Chemical bounds

Some Types of Chemical Bonds close to 99% of the weight of a living cell is composed of just four elements: carbon (C), hydrogen (H), nitrogen (N), and oxygen (O). Almost 50% of the atoms are hydrogen atoms; about 25% are carbon, and 25% oxygen. Apart from water (about 70% of the weight of the cell) almost all components are carbon compounds. Carbon, a small atom with four electrons in its outer shell, can form four strong covalent bonds withotheratoms.But most importantly,carbon atoms can combine with each other to build chains and rings, and thus large complex molecules with specific biological properties.

A. Compounds of hydrogen (H), oxygen (O), and carbon (C)

Four simple combinations of these atoms occur frequently in biologically important molecules: hydroxyl (—OH; alcohols), methyl (—CH 3), carboxyl (—COOH), and carbonyl (C=O; aldehydes and ketones) groups. They impart to the molecules characteristic chemical properties, including possibilities to form compounds.

B. Acids and esters

Many biological substances contain a carbon— oxygen bond with weak acidic or basic (alkaline) properties. The degree of acidity is expressed by the pH value, which indicates the concentration of H + ions in a solution, ranging from 10 —1 mol/L (pH 1, strongly acidic) to 10 —14 mol/L (pH 14, strongly alkaline). Pure water contains 10 —7 moles H + per liter (pH 7.0). An ester is formed when an acid reacts with an alcohol. Esters are frequently found in lipids and phosphate compounds.

C. Carbon–nitrogen bonds (C–N)

C–N bonds occur in many biologically important molecules: in amino groups, amines, and amides, especially in proteins. Of paramount significance are the amino acids, which are the subunits of proteins. All proteins have a specific role in the functioning of an organism.

D. Phosphate compounds

Ionized phosphate compounds play an essential biological role. HPO 4 2– is a stable inorganic phosphate ion from ionized phosphoric acid. A phosphate ion and a free hydroxyl group can form a phosphate ester. Phosphate compounds playan important role in energy-rich molecules and numerous macromolecules because they can store energy.

E. Sulfur compounds

Sulfur often serves to bind biological molecules together, especially when two sulfhydryl groups(–SH)reacttoformadisulfidebridge(– S–S–). Sulfur is a component of two amino acids (cysteine and methionine) and of some polysaccharides and sugars. Disulfide bridges play an important role in many complex molecules, serving to stabilize and maintain particular three-dimensional structures.

Simple flow cytometry protocol to determine

simultaneously live, dead and apoptotic stallion spermatozoa

A simple flow cytometry protocol to determine simultaneously live, dead and apoptotic stallion spermatozoa in fresh and frozen thawed samples.

Flow cytometry is increasingly used in clinical andrology in stallions (Love, 2016; Munoz et al., 2016). Numerous protocols have been developed in the last decade allowing the simultaneous assessment of multiple parameters in the spermatozoa. Developments in flow cytometry have allowed for an enhanced understanding of the biology of spermatozoa, particularly in the fields of clinical spermatology and sperm biotechnology. One of the aspects recently discovered with the aid of flow cytometry, relate to the dependence of the stallion spermatozoa on oxidative phosphorylation to obtain energy for motility and sperm house-keeping functions (Gibb et al., 2014; Pena et al., 2015; Plaza Davila et al., 2015; Davila et al., 2016).

This dependence also results in a greater production of the superoxide anion ($O_2^{-\bullet}$), that if deregulation of redox homeostasis occurs, leads to oxidative stress, accelerated senescence of the spermatozoa and ultimately death of the cell (Aitken et al., 2012b; Morillo Rodriguez et al., 2012; Gallardo Bolanos et al., 2014; Martin Munoz et al., 2015; Munoz et al., 2016; Ortega-Ferrusola et al., 2017). This accelerated sperm senescence has been attributed to changes resembling apoptosis and the term spermptosis was proposed to refer to this biological phenomenon (Gallardo Bolanos et al., 2014; Ortega-Ferrusola et al., 2017).

A similar form of cell death after cryopreservation has also

been described in somatic cells and is termed cryopreservation induced delayed onset cell death (Baust et al., 2001; Baust et al., 2016). These changes have been described in relation to cryopreservation but also may be attributable to stallion subfertility. Due to the importance of these changes, numerous assays have been developed for the identification of cell states using flow cytometry (Martinez-Pastor et al., 2010; Pena et al., 2016b; Ortega-Ferrusola et al., 2017). Many of these assays require the use of three different probes, usually the H-42 probe, to identify spermatozoa and eliminate non-sperm debris from the analysis. If simpler protocols, with minimal spectral overlap, can be developed the assessment of apoptotic changes could be facilitated.

The aim of this study was to develop a simple protocol to assess apoptotic changes, using a combination of two probes with minimal spectral overlap (H-42 and Eth-1). The use of this combination of probes was validated by comparing with use of a classical, and well proven assay for caspase 3 activation (Moran et al., 2008; Ortega-Ferrusola et al., 2008; Ortega Ferrusola et al., 2009; Gallardo Bolanos et al., 2014).

Material and methods

Reagents and media

The H-42 (Excitation: 350 nm, Emission: 461 nm) probe (Ref: H3570); Cell Event Caspase-3/7 Green (Excitation, 502 nm; Emission: 530 nm) (Ref: C10423) and Eth-1 (Excitation, 528 nm; Emission, 617 nm) (Ref E1169) were purchased from Fisher Scientific Spain (Madrid, Spain). All other chemicals were purchased from SIGMA (Madrid, Spain).

Semen collection and processing

Three ejaculates per horse were collected from seven fertile stallions. The samples were obtained on a routine basis (three

collections/week) during the 2016 breeding season. Stallions were maintained according to institutional and European regulations (Law 6/2913 June 11th and European Directive 2010/63/EU). Ejaculates were obtained and processed (fresh and frozen/thawed) using protocols that have been previously published in detail (Rodriguez et al., 2011; Morillo Rodriguez et al., 2012; Martin Munoz et al., 2015; Ortega-Ferrusola et al., 2017).

Staining for determination of live and dead cells and caspase 3 and 7 activity

This protocol has been developed in the laboratory where the present research was conducted and has been extensively described in previous publications (Gallardo Bolanos et al., 2014; Martin Munoz et al., 2015; Munoz et al., 2016; Ortega-Ferrusola et al., 2017). In brief, stock solutions of CellEvent (2 mM in DMSO), Eth-1 (1.167 mM in DMSO), and Hoechst 33342 (1.62 mM in water) were prepared. Spermatozoa (5 × 106 /mL) in 1 mL of PBS were stained with 1 μL of CellEvent, and 0.3 μL of Hoechst 33342 and incubated 25′ in the dark at r.t. Then 0.3 μL of Eth-1 was added to each sample and after incubation for 5 additional minutes the samples were immediately evaluated using the flow cytometer. Representative cytograms of the assay and gating strategy are shown in Fig. 1.

Flow cytometry

Flow cytometry analyses were conducted in a MACSQuant Analyzer 10 (Miltenyi Biotech), equipped with three lasers emitting at 405, 488, and 635 nm and 10 photomultiplier tubes (PMTs): V1 (excitation 405 nm, emission 450/50 nm), V2 (excitation 405 nm, emission 525/50 nm), B1 (excitation 488 nm, emission 525/50 nm), B2 (excitation 488 nm, emission 585/40 nm), B3 (excitation 488 nm, emission 655–730 nm (655LP + split 730)), B4 (excitation 499 nm, emission 750 LP), R1 (excitation 635

nm, emission 655–730 nm (655LP + split 730)), and R2 (excitation 635 nm, emission filter 750 LP). The system was controlled using MACS Quantify software. The quadrants or regions used to quantify the frequency of each sperm subpopulation depended on the particular assay. Forward and sideways light scatter was recorded for a total of 50,000 events per sample. Gating the sperm population after Hoechst 33342 staining eliminated non-sperm events. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. The data were analyzed using Flowjo V 10.2 Software (Ashland, OR, USA). Unstained and singlestained controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications (Pena et al., 2003; Gallardo Bolanos et al., 2014; Martin Munoz et al., 2015).

Statistical analyses

The normality of the data was assessed using the Kolmogorov-Smirnoff test. Because the data were normally distributed, the results were analyzed using an ANOVA followed by use of a Tukey post-hoc test for pair-wise comparisons (using SPSS 19.0 software for Mac). Differences were considered significant when $P < 0.05$. The results are displayed as the mean ± SEM. Bland and Altman Plots, a method agreement analysis technique, of the two staining protocols were also constructed. The differences between paired measurements of the same samples were calculated, and the mean of the differences (d) were used to estimate the average bias of one method relative to the other (Nagy et al., 2003; Pena et al., 2005). The 95% limit of agreement was calculated as d ± 2 SD, where SD is the standard deviation of the differences between paired measurements. These calculations were performed using Microsoft Excel for Mac OS X and SPSS for Mac.

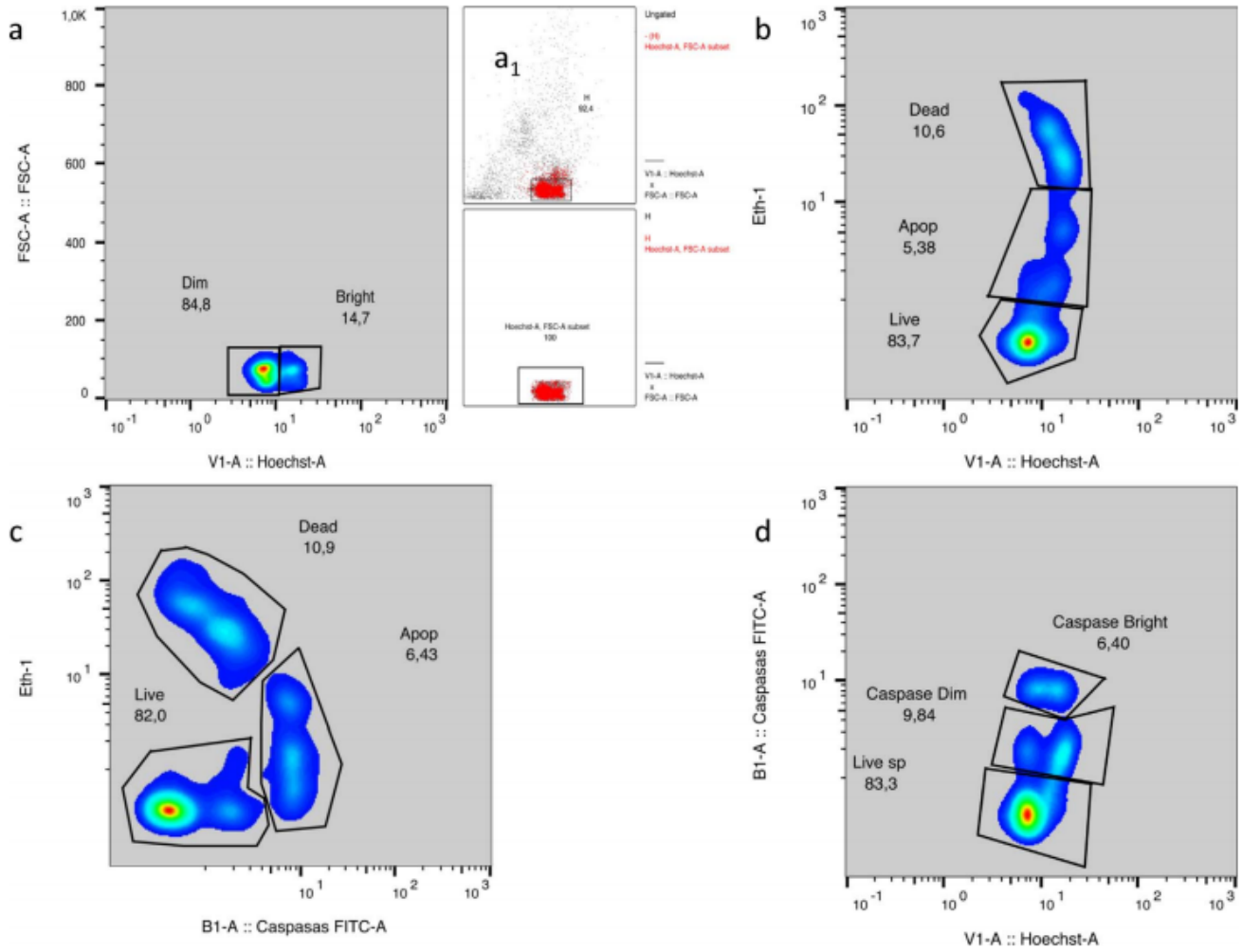


Figure 1. Representative cytograms of the assays reported in the present study. Stallion spermatozoa were obtained and processed as described in material and methods. a) Dot plot showing H33342 fluorescence the region gated in a1 corresponds to spermatozoa and this region is further used set the rest of populations of interest. In a two population of H-42 positive spermatozoa can be easily distinguished. H-42 Dim and H-42 Bright b) Events gated in a1 are now plotted against H-43 and Eth-1 fluorescence showing three populations, Live (H-42 +), apoptotic (H-42 +/Eth-1 +) and dead spermatozoa (Eth-1 +). c) Representative dot plot confronting caspase 3 (X axis) positive vs. ethidium homodimer fluorescence (Y axis); three subpopulations are evident live spermatozoa, negative for caspase 3 and for Eth-1, Apoptotic spermatozoa, positive for caspase 3 and negative for ethidium homodimer, and dead spermatozoa, positive for ethidium homodimer. d) Representative cytograms showing relation between H-42 and

Caspase 3 positivity. Three subpopulations can be detected, live spermatozoa (H-42 positive events), early apoptotic (Caspase 3 dim) and late apoptotic (Caspase bright events).

Experimental design

Semen was obtained and processed for cryopreservation or kept as fresh samples. Split samples from each kind of semen were stained with H-42, Eth-1, and CellEvent for caspase 3 detection. Comparisons were made of the same samples constructing 2D dot plots for each combination of probes assessed.

Conclusion

The bimodal distribution of H-42 positive spermatozoa (faint and bright) can be used to detect live and dead spermatozoa, while the addition of Eth-1 improves the assay allowing also the detection of apoptotic spermatozoa in the same fashion as specific assays detecting active caspases 3 and 7. Additionally, this assay offers the advantages of minimal spectral overlap and easy detection of non-sperm particles.

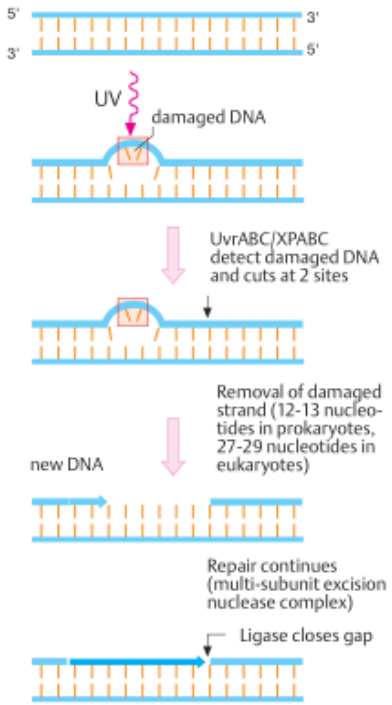
DNA Repair

Life would not be possible without the ability to repair damaged DNA. Since replication errors, including mismatch, and harmful exogenous factors are everyday problems for a living organism, a broad repertoire of repair genes has evolved in prokaryotes and eukaryotes. The following types of DNA repair can be distinguished by their basic mechanisms: (1) excision repair to remove a damaged DNA site, such as a strand with a thymine dimer; (2) mismatch repair to correct errors of

replication by excising a stretch of single-stranded DNA containing the wrong base; (3) repair of UV-damaged DNA during replication; and (4) transcription coupled repair in active genes.

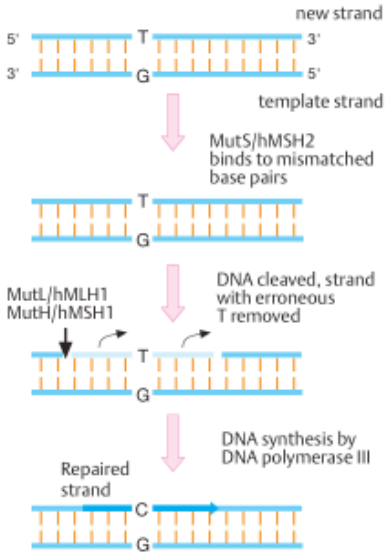
A. Excision repair

The damaged strand of DNA is distorted and can be recognized by a set of three proteins, the UvrA, UvrB, and UvrC endonucleases in prokaryotes and XPA, XPB, and XPC in human cells. This DNA strand is cleaved on both sides of the damage by an exonuclease protein complex, and a stretch of about 12 or 13 nucleotides in prokaryotes and 27 to 29 nucleotides in eukaryotes is removed. DNA repair synthesis restores the missing stretch and a DNA ligase closes the gap.



Excision repair

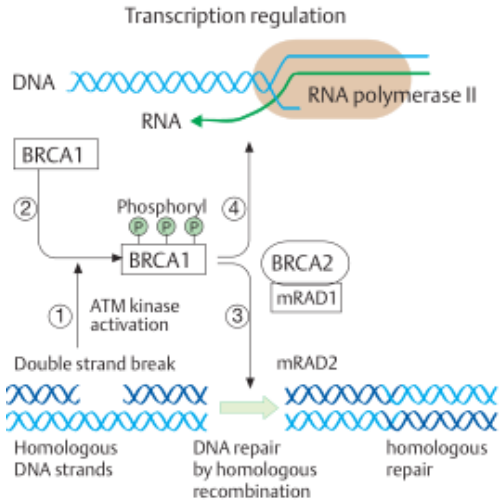
B. Mismatch repair



Mismatch repair

Mismatch repair corrects errors of replication. However, the newly synthesized DNA strand containing the wrong base must be distinguished from the parent strand, and the site of a mismatch identified. The former is based on a difference in methylation in prokaryotes. The daughter strand is undermethylated at this stage. E.coli has three mismatch repair systems: long patch, short patch, and very short patch. The long patch system can replace 1kb DNA and more. It requires three repair proteins, MutH, MutL, and MutS, which have the human homologues hMSH1, hMLH1, and hMSH2. Mutations in their respective genes lead to cancer due to defective mismatch repair. C. Replication repair of UV-damaged DNA DNA damage interferes with replication, especially in the leading strand. Large stretches remain unreplicated beyond the damaged site (in the 3? direction of the new strand) unless swiftly repaired. The lagging strand is not affected as much because Okazaki fragments (about 100 nucleotides in length) of newly synthesized DNA are also formed beyond the damaged site. This leads to an asymmetric replication fork and single-stranded regions of the leading strand. Aside from repair by recombination, the damaged site can be bypassed.

D. Double-strand repair by homologous recombination



Double-strand repair by homologous recombination

Double-strand damage is a common consequence of γ radiation. An important human pathway for mediating repair requires three proteins, encoded by the genes ATM, BRCA1, and BRCA2. Their names are derived from important diseases that result from mutations in these genes: ataxia telangiectasia and hereditary predisposition to breast cancer (BRCA1 and BRCA2). ATM, a member of a protein kinase family, is activated in response to DNA damage (1). Its active form phosphorylates BRCA1at specific sites (2). Phosphorylated BRCA1 induces homologous recombination in cooperation with BRCA2 and mRAD5, the mammalian homologue of E. coli RecA repair protein (3). This is required for efficient DNA double-break repair. Phosphorylated BRCA1 may also be involved in transcription and transcription-coupled DNA repair (4). (Figure redrawn from Ventikaraman, 1999).

Measuring Apoptosis by Flow Cytometry

Mitochondria are double-membraned organelles believed to have been integrated into modern eukaryotes via symbiosis of proteobacteria into an anaerobic pre-eukaryotic (host) cell 1.5–2 billion years ago. According to modern thinking (pioneered by Mitchell;), an essential role of mitochondria is to produce ATP via oxidative phosphorylation (OXPHOS). In this process, the chemical energy stored in nutrients (carbohydrates, fats, etc.) is converted to an electrochemical gradient across the inner mitochondrial membrane via the electron transport chain (ETC) complexes. This electrochemical gradient acts as a store of energy. ATP synthase uses this stored energy to convert ADP to ATP. This bioenergetic picture of the role of mitochondria is now widely accepted. A second role of mitochondria is in the so-called intrinsic apoptosis pathway. This pathway converges (figuratively and literally) at the membrane of the mitochondria. Upon certain cell death signals [such as reactive oxygen species (ROS), DNA damage, etc.], the outer membrane of mitochondria becomes permeable enough to release the soluble hemeprotein cytochrome C (CytC), as well as Smac/Diablo, endonuclease G, and other intermembrane space proteins, which irreversibly activate downstream caspases to carry out the apoptosis process.

Apoptosis is a programmed mode of cell death that is accompanied by numerous morphological as well as biochemical changes to the cellular architecture. This results not only in cell death but also in the effi- cient removal of apoptotic cells by phagocytes. Apoptotic cells display a range of common characteristics that include cell shrinkage, plasma membrane blebbing, cell detachment, nuclear condensation, DNA fragmentation, externalization of phosphatidylserine (PS) and activation of caspases. In contrast, necrotic cell death is

characterised by rapid plasma membrane, organelle swelling and plasma membrane rupture with none of the features of apoptosis. Apart from severe physical stresses, necrotic cell death often betrays the activities of viral infection and the activities of bacterial toxins. While necrotic cell death is characterized by the release of endogenous ‘danger signals’ and subsequent inflammation, apoptosis is largely tolergenic. Therefore, care must be taken when assessing whether cells are dying via apoptosis or necrosis. Here, we highlight a number of assays, utilizing flow cytometry, to determine whether cells have undergone apoptosis or alternative modes of cell death.

Detection of fragmented DNA by flow cytometry as a measure of apoptotic cell death

Intranucleosomal DNA fragmentation is a major hallmark of apoptosis. DNA fragmentation may be assessed by flow cytometry. Analysis of a cell population’s replication state (cell cycle profile) can be readily achieved with the fluorescent dye Propidium iodide (PI), which binds stoichiometrically to nucleic acids resulting in a fluorescence emission proportional to the DNA content of the cell. The rationale behind the approach is as follows: quiescent and G1 cells have two chromosome copies, while cells undergoing mitosis G2/M have double the amount of DNA and so will have double the fluorescence intensity of G1 cells. Cells in S phase will have a fluorescent signal between G1 and G2/M, because these cells are synthesizing DNA on their way to G2/M (Fig. 3A).

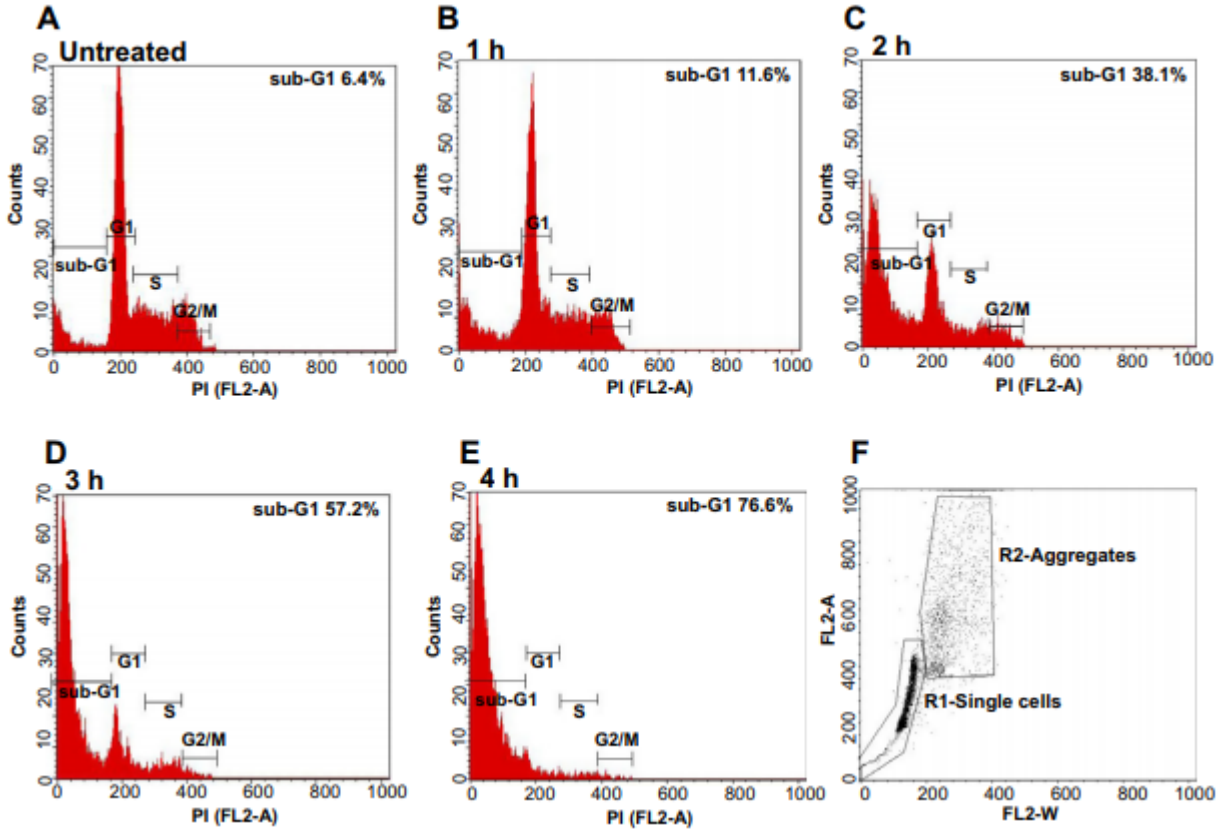


Figure 3. Measure of DNA fragmentation during apoptosis by flow cytometry. Jurkat cells were treated with 200 ng/ml anti-Fas (CH-11). Cells were harvested at indicated timepoints (A–E) and analysed by flow cytometry. (F) Gating strategy to discriminate cells aggregates from single cells.

Due to the generation of lowmolecular weight DNA fragments during apoptosis, cells undergoing apoptosis can be readily identified on DNA content histograms as cells with fractional hypodiploid or ‘‘sub-G1’’ content (Fig. 3B–E). Cellular DNA content is measured using a fluorescent dye after cell fixation with ethanol. Cell fixation does not retain small nuclear fragments in apoptotic cells. These low molecular weight DNA fragments leak out during subsequent wash steps. As a result, apoptotic cells contain a fractional DNA content relative to viable cells that can be readily distinguished by flow cytometry.

Methodology

The following protocol is tailored towards suspension cells, however, if using adherent cells remember to harvest the supernatant (late apoptotic cells become detached and float in the medium) in addition to the adherent/semi adherent cells on the plate and proceed as outlined below:

1.Apoptosis was induced in 2 10^6 Jurkat cells by incubation with 200 ng/ml anti-Fas antibody (CH-11) for 1–4 h. Cells are harvested at the desired time points and centrifuged at 400g for 5 min. Cells are washed with PBS pH 7.2 and centrifuged at 400g for 1 min.

2.Resuspend cells in 1 ml ice-cold 70% ethanol and incubate for at least 1 h at -20 °C to fix (cells can be stored for up to 6 months at -20 °C).

3.Centrifuge cells at 2500g for 5 min (a higher centrifuge speed is required as fixed cells become buoyant and may fail to pellet or stick along the side of the eppendorf). Aspirate off the ethanol without disturbing the cell pellet and resuspend with 1 ml phosphate-citrate wash buffer (200 mM Na2HPO4, 100 mM citric acid) followed by centrifugation at 2500g for 1 min.

4.To stain nuclei, prepare PBS pH 7.2 containing propidium iodide 10 lg/ml and RNase A 100 lg/ml (included to degrade RNA and to prevent PI staining of RNA) and incubate with cells for 30 min.

5.Samples are ready for analysis by flow cytometry (no need to wash out PI/RNase but this can be done if desired).

Setting correct parameters for cell cycle analysis by flow cytometry

A few considerations must be taken into account when using

flow cytometry for cell cycle analysis:

A) Ensure that the fluorescence channel 2 (FL2) is set at linear (LIN) scale. It is harder to distinguish the differential fluorescence between G0/G1 and G2/M peaks on a logarithmic (LOG) scale. LIN amplification allows for clear separation between G0/G1 and G2/M peaks.

B) A common problem to control for during cell cycle analysis is aggregation of cells. For example cells can stick together and pass through the flow cytometer's laser intercept simultaneously. In either case, two cells in G0/G1 that are stuck together or pass through the laser intercept at the same time will have a fluorescence signature equivalent to a cell in G2/ M. Therefore, the number of events recorded as G2/M will be artificially high. A way to exclude these events is by excluding non-single cell events from the analysis using scatter properties (FSC/SSC).

C) To discriminate between cellular aggregation and single cells, select a plot with FL2-A parameter as the y-axis and FL2-W as the x-axis (Fig. 3F). Single cells (G0/G1 or G2/M) will have pulse width values (FL2-W) that are similar, however aggregates will have larger pulse width values (due to increased cell width). In the example (Fig. 3F), single cells have been gated (R1-single cells) and the FL2-A histograms (Fig. 3A–E) have been formatted to display only events within this region (R1-single-cells).

Reference : ScienceDirect Measuring apoptosis by microscopy and flow cytometry

Mutation Due to Different Base Modifications

Mutations can result from chemical or physical events that lead to base modification. When they affect the base-pairing pattern, they interfere with replication or transcription. Chemical
substancees able to induce such changes are called mutagens. Mutagens cause mutations in different ways. Spontaneous oxidation, hydrolysis, uncontrolled methylation, alkylation, and ultraviolet irradiation result in alterations that modify nucleotide bases. DNA-reactive chemicals change nucleotide bases into different chemical structures or remove a base.

A. Deamination and methylation

Cytosine, adenine, and guanine contain an amino group. When this is removed (deamination), a modified base with a different basepairing pattern results. Nitrous acid typically removes the amino group. This also occurs spontaneously at a rate of 100 bases per genome per day (Alberts et al., 1994, p. 245). Deamination of cytosine removes the amino group in position 4 (1). The resulting molecule is uracil (2). This pairs with adenine rather than guanine. Normally this change is efficiently repaired by uracil-DNA glycosylase. Deamination at the RNA level occurs in RNA editing (see Expression of genes). Methylation of the carbon atom in position 5 of cytosine results in 5 methylcytosine, containing a methyl group in position 5 (3). Deamination of 5-methylcytosine will result in a change to thymine, containing an oxygen in position 4 instead of an amino group (4). This mutation will not be corrected because thymine is a natural base. Adenine (5) can be deaminated in position 6 to form hypoxanthine, which contains an oxygen in this position instead of an amino group (6), and which pairs with cytosine instead of thymine.

The resulting change after DNA replication is a cytosine instead of a thymine in the mutant strand.

B. Depurination

About 5000 purine bases (adenine and guanine) are lost per day from DNA in each cell (depurination) owing to thermal fluctuations. Depurination of DNA involves hydrolytic cleavage of the N-glycosyl linkage of deoxyribose to the guanine nitrogen in position 9.This leaves a depurinated sugar. The loss of a base pair will lead to a deletion after the next replication if not repaired in time (see DNA repair).

C. Alkylation

Alkylation is the introduction of a methyl or an ethyl group into a molecule. The alkylation of guanine involves the replacement of the hydrogen bond to the oxygen atom in position 6 by a methyl group, to form 6-methylguanine. This can no longer pair with cytosine. Instead, it will pair with thymine. Thus, after the next replication the opposite cytosine (C) is replaced by a thymine (T) in the mutant daughter molecule. Important alkylating agents are ethylnitroso urea (ENU), ethylmethane sulfonate (EMS), dimethylnitrosamine, and N-methyl-N-nitroN-nitrosoguanidine.

D. Nucleotide base analogue

Base analogs are purines or pyrimidines that are similar enough to the regular nucleotide DNA bases to be incorporated into the new strand during replication. 5-Bromodeoxyuridine (5BrdU) is an analog of thymine. It contains a bromine atom instead of the methyl group in position 5.Thus, it can be incorporated into the new DNA strand during replication. However, the presence of the bromine atom causes ambiguous and often wrong base pairing. E. UV-light-induced thymine dimers

Ultraviolet irradiation at 260 nm wavelength induces covalent bonds between adjacent thymine residues at carbon positions 5 and 6. If located within a gene, this will interfere with replication and transcription unless repaired. Another important type of UV-induced change is a photoproduct consisting of a covalent bond between the carbons in positions 4 and 6 of two adjacent nucleotides, the 4–6 photoproduct.

DNA Amplification by Polymerase Chain Reaction (PCR)

The introduction of cell-free methods for multiplying DNA fragments of defined origin (DNA amplification) in 1985 ushered in a new era in molecular genetics (the principle of PCR is contained in earlier publications). This fundamental technology has spread dramatically with the development of automated equipment used in basic and applied research.

Polymerase chain reaction (PCR)

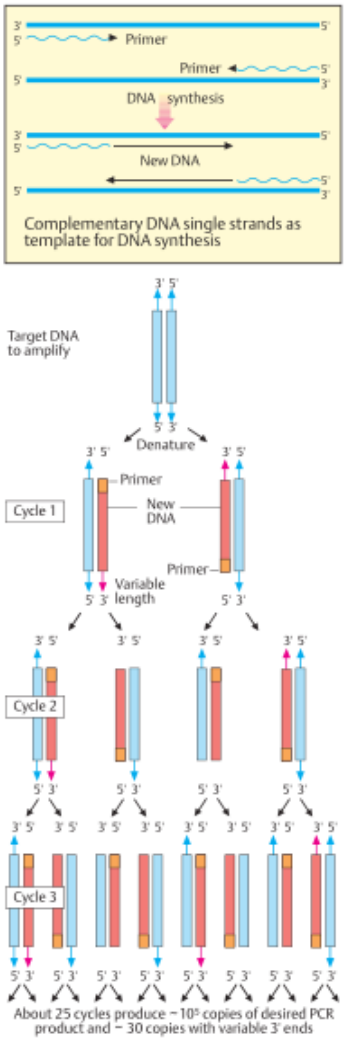
PCR is a cell-free, rapid, and sensitive method for cloning DNA fragments. A standard reaction and a wide variety of PCR-based methods have been developed to assay for polymorphisms and mutations. Standard PCR is an in vitro procedure for amplifying defined target DNA sequences, even from very small amounts of material or material of ancient origin. Selective amplification requires some prior information about DNA sequences flanking the target DNA. Based on this information, two oligonucleotide primers of about 15–25 base pairs length are designed. The primers are complementary to sequences

outside the 3′ ends of the target site and bind specifically to these. PCR is a chain reaction because newly synthesized DNA strands act as templates for further DNA synthesis for about 25–35 subsequent cycles. Theoretically each cycle doubles the amount of DNA amplified. At the end, at least 10^5 copies of the specific target sequence are present. This can be visualized as a distinct band of a specific size after gel electrophoresis. Each cycle, involving three precisely time-controlled and temperature-controlled reactions in automated thermal cyclers, takes about 1–5min. The three steps in each cycle are (1) denaturation of double-stranded DNA, at about 93–95°C for human DNA, (2) primer annealing at about 50–70°C depending on the expected melting temperature of the duplex DNA, and (3) DNA synthesis using heat-stable DNA polymerase (from microorganisms living in hot springs, such as Thermophilus aquaticus, Taq polymerase), typically at about 70–75°C. At each subsequent cycle the template (shown in blue) and the DNA newly synthesized during the preceding cycle (shown in red) act as templates for another round of synthesis. The first cycle results in newly synthesized DNA of varied lengths (shown with an arrow) at the 3′ ends because synthesis is continued beyond the target sequences. The same happens during subsequent cycles, but the variable strands are rapidly outnumbered by new DNA of fixed length at both ends because synthesis cannot proceed past the terminus of the primer at the opposite template DNA.

cDNA amplification and RT-PCR

A partially known amino acid sequence of a polypeptide can be used to obtain the sequence information required for PCR. From its mRNA one can derive cDNA, and determine the sequence of the sense and the antisense strand to prepare appropriate oligonucleotide primers (1). When different RNAs are available in small amounts, rapid PCR based methods are employed to amplify cDNA from different exons of a gene. cDNA is obtained by reverse transcriptase from mRNA, which is then removed by

alkaline hydrolysis (2). After a complementary new DNA strand has been synthesized, the DNA can be amplified by PCR (3). Reverse transcriptase PCR (RT-PCR) can be used when the known exon sequences are widely separated within a gene. With rapid amplification of cDNA ends (RACE-PCR), the 5? and 3? end sequences can be isolated from cDNA.



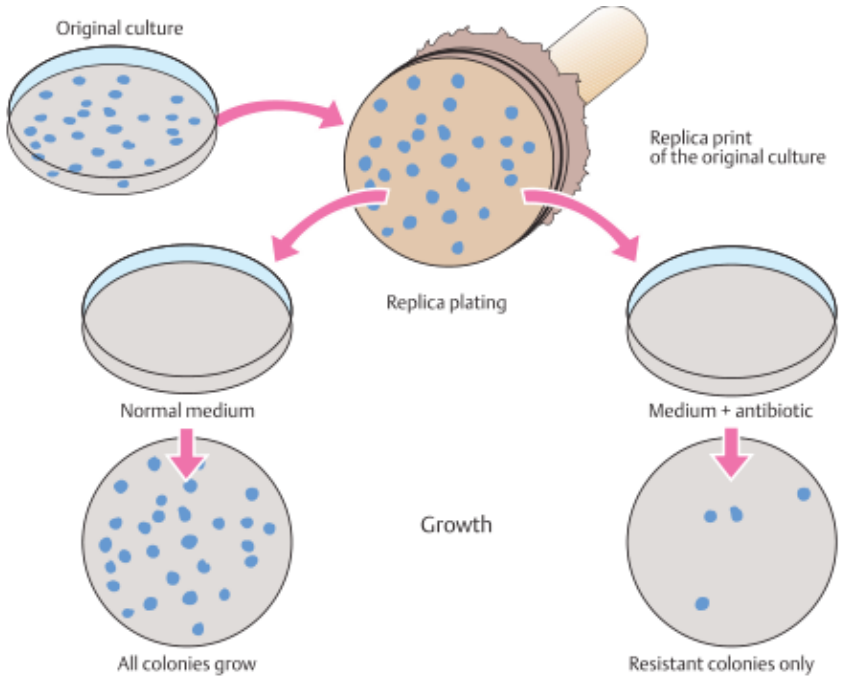
Polymerase chain reaction (PCR)

Isolation of Mutant Bacteria

Important advances in genetics were made in the early 1950s through studies of bacteria. As prokaryotic organisms, bacteria have certain advantages over eukaryotic organisms because they are haploid and have an extremely short generation time. Mutant bacteria can be identified easily. The growth of some mutant bacteria depends on whether a certain substance is present in the medium (auxotrophism). Bacterial cultures are well suited for determining mutational events since an almost unlimited number of cells can be tested in a short time. Without great difficulty, it is possible to detect one mutant in 10e7 colonies. Efficiency to this degree is not possible in the genetic analysis of eukaryotic organisms.

A. Replica plating to recognize mutants

In 1952, Joshua and Esther Lederberg developed replica plating of bacterial cultures. With this method, individual colonies on an agar plate can be taken up with a stamp covered with velvet and placed onto other culture dishes with media of different compositions. Some mutant bacteria differ from non mutants in their ability to grow. Here several colonies are shown in the Petri dish of the initial culture. Each of these colonies originated from a single cell. By means of replica plating, the colonies are transferred to two new cultures. One culture (right) contains an antibiotic in the culture medium; the other (left) does not. All colonies grow in normal medium, but only those colonies that are antibiotic resistant owing to a mutation grow in the antibiotic-containing medium. In this manner, mutant colonies can be readily identified.

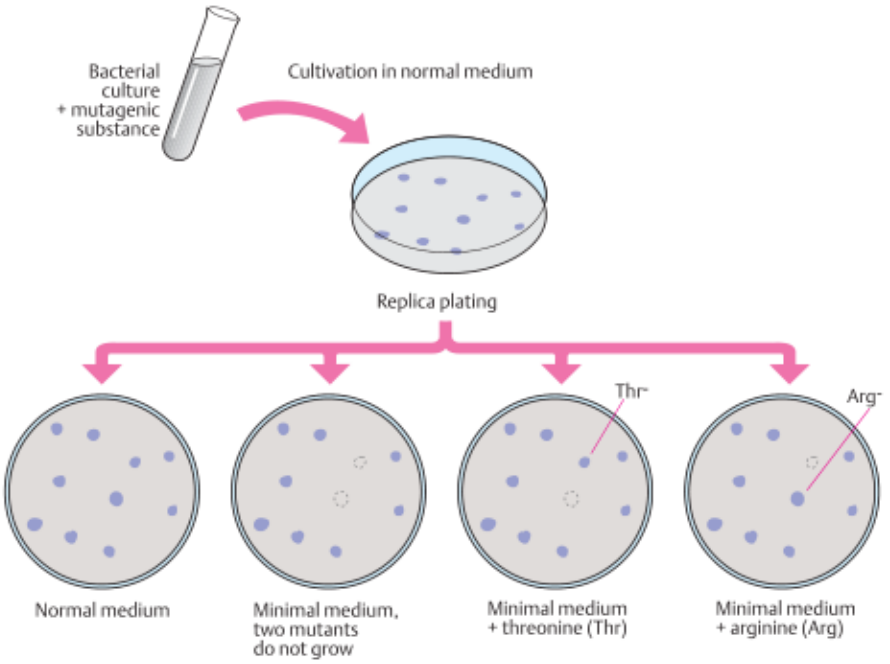


Replica plating to recognize mutants

B. Mutant bacteria identified through an auxotrophic medium

Here it is shown how different mutants can be distinguished, e.g., after exposure to a mutagenic substance. After a colony has been treated with a mutagenic substance, it is first cultivated in normal nutrient medium. Mutants can then be identified by replica plating. The culture with the normal medium serves as the control. In one culture with minimal medium, from which a number of substances are absent, two colonies do not grow (auxotrophic mutants). Initially, it is known for which of the substances the colonies are auxotrophic. If a different amino acid is added to each of two cultures with minimal medium, e.g., threonine (Thr) to one and arginine (Arg) to the other, it can be observed that one of the mutant colonies grows in the threonine-containing minimal medium, but the other does not. The former colony is dependent on the presence of threonine (Thr –), i.e., it is an auxotroph for threonine. The other culture with minimal medium had arginine added. Only here can the other of the two mutant colonies, an auxotroph for arginine (Arg –), grow. After the

mutant colonies requiring specific conditions for growth have been identified, they can be further characterized. This procedure is relatively simple and makes rapid identification of mutants possible. Many mutant bacteria have been defined by auxotrophism. The wild-type cells that do not have special additional growth requirements are called prototrophs (Figures adapted from Stent & Calendar, 1978).



Mutant bacteria identified through an auxotrophic medium

Eukaryotic gene structure

Eukaryotic genes consist of coding and noncoding segments of DNA, called exons and introns, respectively.At first glance it seems to be an unnecessary burden to carry DNA without obvious functions within a gene. However, it has been recognized that this has great evolutionary advantages. When parts of different genes are rearranged on new chromosomal sites during evolution, new genes may be constructed from parts of

previously existing genes.

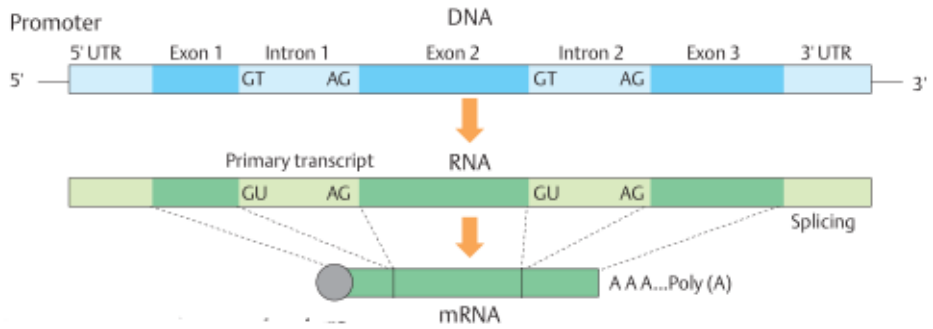
Exons and introns

In 1977, it was unexpectedly found that the DNA of a eukaryotic gene is longer than its corresponding mRNA. The reason is that certain sections of the initially formed primary RNA transcript are removed before translation occurs. Electron micrographs show that DNA and its corresponding transcript (RNA) are of different lengths (1). When mRNA and its complementary single-stranded DNA are hybridized, loops of single-stranded DNA arise because mRNA hybridizes only with certain sections of the single stranded DNA. In (2), seven loops (A to G) and eight hybridizing sections are shown (1 to 7 and the leading section L). Of the total 7700 DNA base pairs of this gene (3), only 1825 hybridize with mRNA. A hybridizing segment is called an exon. An initially transcribed DNA section that is subsequently removed from the primary transcript is an intron. The size and arrangement of exons and introns are characteristic for every eukaryotic gene (exon/intron structure). (Electron micrograph from Watson et al., 1987).

Intervening DNA sequences (introns)

In prokaryotes, DNA is colinear with mRNA and contains no introns (1). In eukaryotes, mature mRNA is complementary to only certain sections of DNA because the latter contains introns (2). (Figure adapted from Stryer, 1995).

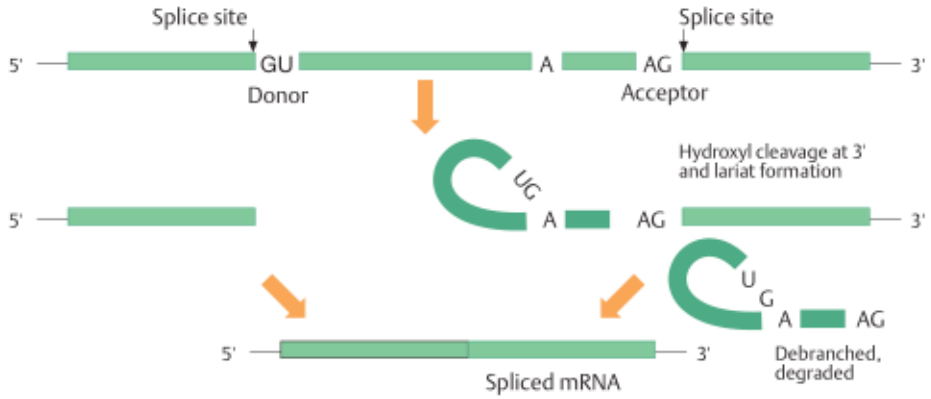
Basic eukaryotic gene structure



Basic eukaryotic gene structure

Exons and introns are numbered in the 5′ to 3′ direction of the coding strand. Both exons and introns are transcribed into a precursor RNA (primary transcript).The first and the last exons usually contain sequences that are not translated. These are called the 5′ untranslated region (5′ UTR) of exon 1 and the 3′ UTR at the 3′ end of the last exon. The non coding segments (introns) are removed from the primary transcript and the exons on either side are connected by a process called splicing. Splicing must be very precise to avoid an undesirable change of the correct reading frame. Introns almost always start with the nucleotides GT in the 5′ to 3′ strand (GU in RNA) and end with AG. The sequences at the 5′ end of the intron beginning with GT are called splice donor site and at the 3′ end, ending with AG,are called the splice acceptor site. Mature mRNA is modified at the 5? end by adding a stabilizing structure called a “cap” and by adding many adenines at the 3’end (polyadenylation).

Splicing pathway in GU–AG introns



Splicing pathway in GU – AG introns

RNA splicing is a complex process mediated by a large RNA-containing protein called a spliceosome. This consists of five types of small nuclear RNA molecules (snRNA) and more than 50 proteins (small nuclear riboprotein particles). The basic mechanism of splicing schematically involves autocatalytic cleavage at the 5'end of the intron resulting in lariat formation. This is an intermediate circular structure formed by connecting the 5′ terminus (UG) to a base (A) within the intron. This site is called the branch site. In the next stage, cleavage at the 3′ site releases the intron in lariat form. At the same time the right exon is ligated (spliced) to the left exon. The lariat is debranched to yield a linear intron and this is rapidly degraded. The branch site identifies the 3′ end for precise cleavage at the splice acceptor site. It lies 18–40 nucleotides upstream (in 5′ direction) of the 3′ splice site. (Figure adapted from Strachan and Read, 1999)

Probiotics

The notion of probiotics has recently developed and most pharmacists have not been trained in these new food supplements.

From birth, our gastrointestinal tract is colonized by many microorganisms that constitute the digestive microbiota. This complex and diversified ecosystem, unique to each individual, contributes to the proper functioning of the intestine through the many activities it carries out. However, the balance of the microbiota is sensitive and its rupture occurs in the pathophysiology of various intestinal disorders, hence the idea of positively modulating a microbiota unbalanced by the administration of probiotics.

The term “probiotic” means “for life” and refers to living microorganisms that, when ingested in appropriate amounts, produce a benefit to the health of the host that goes beyond basic nutritional functions.

Probiotics are often lactic acid bacteria (lactobacilli and bifidobacteria) or yeasts introduced into the diet in the form of fermented milk products or food supplements.

These microorganisms strengthen the intestinal and vaginal flora. Their presence makes it possible to fight against the proliferation of pathogenic bacteria.

Several clinical studies have already demonstrated the efficacy of certain probiotics in the treatment of systemic and infectious diseases such as acute diarrhea and Crohn’s disease.

Other studies have suggested a potential application for the treatment of urogenital infections, colon cancer, atopic dermatitis and allergic diseases including food allergy such as lactose intolerance.

History

The definition of probiotics has evolved over time according to researchers, scientific knowledge and technological advances.

In the 20th century the Nobel Prize winner, Elie Metchnikoff, observed that a surprising number of people in Bulgaria lived for more than 100 years. This longevity could not be explained by the advances in modern medicine, because Bulgaria, one of the poorest countries in Europe at that time, did not benefit from such advances. Dr. Metchnikoff found that Bulgarians consume large quantities of yogurt, he associated the increase in longevity observed with the consumption of living microorganisms from fermented dairy products. Although Metchnikoff saw germs as rather harmful to human health, he considered it beneficial to replace bacteria in the gastrointestinal tract with yogurt, including the Bulgarian bacillus. He then explained the better beneficial effect of this bacteria by the absence of alcohol production (harmful to longevity), compared to bacteria present in other fermented milk such as kefir or kumys. In addition, he assumed that the lactic acid produced, as well as other unidentified factors, would act synergistically to inhibit the growth of putrefaction bacteria in the colon.

At the same time, in 1906, the French pediatrician Henry Tissier observed that the stools of children with diarrhea contained a small number of bifidobacteria compared to the stools of healthy children. He then suggested that these bacteria be administered to diarrheal patients to help them restore a healthy intestinal microbiota.

Metchnikoff and Tissier are therefore the first to put forward the idea of administering exogenous microorganisms to compensate for a possible dysfunction in our intestinal ecosystem. The concept of “probiotics” was born.

Nevertheless, it was not until 1954 that the term probiotics was introduced into the literature by Ferdinand Vergin in a paper entitled “Anti-und Probiotika”. This term derived from the Greek “pro bios”, which literally means “for life” as opposed to the harmful effects of antibiotics

In 1965, Lilly and Stilwell, in the journal Science, defined probiotics as substances produced by microorganisms capable of stimulating the growth of other microorganisms.

In 1989, Fuller highlighted the microbial nature of probiotics by redefining the term as a “living microbial nutritional supplement that has a positive effect on the host animal by improving its intestinal balance”.

In 1992, Havenaar and Huis in’t Velt further refined the term to “a viable culture composed of one or a mixture of bacteria that, when applied to animals or humans, has a beneficial effect on the host by improving the properties of native flora. ».

In 1998, Guarner and Schaafsmaa specified that probiotics are “living microorganisms, which, when consumed in adequate amounts, have a beneficial effect on the health of the host”.

In 2002, the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) formalized the definition of probiotics to avoid any drift.

Probiotics are therefore defined as “living organisms that, when ingested in sufficient quantities, have a beneficial effect on the health of the host”.

History, therefore, underlines that the current definition could still evolve, as there are still many fields of research to better understand and understand the action of probiotics.

Regulation

The conditions and marketing authorization of probiotics are defined according to their drug or food application. Most probiotics are functional foods or are used as food supplements. These “healthy foods” are at the border between the drug and the traditional food and are governed by food legislation.

Probiotic foods

The global market of probiotic foods has been growing rapidly since the early 2000s, particularly in Europe. This dynamic is supported in particular by the link between food and health benefits.

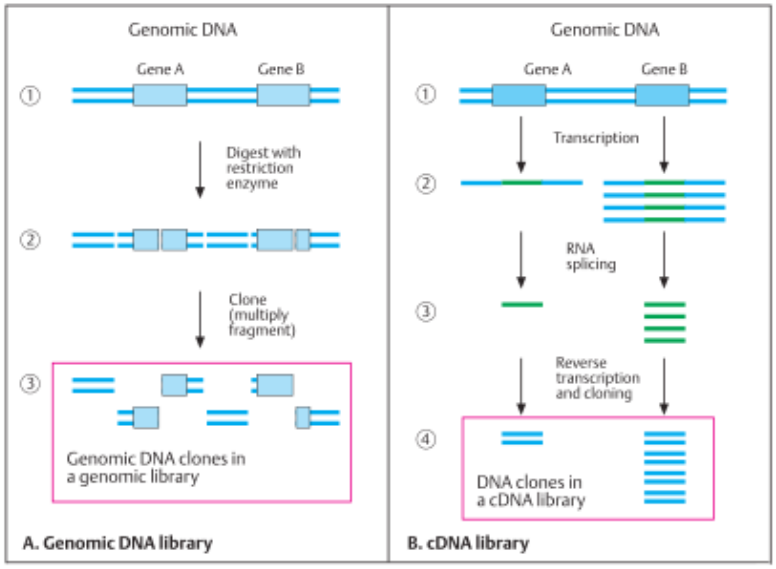
Probiotics used as food supplements, as well as functional foods, are considered as food and are governed by the relevant legislation. They are different from dietary foods that are intended for a particular food and require a specific formulation or manufacturing process to differentiate them from the common food, and from medicinal products.

DNA Libraries

A DNA library is a collection of DNA fragments that in their entirety represent the genome, that is, a particular gene being sought and all remaining DNA. It is the starting point for cloning a gene of unknown chromosomal location. To produce a library, the total DNA is digested with a restriction enzyme, and the resulting fragments are incorporated into vectors and replicated in bacteria. A sufficient number of clones must be present so that every segment is represented at least once. This is aquestion of the size of the genome being investigated and the size of the fragments. Plasmids and phages are used as vectors. For larger DNA fragments, yeast cells may be employed. There are two different types of libraries: genomic DNA and cDNA.

Genomic DNA library

Clones of genomic DNA are copies of DNA fragments from all of the chromosomes (1). They contain coding and non coding sequences. Restriction enzymes are used to cleave the genomic DNA into many fragments. Here four fragments are schematically shown, containing two genes, A and B (2). These are incorporated into vectors, e.g., into phage DNA, and are replicated in bacteria. The complete collection of recombinant DNA molecules, containing all DNA sequences of a species or individual, is called a genomic library. To find a particular gene, a screening procedure is required (see B).



Genomic DNA and cDNA library

cDNA library

Unlike a genomic library, which is complete and contains coding and non coding DNA, a cDNA library consists only of coding DNA sequences. This specificity offers considerable advantages over genomic DNA. However, it requires that mRNA be available and does not yield information about the structure of the gene. mRNA can be obtained only from cells in which the respective gene is transcribed, i.e., in which mRNA is produced (1). In eukaryotes, the RNA formed during

transcription (primary transcript) undergoes splicing to form mRNA. Complementary DNA (cDNA) is formed from mRNA by the enzyme reverse transcriptase (3). The cDNA can serve as a template for synthesis of a complementary DNA strand, so that complete double-stranded DNA can be formed (cDNA clone). Its sequence corresponds to the coding sequences of the gene exons. Thus it is well suited for use as a probe (cDNA probe). The subsequent steps, incorporation into a vector and replication in bacteria, correspond to those of the procedure to produce a genomic library. cDNA clones can only be won from coding regions of an active (mRNA-producing) gene; thus, the cDNA clones of different tissues differ according to genetic activity. Since cDNA clones correspond to the coding sequences of a gene (exons) and contain no noncoding sections (introns), cloned cDNA is the preferred starting material when further information about a gene product is sought by analyzing the gene. The sequence of amino acids in a protein can be determined from cloned and sequenced cDNA. Also, large amounts of a protein can be produced by having the cloned gene expressed in bacteria or yeast cells.

Screening of a DNA library

Bacteria that have taken up the vectors can grow on an agar-coated Petri dish, where they form colonies (1). A replica imprint of the culture is taken on a membrane (2), and the DNA that sticks to the membrane is denatured with an alkaline solution (3). DNA of the gene segment being sought can then be identified by hybridization with a radioactively (or otherwise) labeled probe (4). After hybridization, a signal appears on the membrane at the site of the gene segment (5). DNA complementary to the labeled probe is located here; its exact position in the culture corresponds to that of the signal on the membrane (6). A probe is taken from the corresponding area of the culture (5). It will contain the desired DNA segment, which can now be further replicated (cloned) in bacteria. By this means, the desired segment can

be enriched and is available for subsequent studies.