initiation complex**. By the binding of another transcription factor **TFIIH**, which has a helicase and proteinkinase activity, the RNA polymerase II is phosphoryled and becomes active. By the formation of the **active initiation complex** the promoter is bonded by 6 different transcription factors, out of which all have a complicated quartenary structure. These transcription factors allow only basal transcription. For the change of the intensity of transcription other special transcription factors are necessary (enhancer or silencer). The DNA helix unwinds and the RNA strand starts to be synthesized.

Transcription itself starts at the 5´ UTR (untranslated region), 10 bases before the start triplet, and continues until the end of the 3´ end UTR after the last exon.

The termination of a structural gene transcription is marked by the presence of a polyadenylation signal AATAAA, which rewrites to the hnRNA as AAUAAA. This sequence is recognized by a protein complex with an endonuclease activity. It cleaves the

chain in the distance of 10 to 30 nucleotides from polyadenylation sequence and the newly formed hnRNA is released from the transcription complex.

8.3.1 Post-transcription modification of RNA

The newly synthesized molecule of RNA is called the **primary transcript,** and must be modified. This process is called post-transcription modification or **RNA maturation**. In prokaryotic cells certain mRNAs synthesize as a fully functional primary transcript, rRNA and tRNA are formed from precursor molecules. In eukaryotic cells all types of RNA are formed by the processing of primary transcripts. The precursor form of mRNA is hnRNA, which is synthesized by RNA polymerase II. The transformation of hnRNA is carried out in the nucleus of the cell and has three main steps.

The first step is the binding of a 7-methylguanosine on to the 5´ beginning of the hnRNA chain with a non-typical 5´ – 5´ triphosphate bond, the so called **cap** is formed. The guanine nucleotide is bonded to the RNA through the three remnants of the phosphoric acid, and the 7^{th} atom of nitrogen in the guanine is methylated. All mRNAs in eukaryotic cells have a cap. Its purpose is to protect the 5´ end of mRNA against the influence of exonucleases and to help during the binding of the mRNA to the small subunit of ribosome.

The second step is **splicing**, during which RNA copy of non-coding gene sequences – **introns** – are cutted out from the precursor molecule. In eukaryotic cells, splicing is catalyzed by large enzyme complexes (spliceosomes). They are able (in collaboration with small RNAs) to recognize an intron, cut it out, and connect together the coding sequences – **exons**. A part of the enzyme complexes are ribonucleoproteins, which contain small RNA molecules (Tab. 5). The function of these small RNA molecules is to identify the 5´ beginning and the 3´ end of an intron. On the 5´ end the introns have a GU sequence while on the 3´ end an AG sequence. The sequence allows the enzyme complex together with the ribonucleoproteins to label both ends of the intron, and cut it out (Fig. 65). At first the phosphodiester bond breaks between the 3´ end of the first exon and the 5´ beginning of the intron. In the next step the 5´ "end" of the intron binds to the adenine nucleotide inside the intron with a non-typical 5´– 2´ phosphodiester bond. The intron therefore gets a "lasso" shape. Finally, previous and following exons connect by a phosphodiester bond and the lasso shaped intron releases itself. This process occurs without the addition of ATP energy.

Type of small RNA	Location	Function	Synthesizing enzyme
U1 snRNA	nucleus	management of splicing	RNA polymerase II
U2 snRNA	nucleus	management of splicing	RNA polymerase II
U4 snRNA	nucleus	management of splicing	RNA polymerase II
U5 snRNA	nucleus	management of splicing	RNA polymerase II
U6 snRNA	nucleus	management of splicing	RNA polymerase III
U7 snRNA	nucleus	management of splicing of histone hnRNA	RNA polymerase II
U11 snRNA	nucleus	management of splicing	RNA polymerase II
U12 snRNA	nucleus	management of splicing	RNA polymerase II
7SK snRNA	nucleus	unknown	RNA polymerase III
8-2 snRNA	nucleus	modification of pre-tRNA	RNA polymerase III
U3 snoRNA	nucleolus	modification of pre-tRNA	RNA polymerase II
U8 snoRNA	nucleolus	modification of pre-tRNA	RNA polymerase II
U13 snoRNA	nucleolus	unknown	RNA polymerase II
7SI scRNA	cytoplasm	translocation of proteins	RNA polymerase III

● **Table 5.** Types of small RNA molecules

The third step is the formation of a **polyA tail** on the 3´ end of the mRNA. It is a sequence made up of 100 to 250 remnants of adenine assembled by polyA- polymerase. The polyA-sequence protects the mRNA from the 3´ end against exonucleases, during its transport to the ribosomes. Moreover, it has an important regulatory role its length determines, how many times a single mRNA can be used for translation.

After these modifications a functional mRNA molecule is created, and (after association with regulatory proteins) is transferred from the nucleus to the cytoplasm, where it binds to small subunit of ribosome.

● **Figure 65.** Mature mRNA

8.4 Genetic code

The genetic code is a way of genetic information recording. The smallest particle **(„a letter“)** is a nitrogenous base of nucleotide. The shortest unit **(„a word“)** is a group of three following bases (nucleotides), which determine the placement of one aminoacid, the so called **triplet (codon)**. There are 21 types of amino acids used in proteins and only four different bases in code. If one base will code for one amino acid, then 4^1 is 4 one base is for coding not enough. The combination of two bases also doesn't sustain since 4^2 is 16. A sustaining number of bases is given by the combination of three bases, where 4^3 is 64 possibilities, allowing to code for 21 amino acids. A triplet is always read from the 5´ end of the mRNA, so for example the triplet for tryptophane is:

5´– **U G G** – 3´

There are 64 types of codons, from these only 61 code amino acids. The first, sc. **start triplet**, in mRNA is always AUG, which codes for methionine. Three of these triplets don't code for any amino acid and have an important role during the termination of synthesis of the polypeptide chain. Those are the so called termination codons or **stop codons**. After the transcription to the mRNA they are: **UAA, UAG** and **UGA**. One of them is always at the end of the last exon.

The cell contains an apparatus which is able to read this code and synthesize the given protein with the corresponding sequence of amino acids.

The entire information in genetic code (a "**sentence**") is gene which has the whole information for the primary structure of single protein.

The complete genetic code is demonstrated in table 6. Triplets, which code for the same amino acid, often have the first two bases identical with a variable third base. This means that for the specificity of a codon, the first two bases are most important.

The genetic code is:

- **tripleted** – three nitrogen bases of nucleotides following one after another in the mRNA (or in the DNA, in the coding region of the structural gene) coding for one amino acid (except the stop triplets);
- **universal** – present in all organisms. Only within mitochondria certain deviations from the universal genetic code were observed.

- **not overlapping** – triplets follow one after the other in the DNA (in a linear setting) without interruption;
- **degenerated** – there are too many triplet possibilities (61) for the coding of (21) amino acids. In reality it is a safety measure, since the amino acids that are used the most often also have the highest variation of triplets (4-6), which they can be coded by. It is therefore a protection against the rise of mutations, since the change of one base in the triplet doesn't necessarily mean the change of the entire amino acid inside the protein.

phenylalanine	UUU, UUC, UUA, UUG
leucine	CUU, CUC, CUA, CUG
isoleucine	AUU, AUC, AUA
START + methionine	AUG
valine	GUU, GUC, GUA, GUG
serine	UCU, UCC, UCA, UCG, AGU, AGC
proline	CCU, CCC, CCA, CCG
tyrosine	UAU, UAC
threonine	ACU, ACC, ACA, ACG
alanine	GCU, GCC, GCA, GCG
STOP	UAA (ochre), UAG (amber), UGA (opal)
histidine	CAU, CAC
glutamine	CAA, CAG
asparagine	AAU, AAC
lysine	AAA, AAG
asparagic acid	GAU, GAC
glutamic acid	GAA, GAG
cysteine	UGU, UGC
tryptophane	UGG
arginine	CGU, CGC, CGA, CGG, AGA, AGG
glycine	GGU, GGC, GGA, GGG
selenocysteine	UGA

● **Table 6.** Standard genetic code

8.5 Translation

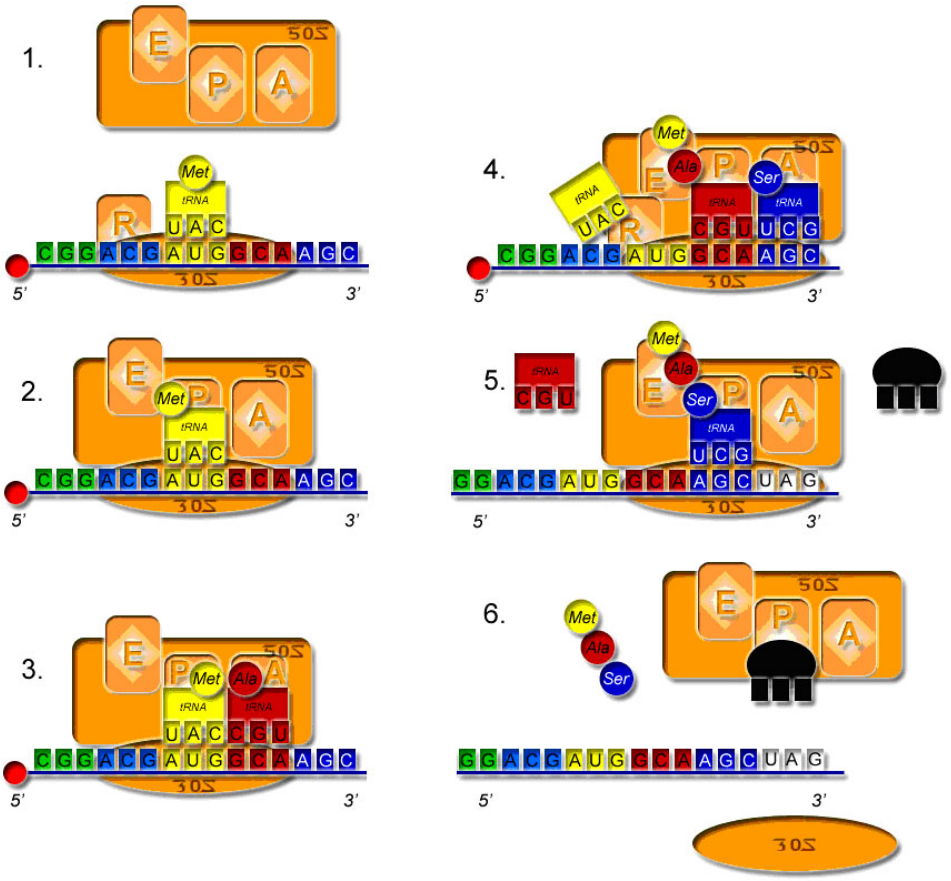
The translation process is the rendering of the nitrogen bases sequence in the mRNA into the sequence of amino acids in the protein. It is the process of synthesis of proteins, during which the amino acids are ordered according to the triplet sequence in a give mRNA.

The proteins are then changed by post-translation modifications, first in the endoplasmatic reticulum and the Golgi apparatus. The correct primary sequence of the amino acids in the chain allows the creation of an active secondary, tertiary, or quarterly structure, which is necessary for protein full biological activity. In certain cells, during differentiation, an alternative usage of a genetic information takes place (e.g. the using of variable start codon, or an alternative splicing), which causes that cells have the same genes, but synthesize different proteins.

The protein synthesis in the cell takes place in the ribosomes, in the cytoplasm and endoplasmic reticulum. First is necessary to build (assembly) a **protein synthetic apparatus**, which consists of: mRNA, small and big subunit of ribosome, aminoacyl-tRNA synthtase, helping molecules and amino acids.

The process of translation can be divided into the following stages (Fig. 66):

- the creation of an initiation complex;
- the activation of an amino acid and its bond on the tRNA;
- the lengthening of the peptide chain;
- the termination of the synthesis of the polypeptide;
- post-translation modification of the protein chain.



● **Figure 66.** The process of translation

8.5.1 The formation of the initiation complex

If simplify the process of translation initiation – the mRNA binds to the small subunit of ribosome by the cap and 5´UTR, with the help of regulatory proteins. The tRNA binding methionine amino acid (the one complementary to the start codon) takes also part. That makes a signal, which binds the large subunit of ribosome and activates it. In each mRNA the coding sequence of the RNA starts with a start codon, which is formed by three bases AUG. In prokaryotes the initiation codon is for tRNA, which binds N-methylformyl methionine. The reason is that only methionin (or its prokaryotic variant) is the only amino acid that can activate – without preliminary amino-acylation – the ribosome, particularly P site in large ribosome subunit.

The synthesis of the peptide chain starts by the formation of the so called **initiation complex.** The formation of the initiation complex is enabled by the helping proteins, which we call the **initiation factors (IF)**. In prokaryotic cells there are three such factors (IF1, IF2 and IF3), in eukaryotes there are 11 of such factors and called as eIF.

In prokaryotic cells the IF3 bonds to the 30S ribosomal subunit. Formylmethionine binds as the first amino acid in the sequence. That is why the **binary complex** (fMet~tRNA+IF2) is formed with the help of IF2. By the binding of GTP

(guanosinetriphosphate) a **ternary complex** (fMet~tRNA+IF2+GTP) is formed. After this the small subunit connects with the ternary complex, to which binds the mRNA forming the **preinitiation complex** (mRNA+30S+IF3+fMet ~tRNA+IF2+GTP+IF1). Then, after GTP hydrolysis, all three initiation factors release and the large (50S) ribosomal subunit binds, forming the **initiation complex**. The initiation complex consists of both ribosomal subunits, and mRNA bond on the small subunit the.

The formation of the initiation complex in ***eukaryotic cell*** is a more complex process. To the 40S subunit connect eIF3 and eIF1AT (40S + eIF3+eIF1A), by which the subunit is activated.

Methionine binds as the first amino acid to its tRNA, and after the connection of GTP the **ternary complex** (Met-tRNA+IF2+GTP) is formed. By the connection of the active form of 40S ribosome subunit and Met~tRNA, another complex is formed (Met~tRNA+IF2+GTP + 40S+eIF3+eIF1A). The mRNA is activated by the connection with three initiation factors - eIF4A, eIF4B and eIF4F. Both complexes connect, and Met~tRNA binds by the anticodon to the start codon in mRNA. This structure is called an **initiation complex**. During GTP hydrolysis, the initiation regulatory factors disconnect and the large (60S) ribosome subunit is bonded. By this a functional **ribosome** is formed.

Four active sites appear in the activated ribosome:

- **A** – to amino acid enter, bonded to the corresponding tRNA;
- **P** – peptide, where the amino acids bind by peptide bonds;
- **E** – for the exit of the newly synthesized protein from the ribosome;
- **R** – for the exit of the tRNA which gave an amino acid.

Methionine doesn't go to the A site but directly to the P site, since only fMet~tRNA in prokaryotes and Met~tRNA in eukaryotes are able to activate the P site – that is why they are always at the beginning of the synthesized protein.

8.5.2 Activation of the amino acid and its binding to the tRNA

The tRNA has an important role in reading of the code. To the adenine on its 3´ oh acceptor arm an amino acid is attached. Amino acids, which are supposed to be build-in into the protein molecule are not able to read the information in the mRNA – by themselves. This is why the amino acid has to connect to the tRNA, which transfers it to a ribosome. This connection is carried out by **aminoacyl tRNA synthetase** – enzyme forming aminoacyl-tRNA. Each of the aminoacids has its own tRNA, which is specific for the connection with only one amino acid. Aminoacyl-tRNA synthetase is specific because it binds to the kind of tRNA, whose anticodon is complementary to the codon.

8.5.3 The lengthening of the peptide chain - elongation

A second complex aminoacyl-tRNA carrying its amino acid enters to the A site. Sequentially tRNA connects to the mRNA, by the codon-anticodon bond. The aminoacyl-tRNA is not able to bind to the mRNA by itself. A so called **elongation factor** EF1 and GTP are required. First, a ternary complex of aminoacyl-tRNA + EF1 + GTP, is formed. If the anticodon in the tRNA is not complementary to the codon in the mRNA, the binding cannot take place and the aminoacyl-tRNA is substituted by another one, which is complementary. The second aminoacyl-tRNA moves from A site to P site of ribosome large subunit and here located enzyme the **peptidyl transferase** connects first and second amino acids by a peptide bond. Then protein elongation factor 2 (EF2) „pushes"the second tRNA. It results to movement of the mRNA by one triplet – following one towards mRNA 3´ end. The tRNA which brought the first amino acid is released from ribosome and liberates its space on

mRNA for a second tRNA – which made a space for a third tRNA (carrying third amino acid). Third aminoacyl-tRNA moves to A site and then to P site, where peptide bond between second amino acid and third one is done. Then again acts EF2, comes fourth aminoacyl-tRNA etc. The newly synthesized peptide exits from the ribosome through the E site. The whole process repeats. The ribosome must always contain two tRNAs. One which is ready to leave and the second one which has just arrived. By the repetition of these steps the peptide chain is slowly contains all the amino acids, according to the sequence of codons in the mRNA molecule. This process is called **elongation**, or the lengthening of the protein product.

8.5.4 The termination of the synthesis of the polypeptide

The process continues until the mRNA reaches to the stop triplet, which signals the termination of the polypeptide chain formation. There is no tRNA for the stop triplet, meaning that the last tRNA remains alone in the ribosome. The outcome is the disintegration of the ribosome and the **termination** of translation.

The pull off of the last amino acid of the protein sequence from the tRNA requires the presence of a **release factor** (RF) and GTP. Simultaneously with the pulling off the polypeptide, the ribosome leaves the mRNA and disintegrates into smaller subunits. During protein synthesis several ribosomes can bind on the same mRNA molecule, such compounds are called polyribosomes or polysomes. On the polysome paralelly several identical proteins are synthesized. The synthesized protein then passes for further modifying to the endoplasmatic reticulum, and from there into the Golgi apparatus.

8.5.5 Posttranslational modifications of the proteins

In eukaryotic cells, initiation and the beginning of translation takes place in the cytoplasm. The beginning of the peptide molecule is universal and is called as an activation sequence (AS). With its help, the ribosome searches for the endoplasmatic reticulum (ER) and binds to the receptor for ribosome on its outside surface. Close to receptor it is the entrance into the ER, where the AS with the entire synthesized polypeptide slides in. In the ER lumen, after the removal of the AS, the protein undergoes the first part of the posttranslational modification. Molecules, which should be integral proteins, jam up in the membrane.

The primary protein chain is modified for instance by the removal of the first methionine or various amino acids, by hydroxylation, glycosylation or phosphorylation, the production of disulfide bridges within the chain, the formation of tertiary structure of the protein etc. A common modification is the removal of the part of the peptide, a good example is pro-insulin. The removal of the polypeptide C, which consists of 33 amino acids, and the connection of the A chain (21 amino acids) and B chain (30 amino acids), functional insulin is formed.

To further modification – proteins are transported in membrane vesicles to Golgi apparatus.

8.6 The mechanism of the effect of antibiotics on protein synthesis in bacteria

Antibiotics inhibit protein synthesis in bacteria. Antibiotics affect different stages of the protein synthesis, but most often they interfere with the protein synthetic apparatus of the bacterias. For example – streptomycin, neomycin and gentamicin bind to the 30 S subunit of the ribosome and block the initiation of translation. Tetracycline prevents the bond of aminoacyl-tRNA onto the site A. Erythromycin inhibits the initiation factor, which is then not able to bond with the 50S subunit. By binding to the 50S subunit, chloramphenicol inhibits peptidyltransferase.

9. Laboratory methods in molecular genetics

9.1 Obtaining of biological material (samples) for DNA analysis

The main prerequisite for a successful molecular DNA analysis is the obtaining of the proper DNA material, from which the DNA is isolated.

The most common method of sample obtaining for DNA analysis is **venepunction** – the taking of peripheral blood (from median cubital vein). For DNA analysis leukocytes are sampled to an anticoagulant (ethylenediaminetetraacetic acid - EDTA) with pH=8 (heparin is not recommended). This kind of modified samples can be stored with the temperature 4° C a few days, under -18° C a several weeks, and under -70° C several months. Considering the risk of infection (e.g. hepatitis B and HIV viruses) it is necessary to follow safety protocols (disposable gloves, surgical mask, etc.) when working with human native material.

For DNA analysis often tissues obtained by **tissue biopsy** are used – the taking of a sample of tissue from a living organism, during surgeries done for therapeutic or diagnostic purposes or by **necropsy** – material taken during autopsy. In both cases it is necessary to carefully consider the part of the body (organ) used for the procedure and the method to be used. For example, during the biopsy of a tissue, it should be done in a way that besides a sample tissue undergoing a pathological process, we should extract a part of the healthy tissue (for comparison). For DNA analysis it is also possible to use archived material – tissues preserved in formalin (10%) or embedded in paraffin. During the determining of fatherhood and the identification of an individual, or even in the case of sampling for gynecology reason – smear is used from the mucous membranes we want to examine. In special cases, especially when analyzing the DNA of viruses and bacteria, **filtration** is used.

9.2 Incubation (cultivation) of biological material

If is amount of material (cells) insufficient, it is necessary to cultivate the cells by *in vitro* multiplication. Optimal conditions for cells reproduction are required – the pH, the composition of the cultivation medium, temperature, atmosphere and sterility. Since in this textbook is a special chapter dealing with the cultivation of eukaryotic cells, only the principles of *in vitro* cultivation of prokaryotes are mentioned here.

For multiplication of microorganisms in laboratory conditions, liquid and half-solid cultivation media, simple, or nourished with amino acids, vitamins and growth factors are used. It is also possible to use special, the so called **selective** media, to determine the success rate of a recombined plasmid vector transfer in recombination techniques. Except this; sterility, optimal temperature and humidity are required for the cultivation of bacteria. Anaerobic bacteria need a special incubator with CO_2 atmosphere, while aerobic bacteria need sufficient oxygen. Bacteria undergoing photosynthesis need enough light. The criterion for observing growth and the propagation of microorganisms population is the concentration of microorganisms in the culture. For the approximation of the amount of bacterial cells in a liquid cultivation medium a colorimetric measurement of absorption in the observable area of light (570-600 nm) is used.

During the isolation of nucleic acids and proteins from microorganisms, it is necessary to know the relationship between its production and the time duration of cultivation. The growth of bacterial cells is observed in certain time intervals. From the obtained data about the quantity of bacterial cells a growth curve is done.

9.3 Storing of biological material

Under storing we mean the short-term holdback of biological material (transport material or the daily accumulation of samples for examination) in portable and commonplace

(kitchen) coolers, in crushed ice or in crystallized CO_2 („dry ice"). The **backing up** of samples for analysis is necessary only in the case of repeating the analysis – material can be stored only several months in commonplace coolers (with temperature under -20° C). **Long-time storing** (archiving) of biological material is done either by deep freezing („deep freeze") in special freezers with temperatures under -80° C, or in liquid nitrogen (-196°C), especially for vital cells, which can be cultivated further. Another possibility is the archiving of fixed tissues in formalin or embedded in paraffin blocks (for retrospective analysis and the application of new diagnostic methods which were developing meanwhile).

9.4 Ways of protection of biological material against contamination

The specificity of DNA analysis requires a strict security against the contamination of individual samples by alien DNA (cells). For instance, the human DNA must not be contaminated neither by material from other human beings, nor bacterial or viral DNA. To fulfill this requirement, three major methods are used. **Disinfection** – the destruction of vegetative forms of bacteria in the air and on object surfaces; **sterilization** – destruction of all microorganisms from the environment and tools, either mechanically (filtration), physically (by heat and/or radiation) or chemically (dilutions) and **decontamination** – the elimination of microorganisms from the equipment (washing by detergents, irradiation). It is done regularly, especially in the case of infection of the environment where people work.

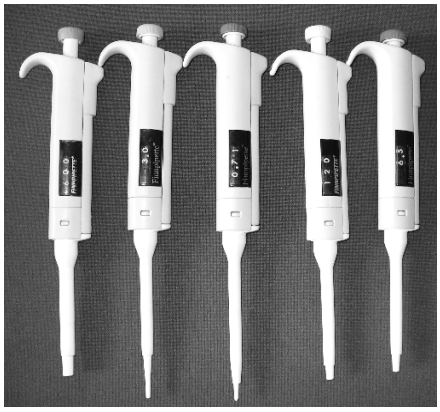
9.5 Apparatuses, tools and material used in a laboratory of molecular biology

9.5.1 Apparatuses

In laboratories of molecular biology the following devices are used:

- incubator – for cultivation of cells;
- laminar flow box ("biohazard") – for working with cells in a sterile environment;
- warm air sterilizer (oven) – for sterilization of metal objects and glass;
- autoclave – sterilization of solutions, objects from cotton, rubber and plastic;
- coolers, freezers – for the storage of material;
- centrifuges – devices for gravitational sedimentation of suspensions;
- magnetic mixers – for the preparation of solutions and gels;
- shakers – for mixing of solutions and suspensions;
- mixers, vortexes – for the homogenization of material;
- water baths – for maintaining the temperature of reaction compounds;
- analytical scales – for the determination of mass of a chemical (with different accuracy);
- spectrophotometer – for determining the amount of a substance (by measuring absorption);
- pH meter – for determining the pH of solutions and reaction compounds;
- device for electrophoresis – for the division of macromolecular compounds in gel (one-way electrical current);
- transilluminator – for the visualization of nucleic acids fragments position of in gel (by UV light);
- thermal cycler – for multiplication (amplification *in vitro*) of DNA by the PCR method;
- hybridization oven – for hybridization of DNA with probes;
- sequencer – for determining the order of bases in a DNA;
- real time PCR – for determining the dynamics of gene expression;
- devices for images documentation ;
- PCR box – for the preparation of reaction mixture for PCR (in non-standard conditions);
- device for preparing of de-ionized water.

9.5.2 Tools



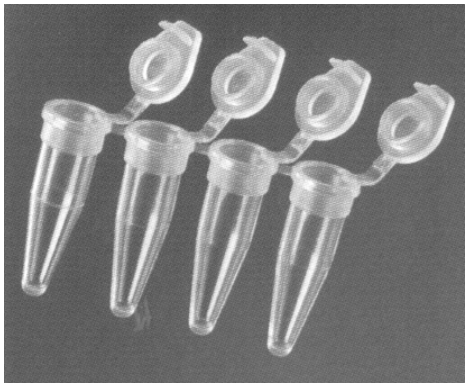
From special tools are most important micropipettes – for dosing of very small volumes of liquids and suspensions (up 0.1µl). They may be calibrated for one specific volume or may be adjusted (Fig. 67). Other tools are scissors, scalpels, pincers, and different kinds of stands for test tubes.

● **Figure 67.** Micropipettes

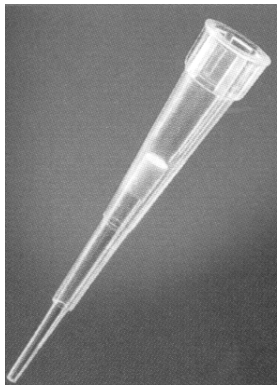
9.5.3 Material and helping tools

From material it is mostly:

- Test tubes (plastic and glass – of different volumes);
- Eppendorf tubes – plastic test tube with a lit (Fig. 68);
- Titration plates (with holes) – for working with small volumes;
- Tips for micropipettes of different volumes (Fig. 69);
- Laboratory glass (beakers, Erlenmayer flasks, cuvettes, flasks, measuring cylinders, pipettes etc.);
- membranes (nylon and nitrocellulose) for the transport of molecules from the gel to the membrane (blotting);
- freezer boxes;
- paper and cotton swabs;
- plastic film for closing of containers



● **Figure 68.** Eppendorf microtubes



● **Figure 69.** Tip for micropipettes

9.6 Processing of biological material

Before the DNA isolation, the tissue needs to be **homogenized** (disorganized, disintegrated). The most simple is the **mechanical** homogenization in a mortar. It is also possible to use homogenizers and different kinds of mixers. For **physical** homogenization are used: ultrasonication, hypotonisation, or repeated freezing and thawing of cells. A simple and efficient way is to use **chemical** homogenization, during which detergents are used (e.g. Triton X100), which by lowering the surface tension cause the rupturing of the cytoplasmatic membrane of cells, or enzymes (e.g. trypsin, pepsin, proteinase K), which predigest the membrane. Often a combination of these methods is used.

The most used method during further processing of biological material is **centrifugation** – the speeding up of solid particles sedimentation in a liquid environment, caused by increased gravitation induced by rotation. Particles sink to the bottom, depending on the properties of the substance (size, form, density) and the environment (density, viscosity). A part of centrifuges with higher rotations have a cooling system (protecting the biological material). A vacuum system is used mainly during ultracentrifugation, where it is necessary to prevent rubbing of the rotor to air and an unwanted increase in temperature – during high rotations. The space where the rotor is located is frozen, for the same reason. A rotor is a symmetrical entity made of metallic alloy, which turns around a central axis inside the centrifuge. It can be angular (for higher rotations) or swinging one. At a certain distance from the middle are located the centrifugal tubes containing samples. The tubes number is usually even and they are always placed into the opposite way positioned openings in the rotor. The necessary parameters (time, temperature, number of rounds/min.) are set on the control panel of the centrifuge.

9.7 Methods of the nucleic acids isolation

Most common sources of DNA to molecular-genetic examination are live cells – peripheral (venous) blood lymphocytes, buccal mucosa, hair roots, skin, chorionic villi, or bacteria. For analysis it is also possible to use degraded DNA, optionally a DNA obtained from old tissue fixed in formalin or from paraffin blocs.

Isolation of nucleic acids belongs to the most common techniques used in a laboratory of molecular biology. The prerequisite of a successful molecular analysis of a human DNA is the obtaining of a sufficiently clean highly molecular weight DNA. The main problem of nucleic acids isolation is the removal of proteins and polysaccharides so, that the primary and secondary structure of DNA or RNA remains unchanged. Reason is, that "free" nucleic acids do not exist in living organisms (except tRNA), because they are always in complexes with supporting and regulatory proteins. To suppress devastating activity of endonucleases, inhibitors (polyamines as spermidine and putrescine) and anticolaguants (e.g. EDTA) are used.

The classical methods of DNA isolation is **phenol-chloroform** extraction from cell lysate (sodium sulfate/proteinase K) and the **salting method**. The method for DNA isolation consists of the following steps:

- **lysis of cells –** by the disturbance of the cytoplasmic membrane (cell wall in bacteria) the content of the cell is loosened;
- **inhibition of nucleases –** by the removal of RNA with the helps of RNases (during RNA isolation DNases is used);
- **breakup of the nucleoprotein complex**– for the break up of the bonds between nucleic acids and proteins, organic reagents are used (phenol, chlorophorm, urea etc.);
- **the removal of proteins and precipitation of the DNA** – proteins are removed by centrifugation. DNA molecules remain in the solution, they are precipitated by salts and alcohol. DNA is "fished-out", left to dry, and dissolves in re-distilled water, – result is formation of so called DNA sample.

Currently – in molecular genetic are preferred kits for isolation DNA or RNA. The DNA isolation kit is a rapid and effective method for isolation of high molecular weight genomic DNA from bacteria, plant, yeast, blood cells and other mammalian cells and tissues of all types. The kit uses RNase mix to eliminate RNA immediately after lysis, and protease mix to rapidly degrade cellular proteins. This is followed by a proprietary salting out technique that precludes the need for phenol, chloroform or other organic extractions.

The concentration and purity of the isolated DNA are ascertained by electrophoresis or spectrophotometry. The principle of **electrophoretic** determination is that the well isolated high-molecular DNA is gathered in a gel on one place – close to the start. If disintegration

(„fracturing“) of DNA happen during its isolation, it will be manifested by the presence of more areas with DNA presence (straps or bands) in gel. **Spectrophotometry** determines concentrations of nucleic acids molecules containing nitrogen bases by absorbing of ultraviolet radiation of a wavelength 200-300 nm – the absorbance of DNA is measured at 260 nm. Absorbance of value 1 (in 260 nm) corresponds to the following concentrations:

- double stranded DNA = 50μg/ml;
- single stranded DNA= 40μg/ml;
- an RNA molecule = 40μg/ml;
- oligonucleotides = 20μg/ml

9.7.1 Principle of isolation of plasmid DNA

The main problem during the isolation of plasmid DNA is the removal of the bacterial chromosome. There are different methods used during their separation. The principle of the most common method is the removal of the DNA chromosome by increasing the pH (to pH=12). Chromosomal DNA of bacteria is denatured by high pH, while plasmid DNA remains native. During high concentrations of salts, the denatured chromosomal DNA precipitates from the solution. After centrifugation the plasmid DNA remains in the supernatant.

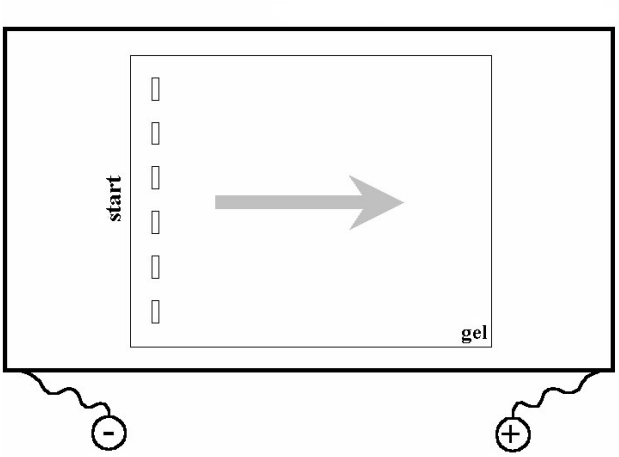
9.7.2 Principle of RNA isolation

RNases (enzymes that non-specifically cut the RNA, that is not protected against their action), considering the preservation of the information flow in cells, are present practically everywhere. During RNA isolation the protection against their effects is the most important condition. Besides sterility, also specific inhibitors of RNases are used (RNasin, diethylpyrocarbonate, vanadyl-dietylcarbonate, vanadyl-ribonucleoside complex, guanidine isothiocyanate, heparin and other). The most commonly used is RNasin, which is obtained from rat liver or human placenta. During the isolation of RNA the sample is ultracentrifugated in a density gradient of CsCl after the destruction of cell membranes. During the isolation of RNA, the sample undergoes centrifugation in a density CsCl gradient after the destruction of cell membranes. RNA forms a pellet, the DNA and proteins remain above the layer of CsCl (this principle is mainly used by commercial kits). Purified RNA is separated by Northern blotting or affinity chromatography and is used for example for the preparation of complementary DNA (cDNA) or for *in vitro* translation.

9.8 Gel Electrophoresis

Electrophoresis belongs to the most commonly used method for the preparation of nucleic acids and proteins, for further analysis. With the help of electrophoresis it is possible to separate mixtures of DNA fragments (and other polar macromolecules), which cannot be separated by other methods (for instance by centrifugation in a dense gradient). Electrophoresis is the movement of electrically charged particles (molecules) towards an electrode with an opposite polarity (charge) (Fig. 70, 71). For practical reasons electrophoresis is not conducted directly inside the solution, but in an adequate carrier. The most commonly used carriers are polyacrylamid or agerose gel, which forms a complicated network structure of polymeric molecules with pores. The size of the pores in the resulting gel can be influenced by the composition of the solution and the concentration of the polymer from which the gel is prepared.

The main carrier of the nucleic acids charge, which enables the movement of the DNA in a electric field, are the negatively charged phosphate groups, so the sample application hollows have to be on the side of the cathode and molecules will be pulled towards the anode.



● **Figure 70.** Scheme of electrophoresis

● **Figure 71.** Equipment for electrophoresis

The speed of the macromolecules movement (path that they undergo) in gel is called electrophoretic mobility. It depends on:

- **Molecular mass of molecule** (fragment). So like bigger a molecule is, so slower it moves in the gel. A estimate of the size of an unknown fragment is conducted by comparing it with a standard of a known size of molecules (fragments);
- **Concentration of gel** – determines the length of the molecules which can be separated in it;
- **Conformation** (spatial – three dimensional structure) of molecule. For instance RNA moves the fastest, circle DNA (e.g. plasmid) moves faster then linear double stranded DNA;
- **Size of the electric potential** – low voltage and highvoltage (above 1000 V);
- **Composition of liquids and temperature** – for electrophoresis in agarose gel the TRIS – borate solution (buffer) with a concentration of 50 mM, pH=7.5–7.8, under laboratory temperature is used most commonly.

DNA in gels is visualized by adding a fluorescent staining agent (ethidium bromide) to mixture, which integrates to a DNA molecule. In UV light with a wavelength of 254 nm on a transluminator (Fig. 72) electrophoretic separation of DNA fragments can be observed. It enables to see lightly orange signals (strands – bands), which represent the DNA with the integrated ethidium bromide.

● **Figure 72.** Transilluminator

9.9 Restriction endonucleases

Restriction endonucleases are enzymes, taking part in sc. restriction-modification system which inside bacterial cells ensures a species-specific protection mechanism against foreign genetic information (mostly bacteriophage DNA) entering the cell.

The restriction-modification system consists of two enzymes:

- **Modification enzyme** – methylase. The principle of modification is the postsynthetic methylation of a certain base. The modified DNA is resistant against cleavage by homologic restriction endonucleases. By modification the cell protects its DNA against cleavage by its own restriction enzymes. The most common method of modification in bacteria is methylation of adenine into sixth nitrogen – methyladenine or cytosine into fifth carbon – methylcytosine;
- **Restriction endonucleases** – specifically cleave the double stranded (linear and circular) DNA, because it recognises sequences of repeating nucleotides with mirror symmetry – the so called **palindromic sequences** (palindromes) (Tab. 7). Cleaving of DNA tends to different ends of molecule – one strand has overhanging end ("sticky") or both strands have equal ("blunt") end.

Microbial origin	restr. enzyme	sequence of recognition site
Artrobacter luteus	Alu I	AG | CT TC | GA
Bacillus amyloliquefaciens H	Bam H I	G|GATCC CCTAG | G
Escherichia coli R I 13	Eco R I	G|AATTC CTTAA | G
Moraxella boris	Mbo I	|GATC CTAG |

● **Table 7.** Commonly used restriction enzymes

After the restriction process, *in vivo* follows rapid degradation of DNA, done by other nucleases to products that are dissolved in acid. The most common and most widely used are enzymes of the II. type, which are important for the formation of recombinant DNA molecules, because they show only restriction enzyme activity. According to how the given restriction enzymes cut the double stranded DNA and which kind of ends it leaves, we divide them into three types:

- 5´ – sticky ends (for example. *Sau 3A I, Cla I, Eco R I*);
- 3´ – sticky ends (for example. *Hba I, PsT I*);
- Non-sticky ("blunt") ends (for example. *Alu I, Sva I, Pva II*).

Restriction endonucleases are used in recombination techniques as „enzyme scissors", by which they create new possibilities for the combination of genetic material. With their help a selected part of a DNA is inserted into a plasmid, a virus or other vector. It is important for the vector and the ends of an inserted (recombinant) foreign DNA to have an identical palindrome present. That is why both molecules are cut by the same restriction enzyme. Endonucleases are used, which form complementary sticky ends inside the DNA. This two molecules hybridize (join) by the sticky ends and ultimately DNA ligase form phosphodiester bonds between vector and inserted (recombinant) DNA. By the vector the insert (recombinant DNA) is transported into the target cell – for DNA replication *in vitro* or biotechnological

utilization. Plasmids containing insert multiplies in the bacterial in multitude of copies (sc. cloning of DNA *in vitro*). Later are bacteria homogenized, and the plasmid DNA is extracted from them. Recombinant DNA is cleaved-out from plasmids by endonuclease, which cleave DNA onto the non-sticky (blunt) ends and it is separated from plasmid DNA in gel – for further use. For biotechnological utilization – conditions are created inside the bacteria, for expression of the gene inserted into the vector and for the cell to start synthesis of the coded protein. The first application of this method was the production of human hormone insulin, by the cells of the bacteria *E. coli.* At present, this method is used for the production of many important proteins, like interleukines, interferones, serum proteins, hormones and recombinant vaccines.

Other usage for the restriction enzymes is in gene therapy. It focuses mainly on tumors and bone marrow cells – the aim is solve the problems on level of DNA – inside the patient's cells. The first successful gene therapy was done on children with severe combined immunodeficiency. By recombinant techniques, with the help of restriction enzymes, the gene able to synthesize the enzyme adenosine deaminase (missing in patient), was incorporated into the genome of retrovirus (RNA-virus). Retroviruses contain reverse transcriptase, an enzyme which can rewrite single strand RNA genome of virus to the double strand complementary DNA. This DNA is then integrated into the hosts chromosomal DNA as sc. provirus. Long living stem cells of patient are washed out of the bone marrow into the blood. Genetically modified viruses and stem cells isolated from the sampled blood are mix together. Viruses carrying the new gene enter the stem cells and insert their provirus in cells genome. By this technique, the stem cells "gained" the ability to synthesize the missing enzyme and they are injected back into the patient to support his immune system. Despite the apparent simplicity, this method contains three groups of problems – the risk of virus genome properties modification, randomness of the provirus containing „therapeutic gene" place of insertion, and also the risk of a malign transformation of the cell.

9.10 DNA-DNA hybridization *(polymorphism)*

DNA-DNA hybridization is classical laboratory method that allows identification of particular segment of DNA (order of nitrogen bases in it) presence in examined DNA. The principle is hybridization – association of nucleic acids strands on the base of complementarity – of the examined DNA (e.g. fragment) and radioactively labeled probe. Probe is single strand of RNA or DNA of known sequence of nitrogen bases. It is mostly used in detection of particular nitrogen bases motifs in the polymorphism of the length of the restriction fragments. Methodological approach allows the finding of the DNA fragment containing motifs, complementary to (labeled) hybridization probe.

There are two main types of DNA polymorphisms (differences in nitrogen bases content or order), that could be examined by DNA-DNA hybridization:

- **point polymorphism** – the exchange of one or more bases. If the mutation creates or destroys a palindrome, it could be detected by the method of – **restriction fragment length polymorphism** (**RFLP**)**;**
- **hypervariable regions** of DNA – they are detected by the **variable number of tandem repeats** (**VNTR**) method. Hypervariable ("minisatellite") regions are segments of DNA, where is present variable number of certain nitrogen bases motifs repetitions – following each other as tandem, i.e. without interruptions. Many types of them are known in human genome – they are called "locuses of polymorphism". Interpersonal variability is in different number of a given sequential motif repetitions, expressed in length of DNA segment. Examination of VNTR requires knowing of sequence of bases that are closely before and after examined VNTR segment. For classical VNTR method it is necessary, that neighboring sequences contain palindromes – so it is possible to "cut-out" segment of repetitions by endonucleases. If even one of palindromes is missing – the copying of this

part is done by the PCR method, which is cheaper – only to buy primers complementary to sequences before and after repetitions, is needed.

9.11 Restriction Fragment Length Polymorphism (RFLP)

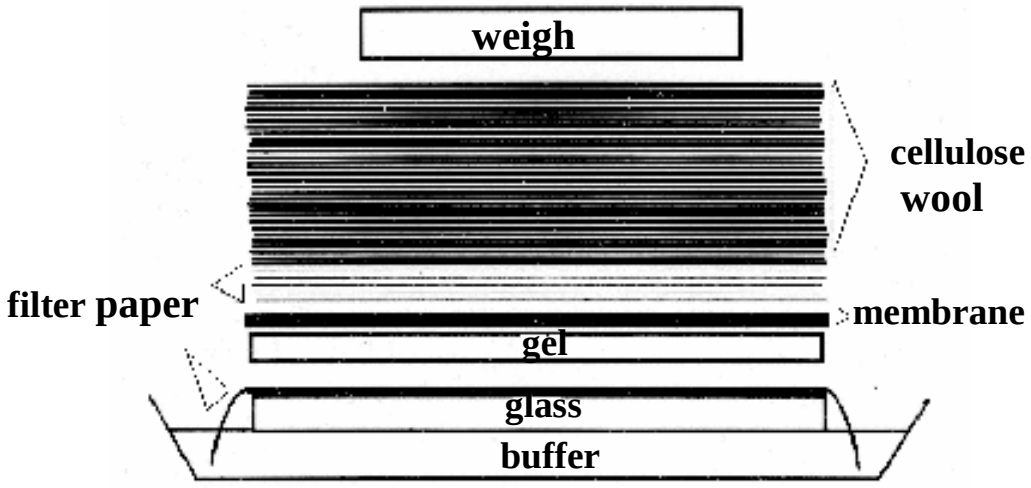
The principle of method is, that mutation in DNA destroys or creates palindrome. Consequence is, that particular endonuclease could not cleave mutated palindrome – or another one can cleave new palindrome, created by mutation. Result is different length of obtained restriction fragments in normal person and mutation owner. Genomic DNA is digested **by one** restriction endonuclease, by which we obtain a big amount (hundreds of thousands) of fragments of different lengths. When DNA sample is in advance prepared (e.g. cutted-out from whole genome) – number of fragments is lower. Length of fragment is found out by help of sc. "length marker" (beforehand prepared DNA fragments of known length) applied in the some gel.

A fragment doesn't need to have the same length in each member of the population. On the contrary, quite often we meet with variability of the length of the defined restriction fragments in the population, what is used during the identification of persons and fatherthood justice.

Double stranded DNA fragments are separated by electrophoresis in agarose gel and denatured. The term **denaturation** of DNA means the detachment of complementary DNA strands by interruption of the hydrogen bonds between nitrogen bases. The unwinding of the double stranded DNA forms two single-stranded molecules. In gel is denaturation done by solution with alkaline pH (close to 12). Alternative method of denaturation by warming the DNA solution to 80° - 100° C cannot be used if the DNA is in the gel, because the heating will destroy it.

During the cooling-down of the solution containing the denatured DNA, **renaturation** occurs. It means the self-renewal of the hydrogen bonds between the strands, which is used in the hybridization with the probe – in dry condition on hybridization membrane.

Single-stranded fragments of the DNA are transferred onto the hybridizing nitrocellulose or nylon membrane by the so called **Southern Blotting (transfer)** (Fig. 73). Principle of this simple method is rising of fragments through capillary attraction – in buffer solution – from gel to membrane. The membrane has microscopic openings that allow buffer to pass-trough (into cellulose wool) and "washed-out fragments stay on membrane. Similar methods are used for extraction of polar macromolecules from gel: Northern blotting – for RNA and Western blotting for proteins.



● **Figure 73.** The configuration of the apparatus used for the blotting of fragments of DNA from the gel to the membrane

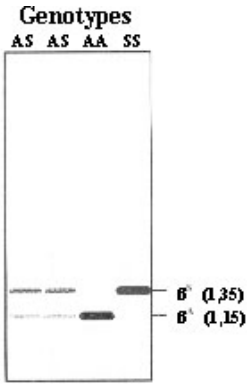
After finishing blotting we need to prepare the probe. The probe is a single-stranded, radioactively or chemically labeled DNA (in some case a RNA) with a known sequence of nitrogen bases. During radioactive labeling nucleotides which contain a radioactive isotope, usually ^{32}P or ^{35}S, are integrated into the sequence of the probe. During non-radioactive labeling (e.g. for FISH), chemically modified nucleotides are added into the probe. For this purpose the molecules of biotin and digoxigenine are often used, which are incorporated into the probe instead of thymine. Fragments attached to the membrane are hybridized with denatured radioactively labeled probe.

The visualization of the presence and location (length) of the analyzed fragments is done autoradiography probe exposes photographic emulsion (e.g. X-ray film).

An example of the usage of RFLP method in single nucleotide polymorphism is the diagnostics of sickle cell anemia. In this disease one of the 146 amino acids is exchanged inside the beta-globin strand of hemoglobin. In an effected individual the glutamic acid in the 6^{th} position is replaced by valine. The cause is the substitution mutation – substitution of adenine to thymine:

A = normal sequence:	CCT G**A**G GAG
coded aminoacids:	Pro **Glu** Glu
S = mutated sequence:	CCT G**T**G GAG
coded aminoacids:	Pro **Val** Glu

The restriction enzyme *Mst II* detects and cleaves the target sequence CCT GAG GAG. If it is not damaged, cleaving happens and the fragment (A) has a length of 1.35 kb. Mutated fragment (S) is cleaved in another position (palindrome) and has length 1.35 kb, because in cells of sickle cell anemia the cleaving position shifts. The detection of fragments is done by electrophoresis and obtained fragment are compared with a length marker. The dominant homozygous (AA) persons have all the fragments on one place – in the 1.15 kb position. The recessive homozygous (SS) persons also have all the fragments on one place, but in the 1.35kb position. Heterozygous (AS) ones have two types of fragments – 1.15kb and also 1.35kb (Fig. 74).



● **Figure 74.** Single nucleotide polymorphism in sickle cell anemia. It is possible to distinguish persons with homozygous SS and AA (they only have one strand) and heterozygous (they have two strands) genotype.

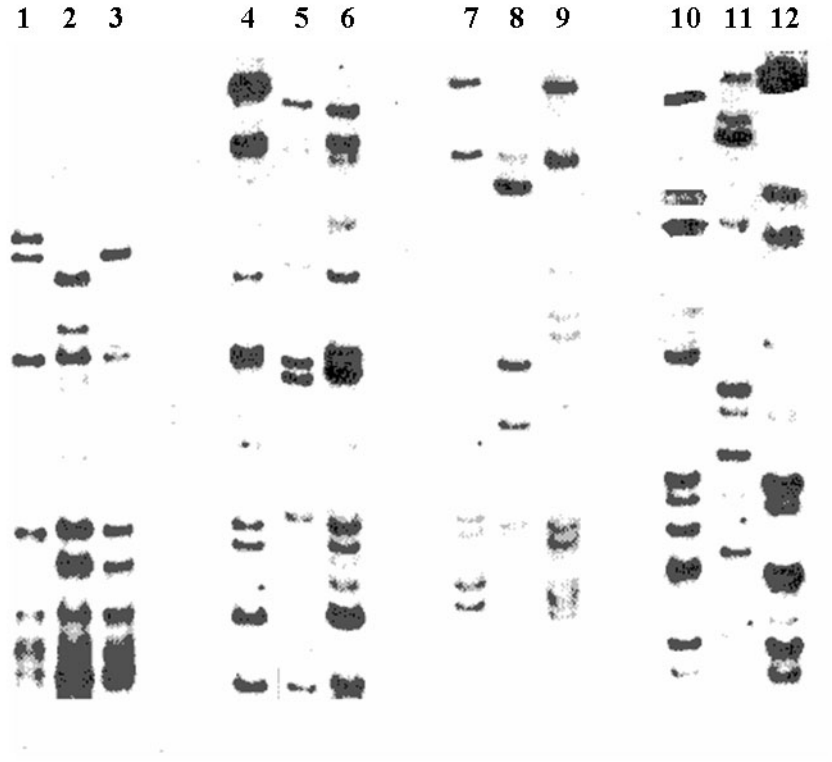
9.12 Method of DNA Fingerprinting

It is based on the examination of a large number of hyper-variable parts of sc. minisatellite DNA. This kind of DNA is composed of short motif – from 3 to 10 nucleotides, that are repeated in, in different number of tandem copies (sc. STR – short tandem repeats). In the human genome there are dozens of such regions there, and each of them, because of the variability in the number of motif repeats, has in different persons different lengths. Each of these STRs can be cleaved by a proper restriction enzyme. Obtained fragments are separated in gel, by Southern blotting brought to the hybridization membrane and then identify by hybridization with a labeled probe detecting different motifs of STRs.

The principle is the same as in the RFLP method, the difference is that by this method you can simultaneously detect several polymorphisms. The autoradiographic image is made up of several strands and is therefore individually more specific then the prints of papillary lines of the fingers. This was probably the reason why A. Jeffreys, who developed this

method, called it DNA fingerprinting. Besides the identification of individuals (in forensic practice), this method is also used in fatherhood justice (Fig. 75).

Currently is identification of person done by 16 with PCR obtained polymorphisms gained with 3-4 fluorescently labeled primers and is analyzed (in regard to color and size) in genetic analyzer.



● **Figure 75.** Identification of twelve subjects by the fingerprinting method

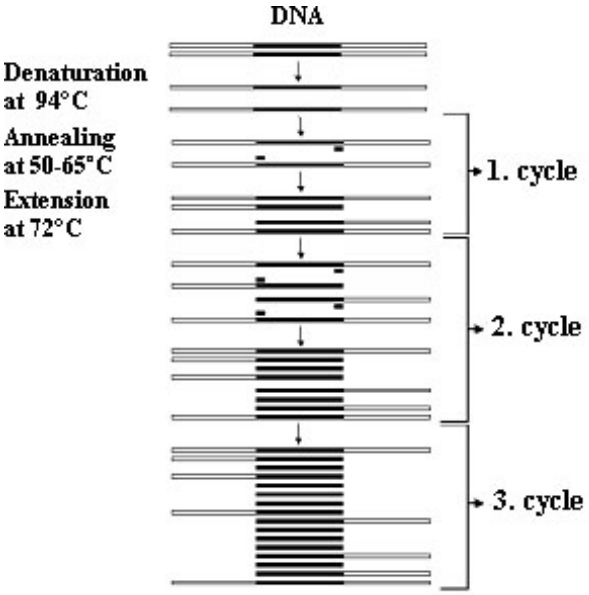
9.13 Polymerase Chain Reaction (PCR)

PCR (polymerase chain reaction) is a method which allows the *in vitro* enzymatic synthesis of specified DNA segments, of defined length and border. It results to multiplication (amplification) of replicated DNA sequence. Nowadays it is one of the most significant and perspective method in molecular genetics. The using of the PCR in practice is very broad.

PCR reaction is simulation of the DNA replication process *in vitro*. Here does not take place the synthesis of the entire DNA molecule, but only of its small defined part - with length to 23kb. For demarcation of replicated part, two short (oligonucleotide) chains of DNA – primers – are required. On the base of complementarity with a target sequence, primers hybridize with complementary sequences (sc. primer binding sites – PBSs) denatured single strands of target DNA, which enables the start of replication. The attached primers are detected by *Taq* DNA polymerase, which – under rules of complementarity – synthesize the complementary strand, until the end of the single strand.

The essential condition for PCR is that the sequence of bases on both ends of the amplified DNA (PBSs) must be known – so it is possible pointed use of proper primers. It is important, that the complete sequence of the inner part of replicated DNA (i.e. between PBSs) doesn't need to be known. For instance, in case of degraded DNA it is enough if only one of the two DNA strands is complete.

The process of DNA amplification by PCR works on a base of exact changes of temperature in the environment of reaction mixture. PCR method consists of repetition of cycles, of which each of them has three steps: denaturation, hybridization of primers into single-stranded DNA (annealing), and the synthesis of complementary DNA strands.



In each cycle the amount of the amplified segment of DNA doubles. The growing number of repetitions leads to an exponential multiplication of the amplified sequence.

In practice, by 25-35 cycles of repetitions it is possible to achieve a 10^4 – 10^6 fold amplification (multiplication) of a specified sequence (Fig. 76).

● **Figure 76.** PCR scheme

Since its discovery, the PCR method underwent many innovations. In the beginning, the Klenow fragment of DNA polymerase I from *E. coli* was used for the synthesis of the DNA strands. Its optimal working temperature is 37° C, so, in each step of denaturation it was inactivated and was necessary to supply it again, for each new cycle. For ensuring of the temperature changes during the each step, three water baths were used with different temperature and the tubes containing reaction mixture had to be replaced after each step. It was time consuming and it didn't allow following an exact time and temperature regime. The development of the **thermal cycler**, neither quickened and simplified the whole process, nor increase accuracy of procedure – since it allowed an automatic adjustment of temperature and followed an exact time and temperature regime necessary for a successful amplification (Fig. 77).

● **Figure 77.** Thermal cycler

Another important invention was the thermostable DNA polymerase, which remains active even during DNA denaturation temperature. Therefore it wasn't necessary to add polymerase during each step. Thermostable DNA polymerase – *Taq* polymerase – is isolated from the thermostable bacteria *Termophillus aquaticus*, living in sulphur hot springs at the Yellowstone National Park (U.S.A). *Taq* polymerase has an optimal working temperature 70 – 75° C. If lasting short, it can endure even higher temperatures, meaning it can successfully overcome the DNA denaturation temperature.

Before starting PCR reaction it is necessary to prepare the reaction mixture. The final volume of the reaction mixture for one amplification is 10 – 100 µl, depending on the further processing of the amplified DNA.

The composition of the reaction mixture – template DNA, pair (couple) of PCR primers, dNTPs (deoxyribonucleic triphosphates), *Taq* polymerase, $MgCl_2$, buffer, deionised water.

The PCR reaction takes place in microtubes with a volume of 0.5 –1ml.

As a DNA template for a PCR reaction, it is possible to use single or two stranded DNA. It can also be a whole genomic DNA or fragments of degraded DNA (isolated drops of blood, hair, pieces of skin, sperms etc.). Theoretically one copy of a given sequence is enough

for undergoing the amplification. The optimal quantity of template DNA for a successful amplification is 50-1000 ng, depending on the volume of the reaction compound and the primers used.

Oligonucleotide primers are artificially synthesized one-stranded DNA sections with an approximate length of 20 nucleotides, which are complementary to the 3' ends of both replicated strands of the DNA. The primers determine a DNA sequence which we want to amplify, and serve as starting points for the *Taq* polymerase. They are chosen in a way, to ensure a specific hybridization of primer with a unique sequence in the genome – not to bind on to multiple places. It is also necessary to choose such sequence of primers bases, which prevents their mutual pairing and the formation of primer dimers. To fulfill all mentioned conditions for successful amplification, the accurate primers are chosen in computer databases of human genome.

dNTP (deoxyribonukleotid triphosphates) are used in equivalent concentrations of each kind of dNTP (d**A**TP, d**G**TP, d**C**TP, d**T**TP) per one reaction.

In the PCR reaction two kinds of ***Taq* polymerase** are used. It is its native form from *T. Aquaticus* or a recombinant enzyme synthesized by *E. coli*. The amount of *Taq* polymerase per one reaction depends on the volume of the reaction mixture. It synthesizes complementary DNA at a speed of 100 – 150 nucleotides per second.

For an optimal course of the PCR reaction a proper quantity of $\mathbf{MgCl_2}$ is required. The concentration of $MgCl_2$ strongly influences the hybridization of the template DNA with primers, the denaturation temperature, the specificity of products, the formation of dimers and also the *Taq* polymerase activity. *Taq* polymerase requires free Mg^{2+} cations for its functioning.

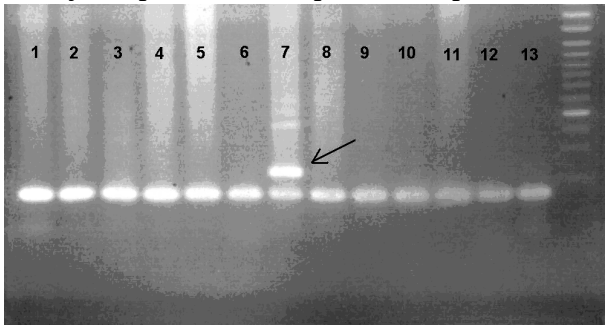
The standard and recommended buffer solution for the *Taq* polymerase is used. The role of the buffer is to increase the activity of the polymerase. That is why its constitution depends on the used polymerase and is usually supplied by the manufacturer together with the polymerase.

On the one hand the PCR reaction is characterized by a high sensitivity and simplicity, on the other hand it requires a very precise manipulation with the different samples. Since the PCR reaction is able to amplify a certain DNA section on the bases of only one template molecule, it is necessary to prevent their contamination during working with them, which could lead to a non-specific amplification.

The temperature and time profile of the PCR reaction depends on the length and sequence of the primers, the amount of template DNA, the length of the amplified section, the polymerase used and also from the type of the thermal cycler.

The PCR takes place in three steps:

- **DNA denaturation** takes place under the temperature of 94° - 96° C and lasts between 30 seconds and 2 minutes.
- **hybridization with primers** (annealing) – primers connect to the single-stranded DNA sections on the principle of complementarity. Hybridization takes place under the temperature 55°C - 65°C, lasting between 30 seconds and 1 minute. The temperature and length of the step depends on the sequence, length and concentration of primers;
- **polymerization** (extension) – The synthesis of the complementary DNA strand, bounded by the primer. The optimal temperature for this step – activity of *Taq*-polymerase is 72°C.



The amplification of the DNA segment is ascertained by electrophoresis in agarose gel or polyacrymalide gel. Visualisation is done by ethidium bromide (Fig. 78).

● **Figure 78.** Photo of gel with DNA 13 fragments amplified by the PCR method

Their size is determined according to the length marker on the right edge of the gel. In the 7th path there is an analyzed mutation, there.

Amplified DNA fragments can by utilized for further analysis, e.g. cleaved by restriction endonucleases which and determine their restriction profile, potentially it can be hybridized by oligonucleotide probes or directly sequenced.

PCR has the following advantages:

- **short time span** of the whole process (around 3 hours), which has a significant meaning, e.g. for prenatal genetic diagnostics.;
- **high sensitivity –** e.g. the detection of a single cell infected by a virus;
- **it requires only a small amount of DNA** – (usually quantities in nanograms), this allows e.g. pre-implantation diagnostics from a single blastomere, DNA diagnostics from hair onions, from single sperm or somatic cell.
- **it also allowed to amplify old and degraded DNA** – e.g. from chromosomal slides, paraffin blocks of tissues, frozen tissues.

Negative consequences of PCR are:

- The sequence of bases for annealing of the primers needs to be known;
- Only relatively short sections can be multiplied – up to 23 kb;
- The contamination (by only single alien DNA molecule) causes a false result of the reaction.

9.13.1 Modifications of PCR method

Reverse PCR (RT–PCR) – assigned for the amplification of RNA molecules, in most cases mRNA. The first step is the isolation of the whole RNA (or particular mRNA), which reverse transcriptase rewrite into the complementary DNA (cDNA), which is consequently amplified by the standard PCR method.

Nested PCR – is used for the amplification of a micro-amount of DNA. The principle are two, consequential amplification reactions. The PCR product of the first reaction is longer and serves as a template for the second reaction, while the used primers are complementary to the sequences inside the first product – in the second reaction only certain part of product of the first PCR reaction is amplified.

Multiplex PCR – in one reaction several primers are used, which allows the amplification of more sequences at the same time. This method is used for the multiplication of several exons of the same gene or for the analysis of DNA polymorphisms.

In situ PCR – is a method which allows the amplification of specific sequences of nucleic acids directly in cells and tissues (cytologic and histologic slides) and in chromosomes (mitotic figures on slides). This method is however far less sensitive then the standard PCR. The main advantage of this method is that it is not necessary to isolate the DNA and it is possible to directly localize the PCR product.

Quantitative PCR – the principle is the determination of the amount of template on the basis of the PCR product. Quantification can be done after the electrophoretic separation of the PCR product in the gel or without the use of electrophoresis. Radioactive and non-radioactive labeling of the PCR product are used, the product is detected with a particular method (autoradiography, the measurement of radioactivity on scintillation computer, by luminescence, fluorescence, etc.). Absolute and relative quantification is used. During **relative quantification** several PCR products are compared between many samples, and to the standard. During **absolute quantification** the result is an amount of DNA sequence (in number of molecules or mass units). By this method it is possible to identify the number of mRNA molecules of an analyzed gene in a cell.

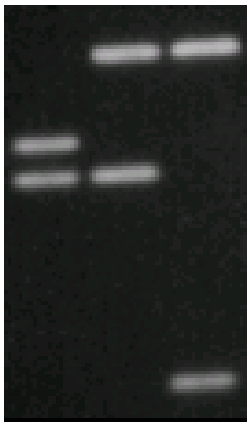
9.13.2 The utilization of the PCR method

PCR is used in many areas:

- **in research** – for example in genetic manipulation (the preparation of recombinant DNA molecules from mRNA for their expression in bacteria), construction and multiplication of probes, the determination of the presence of chosen sequences of DNA including the study of the human genome etc.);
- **in medical genetics** – preimplantational, prenatal and postnatal diagnostics (e.g. of Duchenne muscular dystrophy, fragile chromosome X, hemophilia, thalassemia, cystic fibrosis, phenylketonuria and many more enzymopathies and syndromes). PCR usually serves as the tool for multiplication of an analyzed section, which is than sequenced;
- **in diagnostics of different types of diseases** – mainly cancerous, but also for example infectious caused by viruses, bacteria, fungi and parasites (e.g. chlamydiosis, toxomplasmosis, malaria etc.). With success the PCR is used in the screening (mass searching) of individuals with a genetic predisposition to certain, mostly cancerous, diseases (for instance cancers of breast, prostate, large intestine, ovaries, urinary bladder etc.);
- **in immunology** – for instance in the detection of HLA polymorphism during the finding of an adequate donor and recipient for transplantation;
- **in forensic medicine** – e.g. in the identification of an individual and paternity justice;
- **in breeding** research – e.g. protection of genetic pools of plants and animals;
- **in the food industry** – the detection of genetically modified organisms.

9.14 Variable number of tandem repeats (VNTR)

An example of the PCR usage is the examination of hypervariable areas (e.g. VNTR) for the determination of paternity during paternity justice (Fig. 79).



Hypervariable areas of the DNA are characterized, as mentioned earlier, by a significant variability of their length, which is a result of a various number of tandem repeats of a certain motif. The type and length of a DNA sequence is marked as an „allele“of a given VNTR system. The alleles are inherited from parents to offspring (one allele from the mother and one from the father). Considering the great variability between the members of the population, there is a minimal probability there, that two individuals coincide in bigger amount of VNTR systems (in alleles) at one time.

● **Figure 79.** VNTR – determining paternity – mother (1), child (2) and father (3). Paternity is considered to be excluded, only if the child differs from the father in at least two independent VNTR systems (alleles).

Individual alleles of the mother, child and the concerned man are multiplied with the help of PCR and are then separated by electrophoresis. Visualization is done directly in the gel – in the transilluminator. The length of the different alleles is determined by the comparison of the PCR products with a length marker. The most frequently used VNTR polymorphisms are (Tab. 8)

The name of the VNTR system	Localized on the chromosome	The neighboring gene codes for	Number of alleles in the population
Apo B	2	Apolipoprotein B	15
Col 2A	12	Colagen type II	8
PAH	12	Phenylalanine hydroxylase	8
YNZ 22	17	D17S30	13
MCT 118	1	D1S80	13

● **Table 8.** Use VNTR systems

9.15 Fluorescent in situ hybridization (FISH)

The FISH method is the connection of molecular genetics and cytogenetics. Its principle is the complementary binding of the fluorescently labeled probe to a target DNA section in chromosomes in intact (not homogenized) material (in an interphase nucleus, or chromosomes in mitosis). In the standard method of in situ hybridization (ISH), probes labeled by radioactive isotopes are used. During FISH fluorochromes are used for the visualization (UV light in fluorescent microscope) of the probes binding. These fluorochromes have a specific affinity to intercalation compounds - for example their avidin constituent to biotin). ISH is used mainly during the analysis of fixed archived material (for example from paraffin blocks). FISH also proved useful for the examination of fresh material. The main advantage of the FISH is not only working without the need of contact with radioactive material, but it also allows the use of in color varying probes of different lengths, at the same time. Besides broadening the spectrum of analysis, the expensive and time consuming necessity of cultivation of cells is omitted, which makes the process of getting to a result – by FISH – quicker.

9.15.1 Types of probes for FISH

Centromeric (satellite) probes – they hybridize with areas of satellite sequences near the centromeres, which are unique in each chromosome. They are used mainly in prenatal and preimplantational diagnostics, for instance in determining the number of a certain chromosome (in aneuploidia there is one, three or more signals instead of two) and the origin of a certain chromosome.

Gene (locus specific – minilocus) probes – they hybridize with unique sequences of DNA. They are used mainly for determining the amplification of alleles of a certain gene (for instance oncogenes), finding of translocations and micro-deletions (for example with malignant and microdeletion syndromes).

Whole chromosomal (painting) – probes hybridize with the whole (specific) chromosome or with its important part. They are used for example in diagnostics of chromosomal remodeling and during the diagnostics of aneuploidies – in mitotic figures. In 1996 a special method called „multicolor FISH“ was developed, which allows coloring each pair of chromosomes with a different color.

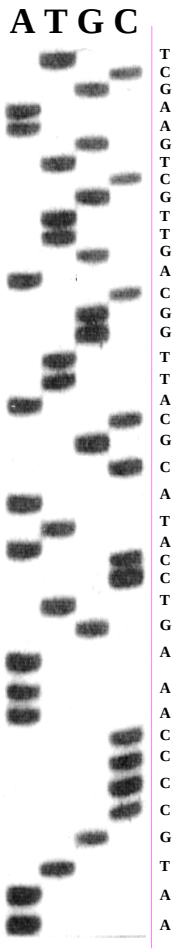
9.15.2 Methodical approach of FISH

The protocol of FISH consists of the following steps:

- the preparation of target DNA (nucleus or chromosomes in mitosis) on a silanized template slide;
- denaturation of target DNA (with alkaline pH);
- denaturation of probe (by heat – in a test tube);

- hybridization of target DNA with a probe (at least 6 hours – under a given temperature), the probe is labeled by avidine, which is bonded by fluorochrome. After the implication of the probe, the slide is covered by a glass and is placed into a humid chamber;
- the clearance of a non-specifically bonded probe (with the help of formamide, under the temperature of 72°C) from places where the probe is not completely complementary bonded;
- addition of antibody against avidin;
- the observation of the slide in a fluorescent microscope – the number of colored signals in 100 nuclei or mitotic figures are recorded.

9.16 DNA sequencing



DNA sequencing is of a collection of methods which allow to recognize the exact order of bases, as they consecutively follow each other in the DNA. Sequencing arises from two standard methods which are independent from each other – Sanger and Maxam-Gilbert. The principle of classical **Sanger method** (1975) is in utilization of 2´–3´**dideoxyribonucleotidetriphosphates** (ddNTP) that – after loading into newly synthesized DNA chain – terminates further synthesis of the strand. This is because they have instead of -OH group in ddNTP pentose third carbon only -H and so are disable to make phosphodiesteric bond with next dNTP.

Classical procedure protocol stars with equal division of sequenced DNA sample into four test-tubes.

In first tube it is placed normal 3´ deoxyribonucleotidetriphosphates (dNTP) of each kind (A, T, ´G, C), while thymine is radioactively labeled. To these tube is also added ddNTP with adenine. Composition of compounds in second tube is equal, but ddNTP is with thymine. In third tube it is guanine and in fourth is cytosine. In each tube DNA polymerase is added and synthesize new DNA (complementary) strand – while randomly using dNTP and ddNTP. After using ddNTP, the further synthesis of the strand is stopped, since the phosphodiester bond between dNTP and ddNTP can't be formed. By this a series of fragments of different length are formed in each test-tube. Their size depends on their distance from the beginning of the chain, in which the ddNTP was loading. The samples from the test-tubes are deposited (each in independent path) into the polyacrylamide gel and are separated by electrophoresis. After autoradiography the order of bases is subtracted in the sequenced sector (gradually – in each column there is only one band in the row) – Fig. 80.

● **Figure 80.** The reading of the autoradiogram during DNA sequencing

At present the Sanger method is used in diagnostics, in connection with the PCR. Used ddNTP are labeled by fluorescent dye and results of amplification are separated by capillary electrophoresis. So like they pass through „reader"a laser beam evokes in the individual ddNTP different color signals. Signals are recorded.

Maxam-Gilbert method (1977) – the sample of sequenced DNA is evenly divided into four test-tubes. DNA is denatured and the single-stranded molecule is then labeled by a radioactive phosphorus. In each test-tube only one type of the nitrogen base is damaged chemically (by methylation). The conditions of the reaction are constructed so they are not entirely destroyed, only their small part. The damaged bases are acted upon by piperidine, which disrupts the DNA chain where they are located. Many fragments arise which have

different sizes, depending on how far was the damaged base located from the end of the molecule. Electrophoresis, autoradiography, and the evaluation are all done in the same way as in the Sanger method.

Presently there is a wide range of application of these methods – ranging from PCR with ddNTP to automatic sequencers, which helped to quicker the pace on the „human genome “project. Most recently a new method is used, called second generation sequencing.

9.17 Biochips

The introduction of biochips in the molecular-genetic laboratory was a revolution. The name came about because the technology used during their manufacture is the same as with computer chips.

Their predecessors are the commercially accessible membranes, which had pre-applied probes on their surface, on which the hybridization with an analyzed DNA took place. On one membrane with the dimensions of 22 x 22 cm there were 36 864 DNA probes, which could detect 18 394 genes. With the coming of the biochips, the membrane was substituted by a silanized glass plate with a size of a post stamp. It can be attached by more then 100 000 different probes and it can identify many different parameters during one examination – for instance all the known polymorphisms and mutations of a certain gene. It is not only used in diagnostics (for example the genetic predisposition to tumours of the mammary gland), in farmacogenetics, but also for determining phylogenetical consequences.

At present automatic biochip machines can separate lymphocytes from a sample of peripheral blood, isolate their DNA, multiply the requested section, and compare it to the probe and evaluate the result.

10. Introduction to the genetics of viruses

Viruses are the most simple forms of life on earth. They are classified as acellular organisms. For their reproduction they need a living host cell (organism). The name virus was formed as the description of an originator of an infectious (transferable) disease which could not be detected by a light microscope. A virion is a particle whose main component is nucleoprotein. Nucleic acid of a nucleoprotein can either be DNA or RNA (never both at once).

A **virion** is a single organism of a virus. The relationship between virion – virus is the same as a single human –mankind. The virion (basically) consists of a protein capsule (capsid) which contains a nucleic acid (genome of the virus).

The shape and reproductive properties of the virus is influenced by the proteins, from which the virion is formed. Proteins, which form the virion, are coded by virus genes. Viral proteins are of two types – the ones which form the subunits of the capsid (capsomeres) and the ones which are located under the capsid (sc. core proteins).

A **capsid** is a protein layer, which surrounds the nucleic acid. A capsid of a virus is made up of two types of proteins, which have two main functions:

- They enable a specific connection of the virion to the membrane (receptors) of the target host cell and by this the start of the life (reproduction) cycle of the virus. They are named as ligands (antireceptors – antigenes). In most cases it concerns glycoproteins and are distinguished according to their molecular weight. They are specific for the individual virions (viruses). In laboratories they are used for determining the originator of the disease;
- for the imune system of the host, the structures which project out from the capsid represent antigenes which it can recognize. It has a large importance for the formation of specific antibodies against them, and so for the survival of target cells and the host.

In certain virions the capsid contain, besides the genomic nucleic acids, different proteins (core proteins), which fulfill specific functions. An example is the human immunodeficiency virus (HIV). Besides different stabilizing and functional (regulatory) proteins, it contains three enzymes (reverse transcriptase, integrase and protease) which are also important for its reproduction cycle.

Capsomeres determine the shape of the virion and its properties. In most cases the capsomeres assemble into a polyhedral crystal which slightly resembles a globe (Fig. 80). Virions can also have two bizarre shapes, either a hollow spiral shape of a mosaic disease of plants or the typical shape of a bacteriophage. In an infected cell the capsomeres accumulate in a predetermined locality (**packaging site**), were the viruses are „assembled“ and which is typical for different kinds of viruses, since it largely relates to the way in which the virions are released from the infected cell.

● **Figure 80.** An example of a virion capsid

Virions leaving the host cells by exocytosis (budding), take the remains of the cytoplasmic membrane of the host cell on the surface of the capsid. This **lipoid covering**, simplifies the process of viral entrance into the host cells and partly protects the virus against the immunity system of the host.

Between the virus and the host cell there is a very narrow and specific relationship, the so called **obligatory cellular biotropism**. This means that only specific virions are able to enter particular type of cells. Except this, there is also an **interspecies barrier**, an occurrence as in the case of the measles virus, which only infects humans and not animals. The transcendence of the interspecies barrier often has dire epidemiologic consequences – for example the crossing of the Simian immunodeficiency virus (SIV) to humans (HIV).

The simple construction of the virions is the cause why they are very resistant. At the same time, unless the viruses are located inside the host cell, they don't show any life manifestations. This has two serious consequences:

- the destruction of viruses is very difficult and the common disinfection methods are uneffective. Virions are able to last out in minimal conditions practically for a limitless amount of time. This makes their spreading easier;
- The methods of virus spreading to the hosts are different with each type. However the predominant method of transfer is by droplets or direct contact. However we also know bizarre methods, as for example the spreading by a vector (mosquito) or an infected white blood cell (with HIV).

The appearance of the disease depends on the **virulence** of the virus and the conditions of its transfer. Virulence is the particular pathogenicity (disease-producing) of the organism causing the infectious disease to a particular host (strain). According to the quantity of sick individuals (morbidity) **epidemias** and **pandemias** are distinguish. During an epidemy the number of infected individuals is limited (countries or regions). Sometimes a focal presence occurs, which is usually related to the life conditions of the vectors – e.g. tick borne encephalitis. During a pandemy, large areas are effected, often whole continents (as with the Spanish flu).

10.1 Types of virus reproduction

The infection of a cell starts by the virus binding to the receptor proteins on the surface of the cell with the help of the glycoproteins protruding from the capsid (ligands). The virus can't infect the cell, if the receptors are not present, or if (as an effect of mutation) they are somehow changed or the ligand is changed (also after the mutation of a gene which codes it) in a way, that it cannot bond to the receptor. After the binding of the virion to the receptor, its nucleic acid (in bacteriophages) enters the cell or the whole virion (or its "nucleus"), from which the nucleic acid separates inside the cell.

The nucleic acids of the virus replicate in the infected cell, while the enzymes of the infected cells serve as polymerases. Sam viruses contain DNA or RNA polymerase, which is able to synthesize specific forms of the nucleic acids of the virus. The genome of the virus doesn't contain genes for the proteins of the ribosomes, rRNA and tRNA.

The course of the reproduction of viruses (life cycle) can be very diverse.

10.1.1 Lytic cycle

After the entering of the virus into the host cell, it usually blocks cells regulatory mechanisms, takes control of its replication and transcription mechanisms and stress it to be intensively reproduced (copied) virions. Usually this leads to the destruction of the cell, relax of new virions and infection of other cells. Adenoviruses are typical example. Sometimes bizarre situations occur, when the viral genome caries the information about the regulation of the type of its reproduction cycle (as it is in bacteriophages), which they undergo inside the cell.

Considering that viruses don't have their own metabolism and for their reproduction they use mechanisms present in cells, the only method (except interpherons) of destroying

them is specific immunity (specific antibodies, which are able to destroy the virions). The host organism survives, if its specific immunity is able to create a sufficient amount of specific antibodies. Antivirus drugs (antivirotics) are still in the stage of development. The treatment with antibiotics only has a preventive significance against superinfection – the spread of bacteria in the damaged (destroyed) tissue by viruses.

10.1.2 Lysogenic cycle

When after entering the cell, the virion (its genome) remains in the cell for a various amount of time apparently in sc. lysogenic stage. When the right conditions arise, the change from the lysogenic cycle to the lytic cycle takes place and the virus starts to reproduce. In certain types of viruses (e.g. retroviruses) the genome (genetic information) of the virus can randomly insert into the DNA of the host. The problem of certain retroviruses (their remains) presence in our genome (sc. "retroelements") is very serious, since they comprise a substantial amount of our genome.

The majority of the viruses cause inflammatory diseases and the lytic form of the cycle predominates, for example: flew, adenoviruses, virus of child poliomyelitis, encephalitis, chicken pox etc. Diseases caused by viruses with the lysogenic cycle belong to the most serious (e.g. acquired immune deficiency syndrome – AIDS).

10.2 Classification of Viruses

Viruses are classified into a system according to many criteria:

According to the organism on which the viruses parasite (in which type of cells they are able to reproduce), viruses are divide to:

- **bacteriophages** (parasite in bacteria);
- **phytotropic** (parasites of the plants);
- **zootropic** (they parasite in the cells of animals and humans).

From a medical point of view it is important to distinguish tropisms of viruses, according to the tissues (organs), which they primarily infect. According to this neurotrophic, hepatotrophic, dermotrophic viruses etc. are recognized.

Examples of medically important viruses are mentioned in table 9.

RNA – viruses				
Family	**Size of virion (in nm)**	**Length of RNA (in kb)**	**Number of genes**	**Examples of diseases, which they inflict**
Retroviruses	80 – 110	8 – 10	8	AIDS
Reoviruses	75 – 80	18	40	encephalitis, meningitis
Picornaviruses	30 – 35	7 – 9	12	poliomyelitis, common cold, hepatitis A
Togaviruses	40 – 60	9 – 12	variable	encephalitis, rubela
Coronaviruses	80 – 220	26 – 30	variable	SARS, gastroenteritis
Paramyxoviruses	150 – 300	4 – 8	30	mumps, chicken-pox
Rhabdoviruses	400	11 – 12	variable	rabies, encephalitis
Orthomyxoviruses	50 – 120	12 – 15	variable	influenza

DNA – viruses				
Family	**Size of virion (in nm)**	**Length of DNA (in kb)**	**Number of Gene**	**Examples of diseases, which they inflict**
Poxviruses	230 – 300	165 – 210	400	variola major
Adenoviruses	75 – 100	36 – 44	50	inflammation of upper respiratory air passages
Herpesviruses	100	125 – 300	180	sheep pox, herpes zoster
Papillomaviruses	40 – 55	5,3 – 8	10	nipple, carcinoma of cervix uteri
Hepadnaviruses	42	3,2	4	hepatitis B, carcinoma of liver

Note: kb = 1000 nitrogenous bases

- **Table. 9.** Examples of medically important viruses according to the genome.

10.2.1 Classification of viruses according to genome

According to the type of nucleic acids which form the genome, viruses are divided to **DNA** and **RNA viruses**. Their nucleic acid can be arranged in different ways, single stranded or double stranded. According to their organization and their genome we divide them to:
Group I.: ds DNA (viruses with double stranded DNA)
Group II.: ss DNA (viruses with single stranded DNA)
Group III.: ds RNA (viruses with double stranded RNA)
Group IV.: (+)RNA (single stranded viruses plus RNA)
Group V.: (-)RNA (single stranded viruses minus RNA)
Group VI.: RNA-RT (RNA viruses with reverse transcriptase)
Group VII.: DNA-RT (DNA viruses with reverse transcriptase)

10.2.1.1 RNA viruses

Their genome is formed by RNA of various polarity (positive or negative), which can be either single stranded – (ss)RNA or double stranded – (ds)RNA. The genome +ssRNA can serve for direct translation (as mRNA), meaning that it is „directly infectious“. -ssRNA viruses must first be rewritten into the +ssRNA (mRNA) and afterwards can the virus reproduce.

10.2.1.1.1 Viruses with double stranded RNA (dsRNA-viruses)

Here belong for example **reoviruses**. During infection the virus dsRNA replicates. The reaction is catalyzed by **RNA-replicase**. The animal cells don’t have this enzyme, and therefore this replication is catalyzed by a different enzyme present in the virion. Replicase creates a new double stranded virus RNA and at the same time the mRNA which is necessary for the synthesis of new viral proteins.

10.2.1.1.2 Viruses with positive single stranded RNA (+ssRNA viruses)

To this category belong for example **togaviruses** and **picornaviruses**. After infection of the cell, the viral RNA serves as a template for the synthesis of the viral proteins (as mRNA). Its RNA replicase synthesizes a minus strand of viral RNA and by its replication the viral genome +ssRNA is formed, which together with the proteins forms new viral particles.

10.2.1.1.3 Viruses with negative single stranded RNA (-ssRNA viruses)

Here belong **orthomyxoviruses** (for example the virus of a influenza) and **paramyxoviruses**. Their genome is segmented – formed by several independent sequences of single stranded RNA of negative polarity. Replication has two phases. In the first phase the +ssRNA is replicated according to –ssRNA. The newly formed +ssRNA serves in the second phase of the replication, where according to +ssRNA the viral -ssRNA is replicated and at the same time is used for the synthesis of the viral proteins.

A serious infectious disease in this category is the **flu** (influenza). The cause are certain peculiarities in the life cycle of the flu virus:

- A relatively large number of cells contain receptors on their membrane which distinguish the ligands (antigenes) on the surface of the capsid, this enable the entrance of the virion of the flew to the cell;
- Penetration is simplified also by the fact that virions leave the cell, which multiplied them, by exocytosis (budding), so virions have a sac from the cytoplasmic membrane on the surface. Therefore when the virus binds to a membrane of another cell, both membranes merge and the virion is „shot“ right into the cell. Besides this, the cytoplasmic layer complicates the recognition of viral antigenes by the specific immunity, which complicates the course of the illness;
- Since the genome of the virus consists of a single stranded RNA, many mutations of it arises and are not repaired, which cause a large degree of **antigen drift**. In one sick person there are often from 4 to 6 variants of one virus which complicate the situation for the specific immunity;
- Virus simple spreading by droplet infection, a large amount of cells which accept the virus, and a large degree of antigen drift cause periodic epidemy of the flu;
- The risk of pandemy, as was mentioned, is increased as a result of possible recombination – mistakes during the packaging (reassembling) of different segments of the viral genome, when the cell is infected by two different types of influenza (e.g. human and avian). This can induce major changes in the virus properties (formation of a new subtype) with a new combination of antigens (**antigen shift**).

10.2.1.1.4 RNA Retroviruses

Typical example – HIV composition is: nucleoprotein („core“) of the retroviruses is the genome (two independent copies of +ssRNA) packaged by p25 protein and another protein layer which is formed by the p17 protein, which is the „matrix“. Besides others it contains enzymes (in two copies) important for the lysogenic cycle of the virus – reverse transcriptase, integrase and protease. The virus is surrounded by a lipid layer (the remains of the cytoplasmic membrane of the previous host cell), to which the gp120 and gp45 proteins are embedded. Glycoprotein gp120 serves for the bonding of the retrovirus to the superficial receptors of the cell (mainly CD4 T-lymphocytes). Gp45 serves as a ligand for the coreceptor on the cytoplasmic membrane of the cell, which causes the tilt of the virion, the contact and fusion of the cytoplasmic membrane of the cell and the capsule of the virus, which causes the „shooting“ of the contents of the capsid into the cell.

Even tough the genome of the retrovirus is made up of a positive single stranded RNA and contains a **reverse transcriptase** enzyme inside the capsid. This enzyme is able (in the cytoplasm of the host cell) to rewrite the single stranded RNA genome of the virus into the single stranded complementary DNA (cDNA) and after that finish the synthesis of the second DNA strand. This double stranded DNA sequence is then randomly inserted (with the help of virus integrase) into the genome of the infected cell and after this double strandede DNA segment is called a **provirus**. Possible side effects for the degree of expression of the consecutive gene (proto-oncogene) are mentioned in the 2. part, page 68.

Integrase is an endonuclease which is able to ensure the integration of the double stranded cDNA strand into the genome of the T lymphocyte. Here it can remain for years, until the right conditions arise for the change into the lytic cycle, during which new HIV virions are formed and consequentially the attacked T lymphocytes are destroyed. As a result of the absence of this kind of T lymphocytes, the patient usually dies as because of a rapidly evolving tumorous disease (sarcoma) or the flare-up of long-date present disease (e.g. toxoplasmosis). This is because one of the main taks of the T lymphocytes is the destruction of alien eukaryotic cells, which they recognize according to their antigens – for example tumorous cells and parasitic protozoa.

The details of the mentioned phenomena will be discussed on lectures and seminars.

10.2.1.2 DNA viruses

Their genome contains a single stranded (ss)DNA or double stranded (ds)DNA. An exception is the family Hepadnaviridae, which contains double stranded and partially single stranded DNA. The replication of DNA and viruses takes place in the following way:

- Transcription of viral genes into the mRNA;
- Translation of viral mRNA on the cells ribosomes;
- The copying of new molecules of viral DNA;
- The construction of new viral subunit;
- The release of virions from infected cells.

Unpackaged DNA viruses (papilloma, polyoma, adenoviruses) have mature capsids. Their formation takes place inside the nucleus. Capsids of herpesviruses, formed inside the nucleus, get their covering on the nuclear membrane or on the membranes of vacuoles in the cytoplasm. Poxviruses are reproduced explicitly in the cytoplasm. Poxviruses code such a substantial amount of their own enzymes, that their replication takes place in the cytoplasm independently from the nucleus of the host cell.

10.2.1.2.1 Viruses with a double stranded DNA (dsDNA viruses)

The replication of the DNA takes place in the nucleus or the cytoplasm of the host cell. Its principle is similar to the way it occurs during replication of the nuclear DNA and takes place in the 5´→ 3´ direction. At the same time the genes of the viral DNA rewrite into the mRNA, which is responsible for the formation of structural proteins of the virus and the enzymes necessary for the replication of viral DNA. The replicated DNA connects with the newly formed proteins during the formation of a new viral subunits. To this group of viruses belong for examples: **herpesviruses**, **adenoviruses**, and **poxviruses**.

10.2.1.2.2 Viruses with a single stranded DNA (ssDNA viruses)

The genome of the virus is formed by a positive (+)DNA strand. The mRNA is not formed according to it. In the nucleus the +ssDNA is formed with a strand that serves for transcription. The formed mRNA‘s are used for the synthesis of structural proteins of the virus. An example are **parvoviruses**.

10.2.1.2.3 DNA retroviruses

The genome consists of a double stranded DNA, which is rewritten in the host cell by RNA polymerase into +ssRNA. The +ssRNA strand serves as an mRNA during synthesis of viral proteins and also as a matrix for the synthesis of the double stranded DNA. The rewriting of the +ssRNA into dsDNA of virus is catalyzed by reverse transcriptase. Here

belong the **hepadnaviruses**, to which also belongs the HBV virus (virus of hepatitis B), which causes „serum“ hepatitis.

10.3 Mutations and recombinations of viruses

With viruses, the same as with other organisms, mutations and recombinations are processes by which they ensure their development. However from a medical point of view, these phenomena are often a source of problems, since not only small changes (result of mutations), but mostly the serious changes in their properties (result of recombination) can significantly higher the pathogenity of viruses.

Eventough **mutations** in viruses appear not more common then in other organisms, but they need not to be detected and to repaired. Primarily it concerns viruses with a single stranded genome, with which it is not possible to repair a mutation, since reparation mechanisms (check 2. part) don’t have a way to repair the change because a complementary strand is not present which could provide a comparison. In viruses with the ssRNA genome there is another factor which plays a role, and this is the fact that RNA polymerases, which synthesizes their genome don’t have a function of “self prove reading” and repairing errors (in complementarity), which they create during their activity. A typical example is the flu virus, which as a result of the mentioned errors has a slight deviation in the structure of antigenes - allready during the replication process in one sick individual. This helps the mass propagation of the disease between susceptible population, with the occurence of periodical **epidemy**. A very interesting figure is the fact (luckily) that during the synthesis of the HIV virus the faultiness is so high, that only one out of a thousand of the newly formed virions is considered fully pathogenic. It is not uncommon to see that a virion with a mutated genome also looses the ability to infiltrate a host cell (loss of host specificity). This means that the number of mutations is much higher then the number of clinically detected **variants** of mutated viruses.

Recombinations in viruses arise as a consequence of new genetic information entering the genome of the virus. The most known situation is when reassortment of parts of the genome occurs **between two familiar types** which infect the cell (organism). A typical example is the virus of A-flew, in which the exchange of segments of its genome between human and animal (bird or pig) type (during packaging of virions) leads to a significant change in its properties – the formation of a new **subtype**. The result is the risk of a fatal infectious disease (**pandemy**). This is how the great flu (Spanish flu) arose, which 90 years ago killed 20 million people worldwide.

Recombination has very serious consequences because it causes acute transformation of retroviruses (see part 2). The genome of the retroviruses is similar to a molecule of mRNA. During the transfer of the newly synthesized (according to provirus) genome of the retrovirus – to the packaging site of the virions on the inner side of the cytoplasmic membrane, they can meet with an cell mRNA and exchange with it a part of the molecule. An absurd mRNA arises and the retrovirus gets a new „gene“(it can be distinguished according to the fact that it doesn’t contain introns). If a virus proto-oncogene arises, the retrovirus becomes capable of an acute malignant transformation of a cell it infected (after reverse transcriptase, integration and expression of this gene).

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Introduction to medical and molecular biology

Published by: Asklepios, Bratislava, 2010

95 pages, 6,7AH, first edition, printing of 300

ISBN: 978-80-7167-151-0