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Genetic Toxicology

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CHAPTER I
Origins and Importance of Genetic Toxicology

1-Definition

Genetic toxicology is a sub-discipline of toxicology, it is the study of the effects of chemical and physical agents on genetic material. It includes the study of DNA damage in living cells that leads to cancer, but it also examines changes in DNA that can be inherited from one generation to the next 1. Genetic toxicology study the processes and mechanisms by which chemicals or physical agents cause damage, including mutations, DNA single-strand breaks and double-strand breaks, gene rearrangements and gene amplifications, and chromosome damage in prokaryotes or eukaryotic cells. This includes aspects of mutagenesis. It includes also chemical carcinogenesis, i.e., the molecular mechanisms by which chemical carcinogens induce mutation in tumor suppressor genes, inactivating them, and induce mutations, rearrangements, or amplifications of oncogenes, deregulating them, causing 15 such changes. These fifteen changes, consisting of inactivation of tumor suppressor genes and activation of oncogenes, lead to further derangements in the expression of numerous genes, which results in the cell acquiring a tumorigenic phenotype and the eventual development of cancer (1).

2-History of genetic toxicology

Genetic toxicology evolved from the initial studies of gene mutability demonstrated first by Muller in 1927 using radiation, followed almost 20 years later by Auerbach et al. using chemicals. Both of these investigators conducted their studies using submammalian species, but within the next 20 years, genetic changes in animals induced by radiation and chemicals were demonstrated by Cattanach and Russell at Oak Ridge. This work created the awareness that some of the "hereditary" diseases observed in human populations might be environmental in origin. Proof in the early 1940s that deoxyribonucleic acid (DNA) is the hereditary material, and the subsequent elucidation of its primary, secondary, and tertiary structures by Watson and Crick in the early 1950s, opened up new avenues of research into the mechanisms of mutagenesis. The period of time from 1953 to 1968 might be considered the first "Golden Era" of molecular genetics. During this time, much of the basic information was developed regarding DNA structure and replication, the genetic code, mechanisms of protein synthesis, and DNA repair processes (Table 1). Cell biologists and biochemists and microbiologists reigned over this Golden Era, and several received Nobel Prizes for their contributions. The foundation established by the scientists in the first "Golden Era" has led to a second burst of information and technology associated with directed manipulation of genetic elements, often referred to as

genetic engineering. This new era started in 1977 with the reports of gene transfer and expression between unrelated organisms (1).

Table 1. Major Advances in Molecular Genetics (1)

Phenomenon	Year
DNA constitutes the hereditary material	1944
Elucidation of DNA structure	1953
Mechanism of protein synthesis-the central dogma hypothesis	1957
Demonstration of semiconservative replication of the DNA molecule	1958
Operon model of gene regulation	1961
Elucidation of the genetic code for protein	1964-1967
Enzymatic synthesis of DNA *in vitro*	1967
Gene cloning (transpecies)	1977

Genetic toxicology was recognized as a discipline in 1969 when the Environmental Mutagen Society was founded under the leadership of Dr. Alexander Hollaender and several other geneticists who were concerned about the potential genetic impact associated with the proliferation of man-made chemicals in the environment. The concept of genetic toxicology was clearly consistent with the intense concern for environmental protection that prevailed at the time. Occurring simultaneously with the growing concern by geneticists over environmental mutagens were the reports from several independent groups of investigators showing a correlative relationship between mammalian carcinogens and mutagens. Earlier attempts to support such a correlation experimentally failed because of the limitations inherent in the genetic assays available. The concept of mutagenic carcinogens was revitalized, however, following the introduction of procarcinogen activation using host-mediated and *in vitro* microsome activation systems developed from 1969 through 1971 (2)..

The fulfIllment of this relationship was the identification of mammalian cancer genes (oncogenes) and the demonstrated presence of mutations in the activated forms of these genes. Thus, genetic toxicology has played a dual role in safety evaluation programs. One function is the implementation of testing and risk assessment methods to define the impact of genotoxic agents found in the environment and whose presence may alter the integrity of the human gene pool. The second function is the application of genetic methodologies to the detection and mechanistic understanding of carcinogenic chemicals. In this latter regard, genetic toxicology has been applied as a front-line screen for potential carcinogens. Genetic toxicology has

continued to grow as a specialty area of toxicology, with significant influence on overall chemical safety evaluation (3).

3-Applied genetics and genetic toxicology

The application of genetic methodology (breeding in plants and animals) to human problems occurred prior to the recent concern over genotoxicity. Technology transfer, the application of basic science methodology to the solution of practical problems, has been essential to the development of genetic toxicology. Many of the methods employed to detect genotoxic substances were initially developed for other purposes. The Ames Salmonella/microsome assay, for example, evolved from early biochemical investigations of histidine biosynthesis in *Salmonella typhimurium*. The UDS (unscheduled DNA synthesis) assays used to measure stimulation of DNA repair processes were originally part of research efforts directed toward the elucidation of enzymes present in DNA repair pathways. As a consequence of numerous scientific and regulatory pressures, interest in understanding the molecular mechanisms for all toxic phenomena has increased (Figure 1). This new orientation of toxicologic investigation has been identified as molecular toxicology. Genetic toxicology, behavioral toxicology, immune-toxicology, teratology, and oncogenesis are disciplines of toxicology in which molecular toxicologists have been most active. Technology transfer from the basic sciences has produced a remarkable turnover in genetic toxicology assay systems during the past several years.

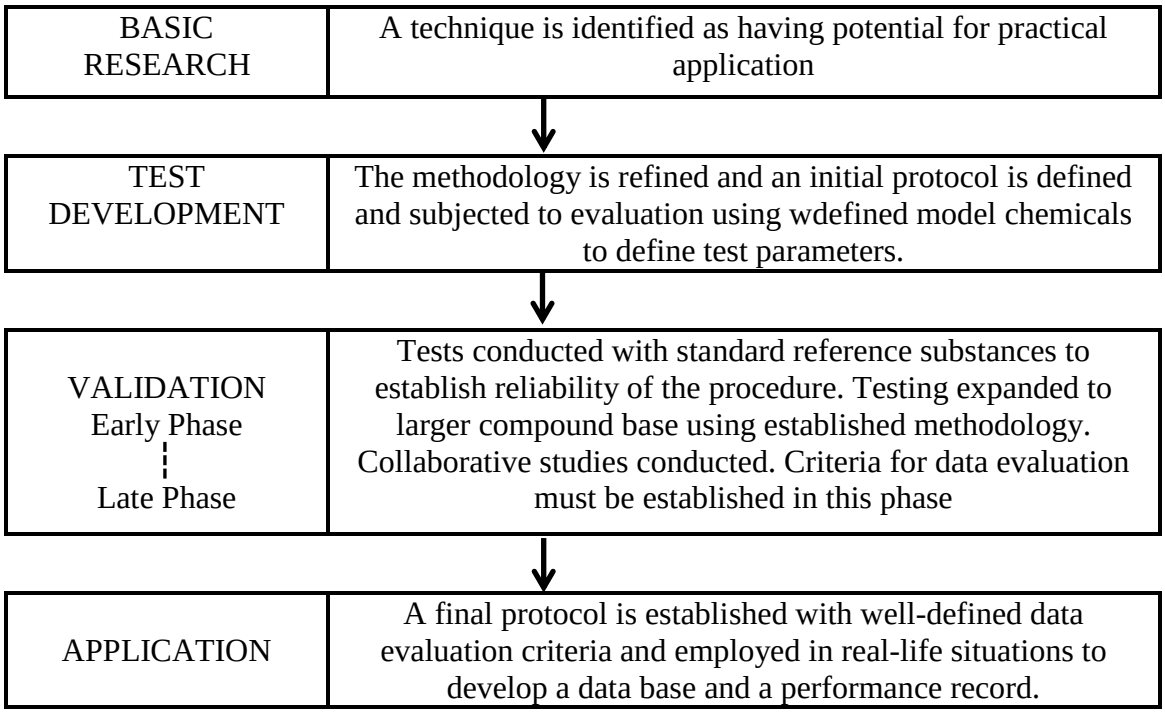

Figure 1. Steps involved in technology transfer for new assays. (1)

New test systems are evaluated for their suitability, and those with promise are often incorporated into the array of tests employed in this science. Unfortunately, as new test systems have been added, few have been retired. Thus, genetic toxicology is cluttered with tests of limited value and relevance for real genotoxic assessment (1).

4-Applications of genetic toxicology in health effects testing

Genetic toxicology, as a subspecialty of toxicology, identifies and analyzes the action of agents with toxicity directed toward the hereditary components of living systems. Some toxicants damage the genetic complex at concentrations also producing acute nonspecific cytotoxicity and death; the primary objective of genetic toxicology, however, is to detect and analyze the hazard potential of those agents that are highly specific for interactions with nucleic acids and produce alterations in genetic elements at subtoxic concentrations. Agents that produce alterations in the nucleic acids and associated components at subtoxic exposure levels, resulting in modified hereditary characteristics or DNA inactivation, are classified as genotoxic (1).

Genotoxic substances usually have common chemical or physical properties that facilitate interaction with nucleic acids. In fact, the universality of the target molecule is the key to the discipline of genetic toxicology. A report of the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC) emphasized that categorization of a chemical as genotoxic is not a priori indication of a health hazard. The term is a general descriptor meant to distinguish chemicals that have an affinity for direct DNA interactions from those that do not (1)..

4-1. *In vitro* and *in vivo* genotoxicity applications

In vitro genotoxicity is applicable in **primary cells** and **cell lines**. However, in cell lines, there is a significant decrease in xenobiotic metabolism specific to the organ. The process of co-culture is also used with *in vitro* implementations, thereby enabling interaction among cells (11). Identifying mutations does not require the observation of pathological changes in the organ or in the whole organism due to the fact that they are cell dependent and so they can be easily determined in *in vitro* cell cultures. Therefore there exists the possibility of using *in vitro* genotoxicity methods as a guide amongst the alternative methods without using test animals. There have been important developments regarding the establishment of alternative methods in some fields such as predicting local toxic effects and genotoxicity. Consequently, many *in vitro* tests have been adopted at OECD (Organisation for Economic Cooperation and Development) level (1).

Mutagenecity tests involving *in vivo* methods are divided into two groups: while germ cell mutations in humans increase the risk of hereditary disease of the next generation, somatic mutations may cause cancer development. In general, *in vivo* genotoxicity tests are used to verify the *in vitro* ones of chromosomal tests. The utilisation of *in vivo* genotoxicity methods on test animals enables the identification of all the interactions in the organism as well. Also, it enables the identification of metabolic conditions regarding the chemical whose genotoxic effect is researched in its natural environment. With *in vivo* methods, as in human biomonitoring studies, genotoxic efficiency of the relevant chemical in exposed groups may be revealed as well (4). Thus, *in vivo* and *in vitro* genotoxicity tests may be used to:

a. Provide information regarding the efficiency of the compound in directly or indirectly inducing mutations and an alert about the probability of the compound's carcinogenicity,
b. Reveal the molecular mechanism underlying the chemicals genotoxic and carcinogenic effects,
c. Identify hazards in risk assessment within molecular epidemiologic research with regards to occupational and environmental chemicals,
d. Determine toxicity profiles of chemicals,
e. Monitor the diseases and effectiveness of clinical treatments,
f. Develop regulations concerning medical, cosmetic and industrial chemicals according to international and national guidelines.

4-2. Use of genotoxicity as a biomarker of effect in the field of molecular epidemiology

Increased efforts are being made to identify the effect of internal and external factors like environment, genetic, nutrition, life style and working conditions over the genomic stability in humans. The use of biomarkers in occupational toxicology and molecular epidemiology provides important information on reflecting specific exposures, to predict a disease risk in individual/populations, to understand the mechanisms in order to assess epidemiologic and cancer risk. In the carcinogenesis, the formation of DNA damage is considered to be the important starting point. However, due to the fact that the development of cancer can take many years, it is not practical to conduct prospective epidemiologic studies that cover such a period. Hence, genotoxicity biomarkers are widely used in molecular epidemiology research (4).

The use of these biomarker methods is based on their genotoxicity in short-term testing of many substances which have been identified as human carcinogens, and capable of inducing chromosomal damage. However, the significance of these indicators in predicting cancer is not completely known. (4).

4-3. Use of genotoxicity methods in revealing toxic effect mechanisms

The effectiveness of short term genotoxicity methods in understanding and revealing the mechanisms which underly the biological activity has become well demonstrated, and it is mostly used *in vitro*. In the first stage of the determination of genotoxicity in cell cultures, there should be the formation of the dose-response curve. In determining the doses for the dose-response curve, the cytotoxicity must be already determined. The genotoxicity mediated mechanisms regarding the chemical/physical agents and particle, nanoparticle and fiber structures can be evaluated with the help of *in vitro* genotoxicity methods. Particularly, it is possible to benefit from the cell lines representing the target tissues in order to elucidate the mechanisms of genotoxicity such as cell lines representing the lung for inhalation toxicants and human keratinocytes and reconstructed skin representing the skin for solar UV. Another example of the use of genotoxicity methods in mechanistic studies is the identification of genotoxic potentials of molecules which have clinical utilisation (5).

4-4. Genotoxicity Methods in Regulations

Genotoxicity methods that enable the early prediction of diseases which generally take a long time from exposure to appearence, is therefore very valuable. The information acquired from genotoxicity methods together with other toxicology data are among the most important tools for regulatory toxicology as well. The function of regulatory toxicology is to conduct and interpret the results of toxicity tests. The purpose of the regulations governing the use of chemicals is to protect humans against the probable dangers of drugs and medicals to ensure the safety of foods and beverages, to ensure the occupational safety of workers during chemical production and to protect people and the environment against possible damages from chemical residues like pesticides. Since there are ethical reasons for testing products prior to human exposure, standardized toxicity testing requirements are systematized by the law. One of the issues that need addressing in future research is the development of standardized tests that could be used for regulatory purposes. Thus it can be seen that there are various studies of genotoxicity requested or recommended by the relevant authorities in the sale and registration of chemicals such as medicine, cosmetics, industrial chemicals and pesticides in the world in general. There is also a separate regulation for which methods of genotoxicity shall be applied to which type of chemicals and in which type of system (4).

In the assessment of safety of compounds, in terms of genotoxicity of a specific substance (or mixture) some aspects should be covered. These are;

a- Chemical structure and class of the substance (probable structure-activity relationships) and physicochemical features (solubility, stability, pH etc.)

b- Expected metabolism pathways, chemical and biological reactivity/activity and known relations with genotoxic substances

c- Exposure routes, bioavailability and target tissues

CHAPTER II
Fundamentals of Genetic Toxicity

The purpose of this chapter is to describe the genetic background and terminology essential for an understanding of genetic toxicology. Since the types of molecular lesions that chemicals induce in DNA and the resultant genetic damage are intimately tied to the structure and function of this molecule, it is essential to appreciate the basic structure of DNA and understand how it operates. Because of the unique position held by DNA in storing and processing cellular information, an intrinsic capacity for self-repair appears to have evolved simultaneously with the environmental adaptation of this molecule. This repair process is believed to influence the kinetics of mutation induction by preventing many induced lesions from becoming fixed as permanent damage. The first portion of this chapter presents some of the fundamentals of the science of genetics. The second section is devoted to a presentation of the categories and mechanisms of DNA alterations that generate genotoxic effects in cells and the mechanisms of DNA repair.

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1-Basic genetics for toxicologists

1-1. Gene Structure

The hereditary informational molecules of all living systems, with the exception of some viruses that use RNA, are composed of DNA, and those organisms that store their hereditary information in RNA go through a DNA-intermediate during replication. Some common characteristic features of DNA molecules are listed in Table 2.(2) DNA is the macromolecule from which all characteristics of life ultimately derive (Figure 2) (1).

Table 2. Basic Biochemical Characteristics of All Double-Stranded DNA (1).

I. DNA consists of two different purines (guanine, adenine) and two different pyrimidines (thymine and cytosine) **2**. A nucleotide pair consists of one purine and one pyrimidine [adenine/thymine (AT) or guanine/cytosine (GC)] **3**. Nucleotide pairs are connected into a double-helix molecule by sugarphosphate backbone linkages and hydrogen bonding (Figure 2.1) **4.** The AT base pair is held by two hydrogen bonds, while the GC is held by three **5.** The distance between each base pair in a molecule is 3.4 A, producing ten nucleotide pairs per tum of the DNA helix **6.** The number of adenine molecules must equal the number of thymine molecules in a DNA molecule; the same relationship exists for guanine and cytosine molecules; the ratio of AT to GC base pairs, however, may vary in DNA from species to species	**7**. The two strands of the double helix are complementary and antiparallel with respect to the polarity of the two sugar-phosphate backbones, one strand being 3'-5' and the other being 5'-3' with respect to the terminal OH group on the ribose sugar **8.** DNA replicates by a semiconservative method in which the two strands separate and each is used as a template for the synthesis of a new complementary strand **9.** The rate of DNA nucleotide polymerization during replication is approximately 600 nucleotides/sec; the helix must unwind to form templates at a rate of 3600 rpm to accommodate this replication rate **10.** The DNA content of cells is variable (1.8 x 109 daltons for Escherichia coli to 1.9 x 1011 daltons for human cells)

The simplest complete functional unit in a DNA molecule is termed a "gene". The differences between the genes of prokaryotic organisms (bacteria) and eukaryotic organisms (plant and animal cells) center primarily on their number, location on the respective chromosomal entities, and mechanisms of gene regulation (Table 3). In prokaryotic cells there is a single chromosomal entity with little or no differentiation along the DNA molecule as far as function is concerned. Eukaryotic cells, on the other hand, have DNA with non-functional, repeated sequences of some genes; these cells also have regions of noncoding DNA, called introns, between coding sequences called exons. The function of repeated DNA sequences and intron regions is not known. The nucleotide composition and the mechanisms by which information encoded in a gene are transformed into gene products appear to be universal (2).

Table 3. Characteristics of DNA in Prokaryotic and Eukaryotic cell types (2)

Prokaryotic cells	Eukaryotic cells
Primarily haploid DNA uncomplexed DNA nonlocalized in the cell cytoplasm No morphologic stages in DNA replication DNA often found as a closed circle Replication not associated with cellular organelles All genes encoded in the DNA are functional Spacer sequences have not been identified.	Primarily diploid DNA complexed with proteins forming chromosomes DNA localized primarily within the nucleus of the cell DNA replication described by mitotic cycle consisting of specific cytologic stages DNA found in linear chromosomes Replication and separation of chromosome associated with cellular organelles called centrioles Repetitive, nonfunctional gene sequences are common Noncoding spacer sequences identified as introns occur along the DNA model

1-2. Somatic and germ-cell characteristics

Mammals and many other multicellular organisms are composed of two cell types, **somatic cells** and **germ cells**. Somatic cells constitute the major portion of the mammalian organism. The genomes of somatic cells are generally diploid (i.e., having two complete sets of chromosomes). While genetic damage to somatic cells may be transmitted to daughter cells following mitotic

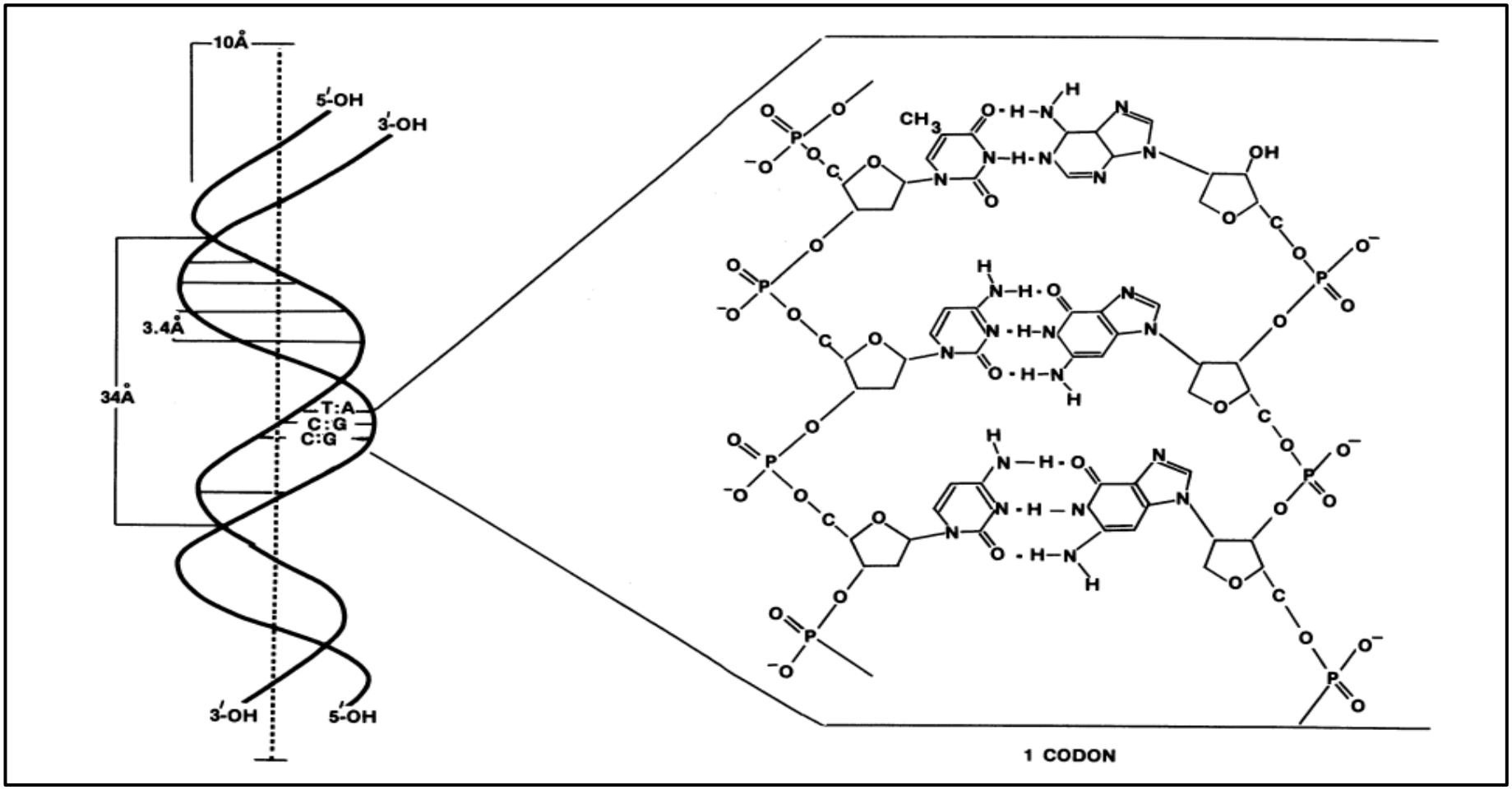

Figure 2. The basic structure of DNA. An enlarged codon consisting of three base pairs is shown. This enlarged view illustrates the base configuration and hydrogen bonding relationships for DNA. Hydrogen bonding involves weak interactions between N, O, and H atoms and allows the two strands to separate from each other over short regions or during replication. One codon specifies an amino acid (1).

cell division, it is not transmissible to subsequent generations. Virtually all in vitro mammalian cell assays used in genetic toxicology employ somatic cell types as target organisms. Germ cells, the second cell type, form a unique cell population in multicellular organisms and play a specialized role in sexual reproduction. Mature germ cells are derived from diploid stem cells in gonadal tissues, and following meiosis, carry a haploid set of chromosomes. Mutations in germ cells result in a broad array of heritable genetic diseases, congenital malformations, and other disorders in subsequent generations. The incidence of heritable genetic damage in the total number of germ cells residing in a population (species) is called the genetic load. Mutations are classified as recessive or dominant, depending upon their expression in the diploid state. Dominant mutations are expressed when a single mutant allele is present (2).

A large proportion of human genetic diseases, however, are associated with recessive mutant genes and are expressed only when two mutant alleles (one contributed by each parent) are present in the homozygous condition. Recessive mutations are maintained in the gene pool in the heterozygous state and are carried in that configuration by individuals who are phenotypically normal. The inability to easily identify heterozygous carriers of mutant alleles is one of the primary factors preventing the human genetic burden from being eliminated or even reduced (2)

1-3. Gene function

With only minor exceptions, it is assumed that gene function at the molecular level parallels DNA structural similarity and is identical in all organisms. The following characteristics represent the basics of gene function (1).

1- A gene is composed of a segment of a DNA molecule containing sufficient information to produce a functional product. The products of almost all genes are polypeptides (amino acid heteropolymers). The function of these polypeptides can be structural, enzymatic, or regulatory. All visible and functional qualities of an organism, defined as the phenotype, are dependent upon the accurate structure and expression of these gene products.
2- The information required to specify a single amino acid is contained in a triplet of base pairs called a codon. The sequence of the bases in the codons is important for translation of information. Table 2.2 gives the codon dictionary for the naturally occurring amino acids used to synthesize protein. More than one codon can specify a given amino acid. For example, 5' CGU 3', 5' CGA 3', and 5' AAG 3' all code for the amino acid arginine. The sequence of codons in a gene specifies the sequence of amino acids in the polypeptide and thus its ultimate role in cellular processes (1).

3- Eventually be expressed by cell or organism death, or in an altered form of the cell or organism (Figure 2.2). Thus, these changes form the basis for genetic alterations, or "mutations."

4- The production of a polypeptide gene product involves two types of RNA: messenger RNA (mRNA), a short-lived copy of the gene being expressed; and transfer RNA (tRNA), which contributes to both the transportation and coupling of amino acids into the polypeptide gene product. This latter process also involves an RNA-protein structure called a ribosome. The basic process identified in prokaryotic (bacterial) cells is illustrated in Figure 4

5- The initiation and termination of the transcription of a gene into a polypeptide are regulated by a separate set of regulatory (promoter) genes. Most regulatory genes respond to chemical or temporal cues such that only those genes needed at a given time are expressed. The remaining genes are in an inactive state. The processes of gene activation and inactivation are believed to be critical to cellular differentiation in multicellular organisms.

Table 4. Dictionary of the Genetic Code for Proteins[a]

1st base in codon	2nd base in codon: U	C	A	G	3rd base in codon
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

[a] U substitutes for T in RNA. The DNA code sequence would be complementary to each RNA code [e.g., (DNA sequence/CAG) → (RNA sequence/GUC) → (amino acid/valine)]. Codon words read from 5'-OH end (left) to 3'-OH end (right). Codons UGA, UAA, and UAG are all terminating sequences and do not code for any amino acids.

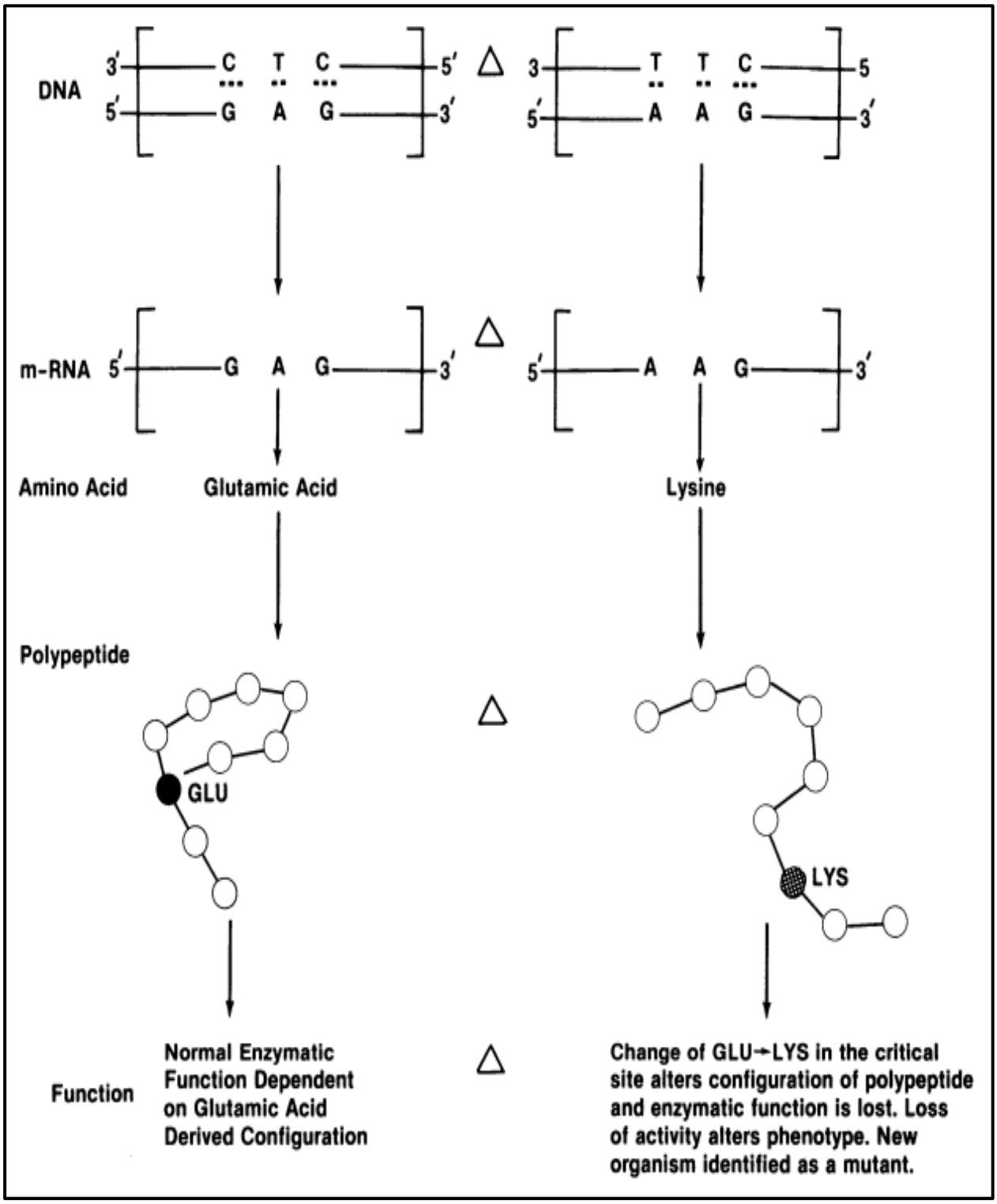

Figure 3. Gene-protein relationships in mutation induction. A change in the base sequence of one codon may produce an amino acid replacement in the gene product that results in a nonfunctional polypeptide (1).

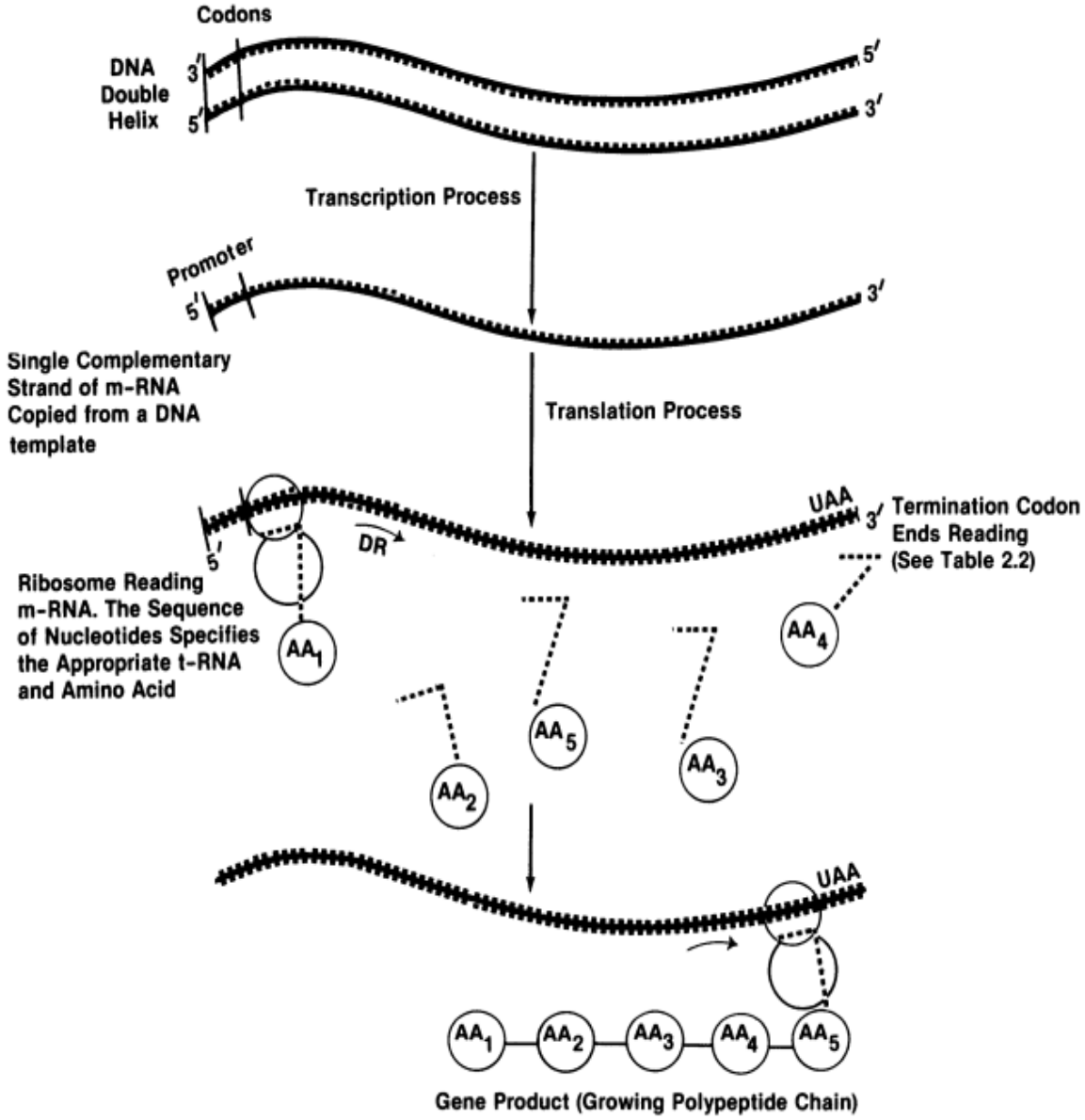

Figure 4. Protein synthesis steps (1)

2-Cell cycle in eukaryotes

The cell is the basic structural and functional unit of any living being. It is the fundamental building block, which when combined with similar cells forms a tissue and organs. Mammalian organisms consist of several distinct cell types that have acquired specific functions during differentiation. All of the cell types in the mature organism originated from one of the three embryonic cell types (mesodermal, entodermal, or ectodermal). Histopathologic diagnosis of toxic effects relies upon examination of the morphology and histochemistry of differentiated cell types. Issues relevant to genetic toxicology focus more on the hereditary status of the target cell. Somatic cells constitute the major portion of the mammalian organism. The genomes of most

somatic cells are diploid. Damage to somatic cells is not transmissible to subsequent generations and may have toxicologic consequences only to the exposed cell population. Virtually all *in vitro* assays measure damage in somatic cells. Germ cells are those cells that undergo meiotic division to haploid gametes. Damage to germ cells has the potential for transmission to the next generation. The cell undergoes a series of events that result in the duplication of cell along with the DNA. This is known as the cell cycle (2).

2-1.Definition

Cell cycle refers to the series of events that take place in a cell, resulting in the duplication of DNA and division of cytoplasm and organelles to produce two daughter cells. It was discovered by Prevost and Dumas (1824) while studying the cleavage of zygote of Frog. It is a series of stages a cell passes through to divide and produce new cells (3).

This entire process where with the help of one single parent cell, a new cell population grows and develops is known as the cell cycle.

2-2.Phases of somatic Cell Cycle

Cell cycle takes place in a cell leading to its maturity and subsequent division. This event includes duplication of its genome and synthesis of the cell organelles followed by division of the cytoplasm.

Human cells exhibit typical eukaryotic cell cycle (figure X) and take around 24 hours to complete one cycle of growth and division. The duration of the cycle, however, varies from organism to organism and cell to cell (6).

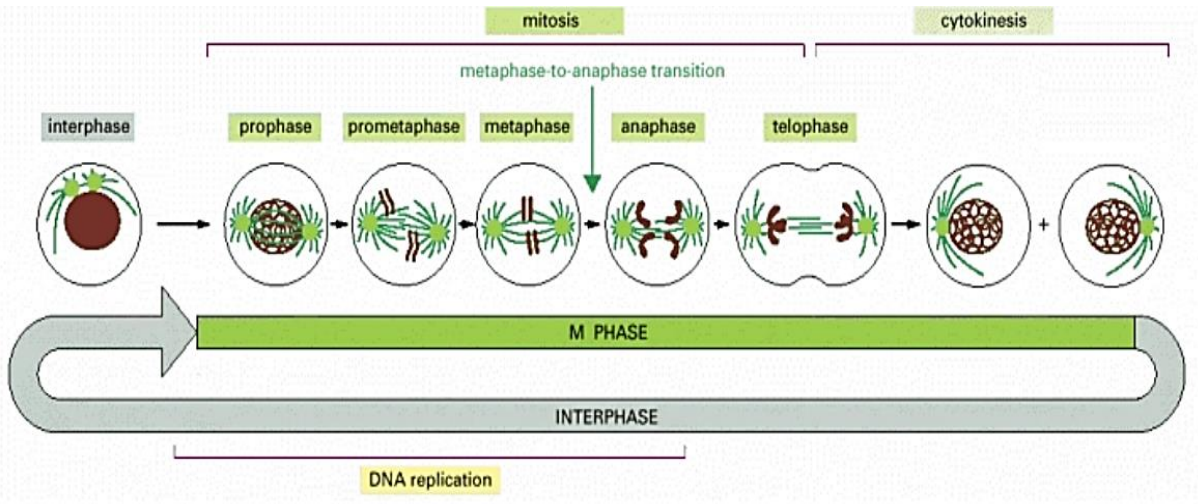

Figure 5. The events of eukaryotic cell division [1]

The cell cycle is composed of the **mitotic (M) phase** and **interphase** (Figure 6). The mitotic phase, which includes both mitosis and cytokinesis, is the shortest part of the cell cycle. Most of the time, the cells are in interphase, which is composed of three different subphases: the **G_1 phase** (first gap), the **S phase** (synthesis), and the **G_2 phase** (second gap).

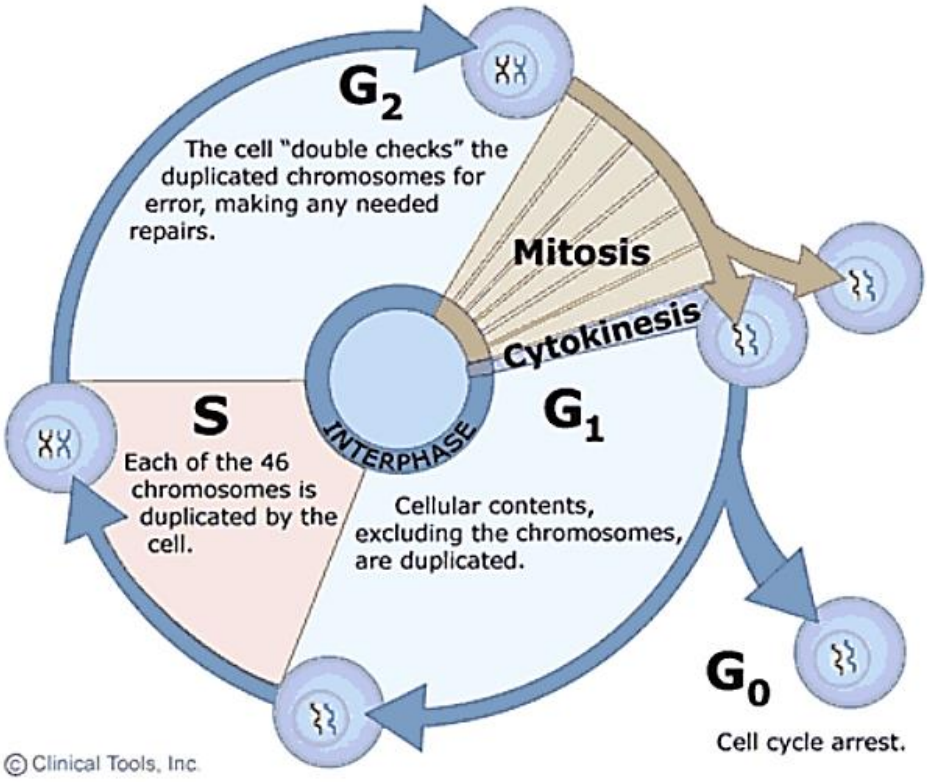

Figure 6. Phases of the cell cycle: the key steps a cell must go through in order to divide [2]

1-Interphase

Also known as the resting phase of the cell cycle; The goal of interphase is to grow by producing proteins and cytoplasmic organelle such as the endoplasmic reticulum. DNA is replicated during the S phase only (2). It occupies around 95% time of the overall cycle. The interphase is divided into three phases:

- **G1 phase (Gap 1)** – G1 phase is the phase of the cell between mitosis and initiation of replication of the genetic material of the cell. During this phase, the cell is metabolically active and continues to grow without replicating its DNA.
- **S phase (Synthesis)** – DNA replication takes place during this phase. If the initial quantity of DNA in the cell is denoted as *2N*, then after replication it becomes *4N*. However the number of chromosomes does not vary, viz., if the number of chromosomes

during G1 phase was *2n*, it will remain *2n* at the end of S phase. The centriole also divides into two centriole pairs in the cells which contain centriole (6)

During the S phase, the chromosomes and chromatin protein that govern various aspects of chromosomes are duplicated accurately. Chromosome duplication is triggered by the activation of S-Cdk.

- **G2 phase (Gap 2)** –During this phase, the RNA, proteins, other macromolecules required for multiplication of cell organelles, spindle formation, and cell growth are produced as the cell prepares to go into the mitotic phase.

Some cells like cardiac cells in the adult animals do not exhibit division and some others only divide to replace those cells which have been either damaged or lost due to cell death. Such cells which do not divide further attain an inactive G0 phase also known as quiescent phase after they exit the G1 phase. These cells remain metabolically active but do not divide unless called upon to do so (6).

2-M phase (mitosis + cytokinesis)

During the M phase or the phase of the equational division, the cell undergoes a complete reorganization to give birth to a progeny, the replicated chromosomes are segregated into individual nuclei (mitosis) that have the same number of chromosomes as the parent cell, and the other organelles are also splited equally by the process of cytokinesis which is preceded by mitotic nuclear division. (6, 7)

a-Mitosis or mitotic phase is divided into four overlapping stages:

2. Prophase, the first stage of mitosis.
    - The chromosomes condense and become visible
    - The centrioles form and move toward opposite ends of the cell ("the poles")
    - The nuclear membrane dissolves
    - The mitotic spindle forms (from the centrioles in animal cells)
    - Spindle fibers from each centriole attach to each sister chromatid at the kinetochore
3. Metaphase,
    - The Centrioles complete their migration to the poles
    - The chromosomes line up in the middle of the cell ("the equator")
4. Anaphase,
    - Spindles attached to kinetochores begin to shorten.

- This exerts a force on the sister chromatids that pulls them apart.
- Spindle fibers continue to shorten, pulling chromatids to opposite poles.
- This ensures that each daughter cell gets identical sets of chromosomes.

5. Telophase,

- The chromosomes decondense
- The nuclear envelope forms

b-Cytokinesis

In this phase, the cytoplasm of the cell divides. It begins as soon as the mitosis ends. Plant cells are much tougher than animal cells, as they have a rigid cell wall and high internal pressure. Thus, cytokinesis occurs in plant and animal cells differently. Cytokinesis is slightly different in plant and animal cells. In animal cells, a contractile ring of actin and myosin divides the cytoplasm into two. By contrast in plants, a growth plate comprised of fused golgi vesicles grows outward from the middle of the cell, dividing the old cell in two (6, 7).

A cell doesn't continuously divide. Some cells, such as nerve cells and muscle cells, do not divide at all or they do it a very low rate in a mature humans. Cell division rates and timing are crucial for development, growth and maintenance. Breakdown in cell cycle control plays a major role in cancer development.

2-3.Germ cells cycle "Meiosis"

Meiosis is a process where a single cell divides twice to produce four cells containing half the original amount of genetic information.

- During meiosis one cell divides twice to form four daughter cells.
- These four daughter cells only have half the number of chromosomes of the parent cell – they are haploid.
- Meiosis produces our sex cells or gametes – egg and sperm cells.

What happens in meiosis?

Meiosis can be divided into nine stages. These are divided between the first time the cell divides (meiosis I) and the second time it divides (meiosis II).

Meiosis I [5]

1.Interphase:

- The DNA in the cell is copied resulting in two identical full sets of chromosomes.

- Outside of the nucleus are two centrosomes, each containing a pair of centrioles; these structures are critical for the process of cell division.
- During interphase, microtubules extend from these centrosomes.

2.Prophase I:

- The copied chromosomes condense into X-shaped structures that can be easily seen under a microscope.
- Each chromosome is composed of two sister chromatids containing identical genetic information.
- The chromosomes pair up so that both copies of chromosome 1 are together, both copies of chromosome 2 are together, and so on.
- The pairs of chromosomes may then exchange bits of DNA in a process called recombination or crossing over.
- At the end of Prophase I the membrane around the nucleus in the cell dissolves away, releasing the chromosomes.
- The meiotic spindle, consisting of microtubules and other proteins, extends across the cell between the centrioles.

3.Metaphase I:

- The chromosome pairs line up next to each other along the centre (equator) of the cell.
- The centrioles are now at opposite poles of the cell with the meiotic spindles extending from them.
- The meiotic spindle fibres attach to one chromosome of each pair.

4.Anaphase I:

- The pair of chromosomes are then pulled apart by the meiotic spindle, which pulls one chromosome to one pole of the cell and the other chromosome to the opposite pole.
- In meiosis I the sister chromatids stay together. This is different to what happens in mitosis and meiosis II.

5.Telophase I and cytokinesis:

- The chromosomes complete their move to the opposite poles of the cell.
- At each pole of the cell a full set of chromosomes gather together.
- A membrane forms around each set of chromosomes to create two new nuclei.

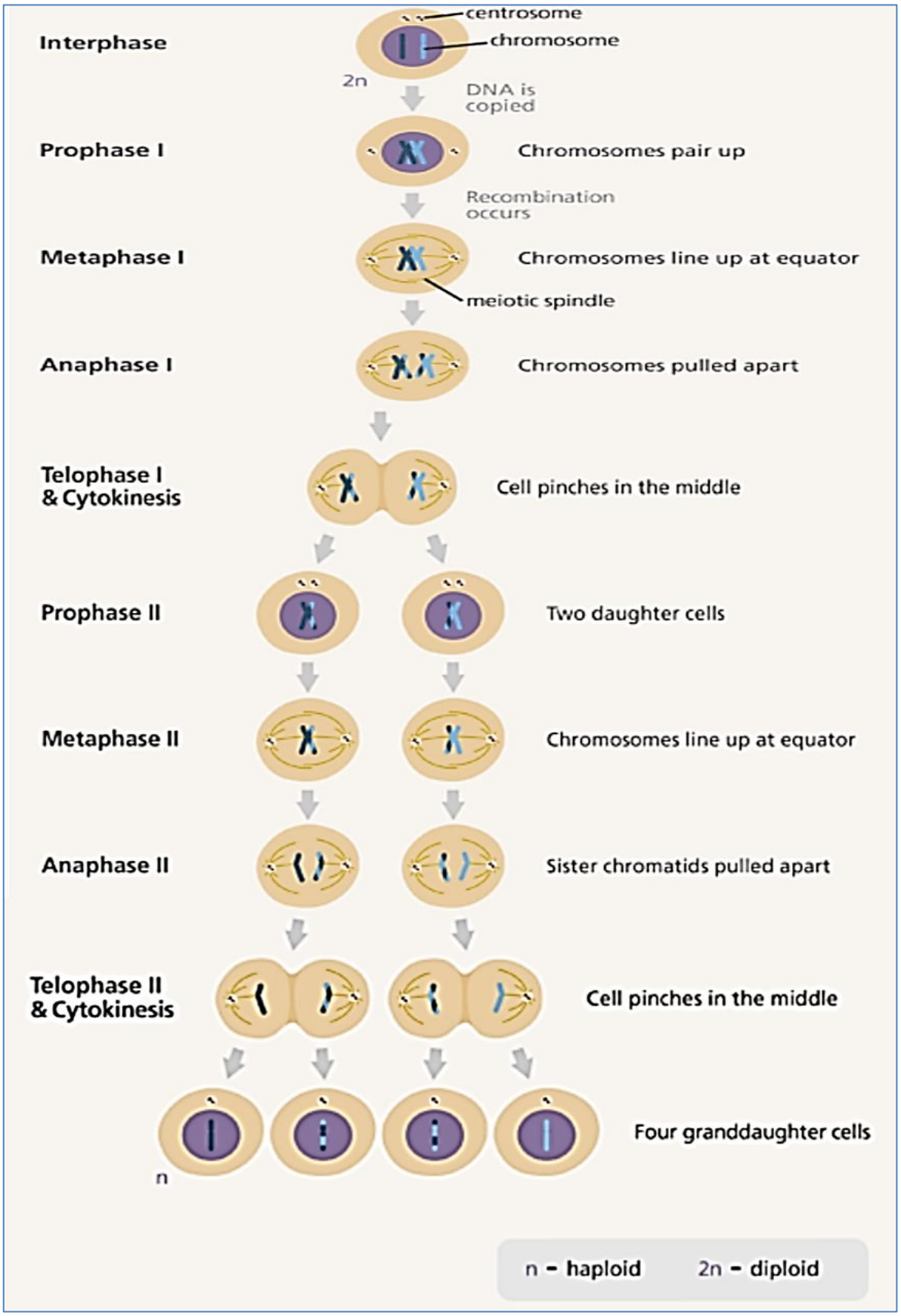

Figure 7: Illustration showing the nine stages of meiosis [3].

- The single cell then pinches in the middle to form two separate daughter cells each containing a full set of chromosomes within a nucleus. This process is known as cytokinesis.

6.Prophase II:

- Now there are two daughter cells, each with 23 chromosomes (23 pairs of chromatids).
- In each of the two daughter cells the chromosomes condense again into visible X-shaped structures that can be easily seen under a microscope.
- The membrane around the nucleus in each daughter cell dissolves away releasing the chromosomes.
- The centrioles duplicate.
- The meiotic spindle forms again

7.Metaphase II:

- In each of the two daughter cells the chromosomes (pair of sister chromatids) line up end-to-end along the equator of the cell.
- The centrioles are now at opposites poles in each of the daughter cells.
- Meiotic spindle fibres at each pole of the cell attach to each of the sister chromatids.

8.Anaphase II:

- The sister chromatids are then pulled to opposite poles due to the action of the meiotic spindle.
- The separated chromatids are now individual chromosomes.

9.Telophase II and cytokinesis:

- The chromosomes complete their move to the opposite poles of the cell.
- At each pole of the cell a full set of chromosomes gather together.
- A membrane forms around each set of chromosomes to create two new cell nuclei.
- This is the last phase of meiosis, however cell division is not complete without another round of cytokinesis.
- Once cytokinesis is complete there are four granddaughter cells, each with half a set of chromosomes (haploid):
- in people with XY chromosomes, these four cells are all sperm cells
- in people with XX chromosomes, one of the cells is an egg cell while the other three are polar bodies (small cells that do not develop into eggs) [3]

3-DNA alterations resulting in genotoxic effects in the cell

Mutation Most biological molecules have a limited lifetime. Many proteins, lipids and RNAs are degraded when they are no longer needed or damaged, and smaller molecules such as sugars are metabolized to compounds to make or store energy. In contrast, DNA is the most stable biological molecule known, befitting its role in storage of genetic information. According to the classic definition mutations are sudden heritable changes in the DNA (8).

The process (change) itself is still called mutation, but due to the fast development of genetics and genomics, two terms related to the variations in the sequence had to be modified. Next to the above mentioned definition the term mutation is also used to indicate a disease-causing change or sometimes rare change. Similarly, the term polymorphism is used both to indicate a non disease-causing change or a change found at a frequency of 1% or higher in the population. In the era of advanced DNA sequencing tools and personal genomics, these earlier definitions of mutation and polymorphism are antiquated. a mutation would be a "DNA variant" acquired over the lifetime of an organism, i.e. a somatic mutation. In this sense, mutations are the principal causes of many diseases like cancer but are typically not inherited by their offspring. Alterations in the DNA of germ cells – sperms and eggs – can be inherited by offspring and are currently called germline mutations. In this case, the term mutation should be used only if the germline "variant" has been detected using as a reference the germline DNA of the same individual (9).

3-1-Mutagens and types

Mutations that are caused by agents that damage the DNA are known as induced mutations. Agents that mutate DNA are called mutagens and are of three main types: mutagenic chemicals, radiation and heat. Even if there are no dangerous chemicals or radiation around, mutations still occur, though less frequently. These are spontaneous mutations. Some of these are due to errors in DNA replication. The enzymes of DNA replication are not perfect and sometimes make mistakes. In addition, DNA undergoes certain spontaneous chemical reactions (alterations) at a low but detectable rate and this rate goes up with increasing temperature (9).

3-1-1.chemicals mutagens

They are toxic that react with DNA and alter the chemical structure of the bases. For example, EMS (ethyl methane sulfonate) is widely used by molecular biologists to mutagenize growing cells. It adds an ethyl group to bases in DNA and so changes their shape and their base-pairing properties. Nitrite converts amino groups to hydroxyl groups and so converts the base cytosine to uracil. Nitrite is used experimentally to mutate purified DNA, such as a cloned gene carried on a

plasmid, while the plasmid is in the test tube. The mutagenized DNA is then transferred back into a cell to identify the mutations that were generated. During DNA replication, the DNA polymerase misidentifies these altered bases and puts in the wrong bases in the new complementary strand of DNA it is making (9).

a-**Base analogs** are chemical mutagens that mimic the bases found in natural DNA. For example, bromouracil resembles thymine in shape. It is converted by the cell to the DNA precursor, bromouridine triphosphate, which DNA polymerase inserts where thymine should go. Unfortunately, bromouracil can flip-flop between two alternative shapes .

In its alternate form, bromouracil resembles cytosine and pairs with guanine. If bromouracil is in its misleading form when DNA polymerase arrives, a G will be put into the new strand opposite the bromouracil instead of A. Some mutagens imitate the structure of a base pair rather than a single base. abnormal development of the embryo, which results in gross structural defects (9).

3-1-2.Physicical mutagens

Some types of radiation cause mutations. High frequency electromagnetic radiation, ultraviolet radiation (UV light), X-rays and gamma rays (g-rays), directly damage DNA. X-rays and g-rays are ionizing radiation; that is, they react with water and other molecules to generate ions and free radicals, notably hydroxyl radicals. Ionizing radiation is responsible for about 70 percent of the radiation damage to DNA. The other 30 percent of the radiation damage is due to direct interaction of X-rays and g-rays with DNA itself (9).

In the early days of molecular biology, X-rays were often used to generate mutations in the laboratory. X-rays tend to produce multiple mutations and often yield rearrangements of the DNA, such as deletions, inversions and translocations.

Ultraviolet radiation is electromagnetic radiation with wavelengths from 100 to 400 nm. It is nonionizing and acts directly on the DNA. The bases of DNA show an absorption peak at around 254 nm and UV close to this wavelength is absorbed very efficiently by DNA. In particular, UV causes two neighboring pyrimidine bases to cross-react with each other to give dimers. Thymine dimers are especially frequent . Although DNA polymerase can proceed by skipping over thymine dimers, this leaves a single-stranded region that needs repairing (9).

In addition to electromagnetic radiation, there are other forms of radiation, such as the A-particles and B-particles emitted by radioactive materials along with G-rays. Most A-particles are too weak even to penetrate skin but B-particles may cause significant damage to DNA and other biological molecules. However, A-emitters can be mutagenic if they have entered the body, for example by being breathed in or swallowed (figure 8).

3-1-3.- Biological mutagens:

Such as virus and transposons that can isert themselves within a gene and destroy its function

3-2-Phenotypical classification of mutation

Although all mutations represent biochemical changes, they have various manifestations such as **a- Colour mutation ;** these change in the colour e.g. red eyes to white eyes in Drosophila , red flowers to whith flowers in peas plant (10)..

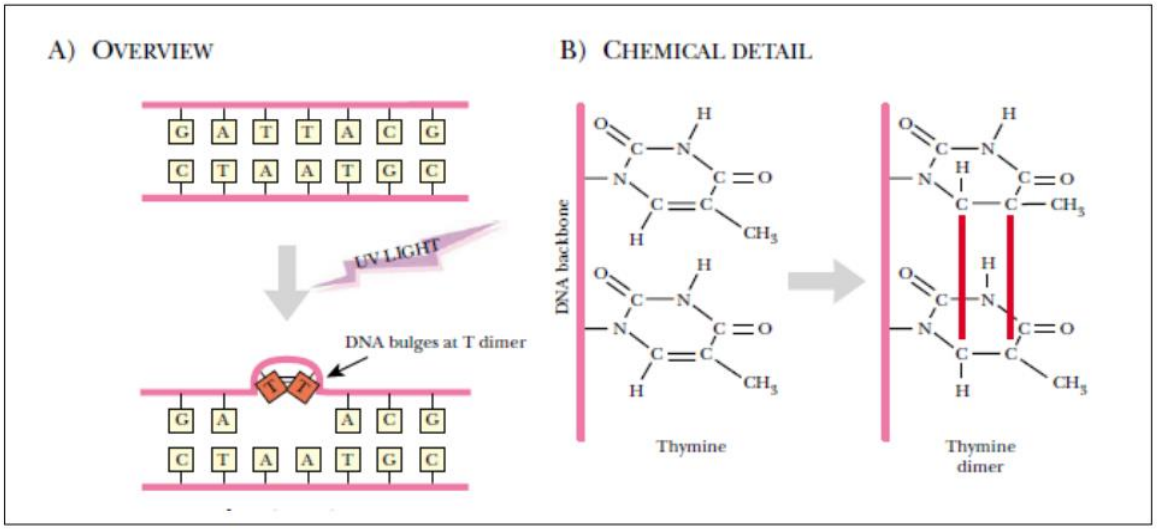

Figure 8. Thymine Dimers A) Ultraviolet light (UV) sometimes results in the formation of a thymine dimer (red). B) The detailed chemical structure of the thymine dimer is shown [4].

b- Morphological mutation; these change the morphology of the mutant e.g normal wings to curly wings in Drosophila , normal coloniesto fluffy colonies in the fungus *Aspergillus*.

c- Resistant mutation ; these make mutant capable of growth on chemical or antibiotic that are toxic to the wild type e.g drug resistant mutations in microorganisms.

4- Auxtrophic (nutritional) mutations ; these make mutant unable to grow on the minimal medium (MM) unless a certain nutrient is added to the medium.The wild type can grow on MM and is called prototrophic .

3-3-The Major Types of Mutation

A single mutation is a single event and a multiple mutation is the result of several events. A single mutational event, however large or complex its effect, is regarded as a single mutation. A mutation that involves only a single base is known as a point mutation. A null mutation totally inactivates a gene; the expression "null mutation" is a genotypic term. Complete absence of a gene product may or may not cause a detectable phenotype.

A tight mutation is one whose phenotype is clear-cut. The complete loss of a particular enzyme may result in no product in a particular biochemical pathway. For example, the complete inability of a bacterium to grow if provided with a certain sugar is an example of a tight mutation. A leaky mutation is one where partial activity remains. For example, 10 percent residual enzyme activity might allow a bacterium to still grow, albeit very slowly. The major types of sequence alteration are as follows, and will be discussed separately below (10).:

1. Base substitution: one base is replaced by another base.If one base is replaced by another, a base substitution mutation has occurred. These may be subdivided into transitions and transversions. In a transition a pyrimidine is replaced by another pyrimidine (i.e., T is replaced by C or vice versa) or a purine is replaced by another purine (i.e., A is replaced by G or vice versa). A transversion occurs when one base is replaced by another of a different type; for example, a pyrimidine is replaced by a purine or vice versa (10)..

2. Insertion: one or more bases are inserted into the DNA sequence. Genes may also be inactivated by insertions of DNA. If a foreign segment of DNA is inserted into the coding region, then the gene is said to be disrupted. (Fig. 7.3).The cause of insertion mutations may be divided into two distinct categories. Some of these mutations are the result of mobile genetic elements, usually thousands of bases long, inserting themselves into a gene. Other insertion mutations, usually only one or a few bases long, are caused by mutagenic chemicals or by mistakes made by DNA polymerase (10)..

Occasionally, insertions may activate genes. If an insertion occurs in the recognitionsite for a repressor, binding of the repressor will be prevented and activation of the gene may result.

3. Deletion: one or more bases are deleted from the DNA sequence. In particular, we should distinguish between point mutations where one (or a very few) bases are affected, and gross deletions and insertions that affect long segments of DNA. Point deletions and insertions may have major effects due to disruption of the reading frame—see below. Here we will consider the

effects of larger deletions. Large deletions may remove part of a gene, an entire gene or several genes. Deletions may also remove part or all of the regulatory region for a gene. Depending on the precise region removed, gene expression may be decreased or increased. For example, a deletion that removes the binding site for a repressor may result in a large increase in activity of the gene in question. Thus loss of DNA may result in elevated activity (10).

4. Inversion: a segment of DNA is inverted, but remains at the same overall location. 5. Duplication: a segment of DNA is duplicated; the second copy usually remains at the same location as the original. 6. Translocation: a segment of DNA is transferred from its original location to another position either on the same DNA molecule or on a different DNA molecule (10).

4-DNA Repair mechanisms

Many of these processes were first studies in bacteria such as E. coli, however only a few are limited to this species. For instance, nucleotide excision repair and base excision repair are found in virtually all organisms, and they have been well characterized in bacteria, yeast, and mammals. Like DNA replication itself, repair of damage and misincorporation is a very old process.

4-1. Reversal of damage

Some kinds of covalent alteration to bases in DNA can be directly reversed. This occurs by specific enzyme systems recognizing the altered base and breaking bonds to remove the adduct or change the base back to its normal structure (11).

4-1.1.Photoreactivation is a light-dependent process used by bacteria to reverse pyrimidine dimers formed by UV radiation. The enzyme photolyase binds to a pyrimidine dimer and catalyzes a second photochemical reaction (this time using visible light) that breaks the cyclobutane ring and reforms the two adjacent thymidylates in DNA. Note that this is not formally the reverse of the reaction that formed the pyrimidine dimers, since energy from visible light is used to break the bonds between the pyrimidines, and no UV radiation is released.

A second example of the reversal of damage is the removal of methyl groups. For instance, the enzyme O6-methylguanine methyltransferase, encoded by the ada gene in *E. coli*, recognizes O6-methylguanine in duplex DNA. It then removes the methyl group, transferring it to an amino

acid of the enzyme. The methylated enzyme is no longer active, hence this has been referred to as a suicide mechanism for the enzyme (11).

4-2.Excision repair

The most common means of repairing damage or a mismatch is to cut it out of the duplex DNA and recopy the remaining complementary strand of DNA, as outlined in Fig. 9. Three different types of excision repair have been characterized: nucleotide excision repair, base excision repair, and mismatch repair. All utilize a cut, copy, and paste mechanism. In the cutting stage, an enzyme or complex removes a damaged base or a string of nucleotides from the DNA. For the copying, a DNA polymerase (DNA polymerase I in E. coli) will copy the template to replace the excised, damaged strand. The DNA polymerase can initiate synthesis from 3' OH at the single-strand break (nick) or gap in the DNA remaining at the site of damage after excision. Finally, in the pasting stage, DNA ligase seals the remaining nick to give an intact, repaired DNA (11).

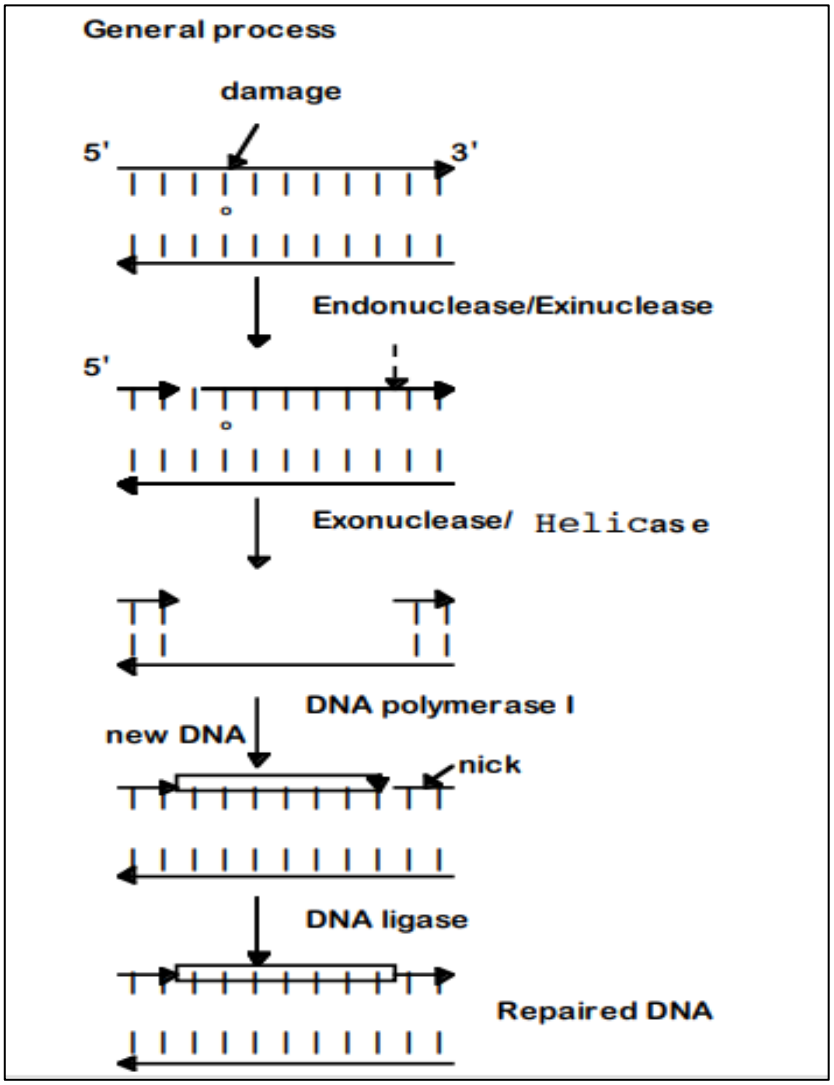

Figure 9. A general scheme for excision repair (11).

4-3-Nucleotide excision repair

In nucleotide excision repair (NER), damaged bases are cut out within a string of nucleotides, and replaced with DNA as directed by the undamaged template strand. This repair system is used to remove pyrimidine dimers formed by UV radiation as well as nucleotides modified by bulky chemical adducts. The common feature of damage that is repaired by nucleotide excision is that the modified nucleotides cause a significant distortion in the DNA helix. NER occurs in almost all organisms examined. Some of the best-characterized enzymes catalyzing this process are the UvrABC excinuclease and the UvrD helicase in *E. coli*. The genes encoding this repair function were discovered as mutants that are highly sensitive to UV damage, indicating that the mutants are defective in UV repair (11).

4-4-Base excision repair

Base excision repair differs from nucleotide excision repair in the types substrates recognized and in the initial cleavage event. Unlike NER, the base excision machinery recognizes damaged bases that do not cause a significant distortion to the DNA helix, such as the products of oxidizing agents. For example, base excision can remove uridines from DNA, even though a G:U base pair does not distort the DNA. Base excision repair is versatile, and this process also can remove some damaged bases that do distort the DNA, such as methylated purines. In general, the initial recognition is a specific damaged base, not a helical distortion in the DNA. A second major difference is that the initial cleavage is directed at the glycosidic bond connecting the purine or pyrimidine base to a deoxyribose in DNA. This contrasts with the initial cleavage of a phosphodiester bond in NER.

4-5-Mismatch repair

The third type of excision repair we will consider is mismatch repair, which is used to repair errors that occur during DNA synthesis. Proofreading during replication is good but not perfect. Even with a functional ε subunit, DNA polymerase III allows the wrong nucleotide to be incorporated about once in every 108 bp synthesized in E. coli. However, the measured mutation rate in bacteria is as low as one mistake per 10^{10} or 10^{11} bp. The enzymes that catalyze mismatch repair are responsible for this final degree of accuracy. They recognize misincorporated nucleotides, excise them and replace them with the correct nucleotides. In contrast to nucleotide excision repair, mismatch repair does not operate on bulky adducts or major distortions to the DNA helix. Most of the mismatches are substitutes within a chemical class, e.g. a C incorporated instead of a T. This causes only a subtle helical distortions in the DNA, and the misincorporated

nucleotide is a normal component of DNA. The ability of a cell to recognize a mismatch reflects the exquisite specificity of MutS, which can distinguish normal base pairs from those resulting from misincorporation. Of course, the repair machinery needs to know which of the nucleotides at a mismatch pair is the correct one and which was misincorporated. It does this by determining which strand was more recently synthesized, and repairing the mismatch on the nascent strand (11).

4-6-**Recombination repair (Retrieval system)**

In the three types of excision repair, the damaged or misincorporated nucleotides are cut out of DNA, and the remaining strand of DNA is used for synthesis of the correct DNA sequence. However, this complementary strand is not always available. Sometimes DNA polymerase has to synthesize past a lesion, such as a pyrimidine dimer or an AP site. One way it can do this is to stop on one side of the lesion and then resume synthesis about 1000 nucleotides further down. This leaves a gap in the strand opposite the lesion (Fig 9). The information needed at the gap is retrieved from the normal daughter molecule by bringing in a single strand of DNA, using RecA-mediated recombination. This fills the gap opposite the dimer, and the dimer can now be replaced by excision repair (Fig. 9). The resulting gap in the (previously) normal daughter can be filled in by DNA polymerase, using the good template (11).

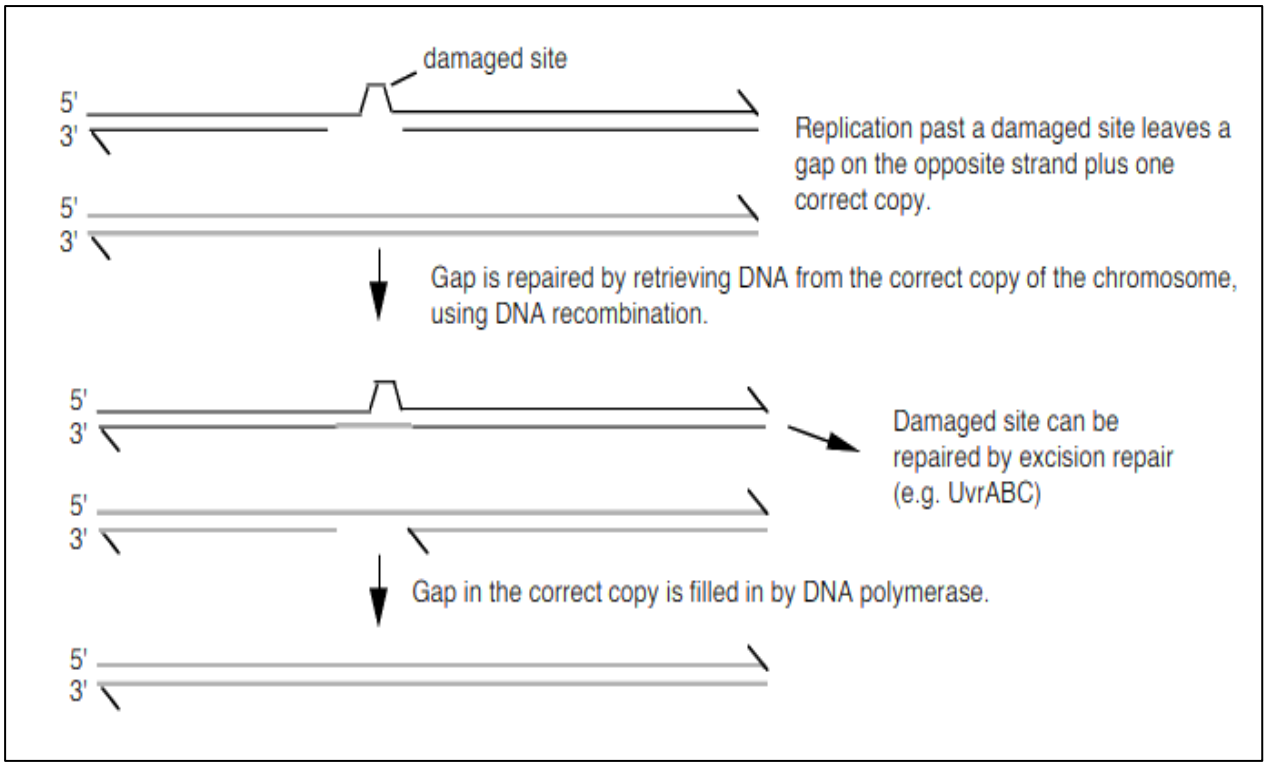

Figure 9. Recombination repair, a system for retrieval of information [5]

4-7-Translesion synthesis

As just described, DNA polymerase can skip past a lesion on the template strand, leaving behind a gap. It has another option when such a lesion is encountered, which is to synthesis DNA in a non-template directed manner. This is called translesion synthesis, bypass synthesis, or error-prone repair. This is the last resort for DNA repair, e.g. when repair has not occurred prior to replication. In translesion replication, the DNA polymerase shifts from template directed synthesis to catalyzing the incorporation of random nucleotides (10, 11).

These random nucleotides are usually mutations (i.e. in three out of four times), hence this process is also designated errorprone repair. Translesion synthesis uses the products of the umuC and umuD genes. These genes are named for the UV nonmutable phenotype of mutants defective in these genes (10, 11).

4-8-The SOS response

A coordinated battery of responses to DNA damage in E. coli is referred to as the SOS response. This name is derived from the maritime distress call, “SOS” for "Save Our Ship". Accumulating damage to DNA, e.g. from high doses of radiation that break the DNA backbone, will generate single-stranded regions in DNA. The increasing amounts of singlestranded DNA induce SOS functions, which stimulate both the recombination repair and the translesional synthesis just discussed (11).

Key proteins in the SOS response are RecA and LexA. RecA binds to single stranded regions in DNA, which activates new functions in the protein. One of these is a capacity to further activate a latent proteolytic activity found in several proteins, including the LexA repressor, the UmuD protein and the repressor encoded by bacteriophage lambda (Fig. 10). RecA activated by binding to single-stranded DNA is not itself a protease, but rather it serves as a co-protease, activating the latent proteolytic function in LexA, UmuD and some other proteins (11).

In the absence of appreciable DNA damage, the LexA protein represses many operons, including several genes needed for DNA repair: recA, lexA, uvrA, uvrB, and umuC. When the activated RecA stimulates its proteolytic activity, it cleaves itself (and other proteins), leading to coordinate induction of the SOS regulated operons (Fig. 10).

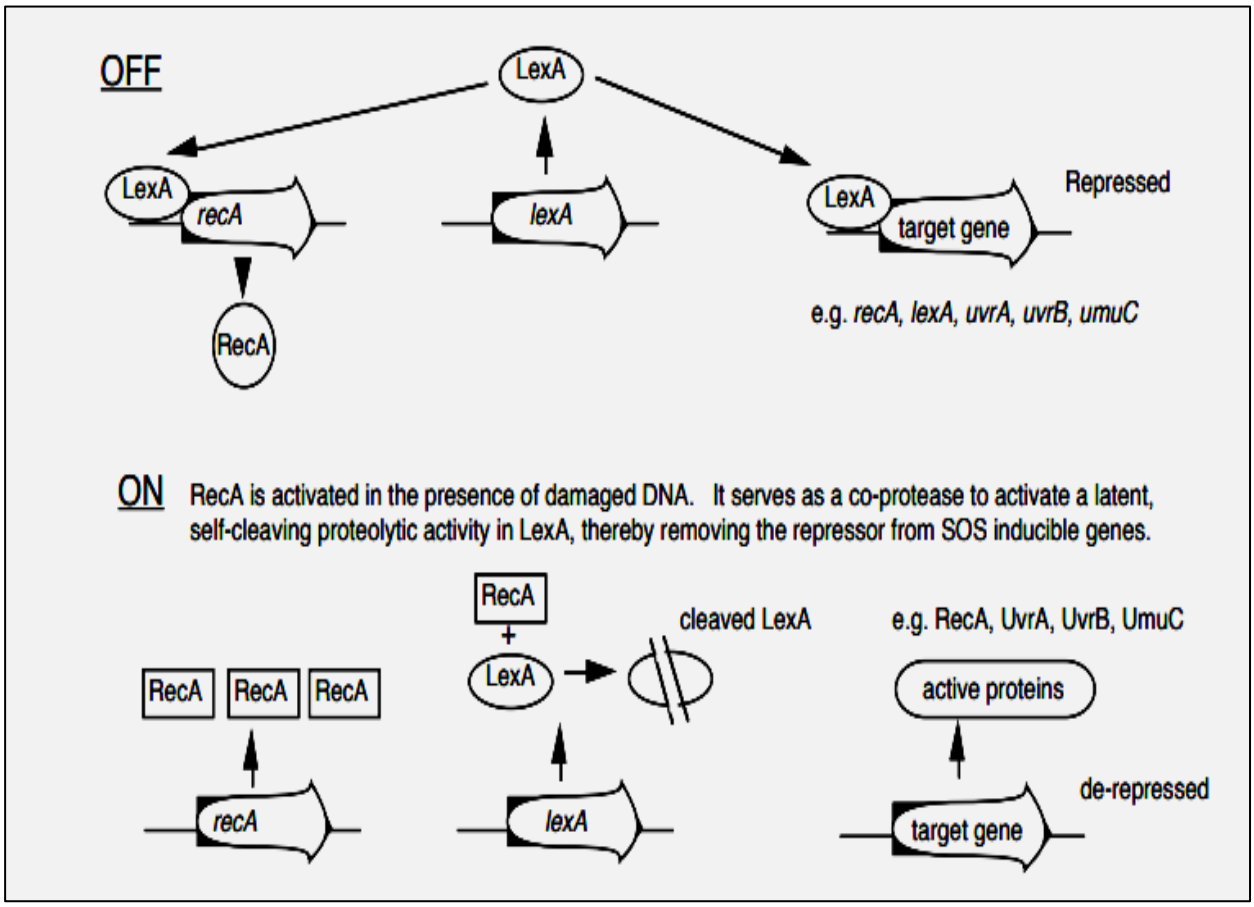

Figure 10. RecA and LexA control the SOS response [6]

CHAPTER III
Genotoxicity Assays

Introduction

Genotoxicity testing, a technique which helps identify chemicals that cause genetic alterations includes tests for mutagenicity and DNA damage. Mutagenicity refers to the capability of a substance to induce a genetic mutation, an irreversible event that alters DNA and/or chromosomal number or structure, and may be passed onto subsequent cell generations. Mutations in somatic cells may cause cancer, while mutations in germ cells may be passed on to offspring. DNA damage may not result in permanent alterations since it may be reversed by a DNA repair process. However, accumulation of DNA damage in somatic cells is associated with the development of certain degenerative conditions, such as accelerated aging, immune dysfunction, cardiovascular and neurodegenerative diseases, while such damage in germ cells may be associated with spontaneous abortions, infertility, malformation, or heritable mutations in the offspring. Therefore, genotoxicity testing aids the determination of not only irreversible genetic alteration, but also helps evaluate indirect evidence of mutations, such as DNA strand breaks and DNA adduct formation. A combination of multiple tests (usually referred to as a test battery) is commonly used to comprehensively evaluate the ability of a chemical to induce genotoxicity, because an individual test does not provide information on all end points.

As individual genotoxicity tests have been optimised for use in a specifc species and for the detection of a specifc end point, a specifc test cannot be used to detect all genotoxicities. Therefore, genotoxicity tests are conducted via a combination of various complementary tests for the detection of a wide range of potential genotoxicities of chemical substances. In 2012, the International Council of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) established a "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (S2(R1))." In this guidance, two approaches have been provided as standard combinations of genotoxicity tests (ICH, 2011). The first approach is as follows (12):

1. A bacterial reverse mutation assay (Ames test) (*in vitro* test).
2. A chromosome aberration test, micronucleus test, or mouse lymphoma test using mammalian cells (*in vitro* test).
3. A micronucleus test or chromosome aberration test targeting a hematopoietic tissue of rodents (*in vivo* test).

The second approach is as follows:
1. An Ames test (*in vitro* test).
2. A micronucleus test or chromosome aberration test targeting a hematopoietic tissue of rodents (*in vivo* test).
3. A DNA strand break assay (comet assay or alkaline elution assay), a transgenic rodent gene mutation assay, or a UDS test targeting liver of rodents (*in vivo* tests).

1-*In vitro* tests

1.1. Bacterial reverse mutation assay (Ames test)

The basic structure and function of DNA or the mutation induction mechanism in bacteria is considered to be shared across higher animals. Mutagenicity testing methods that use bacteria have the following advantages:

- The results can be more easily interpreted than those in mammalian tests because of advances in the study of DNA damage and its repair mechanisms in bacteria.
- These testing methods use simpler procedures than those for higher organisms, allowing many samples to be tested over a short time period at a low cost;
- Databases are readily available because many tests have already been conducted.

The most widely used testing method is the bacterial reverse mutation test (Ames test) (Figure 11). This test detects a mutagen by using the reverse mutation of an amino acid-requiring strain of *Salmonella typhimurium* or *Escherichia coli* as an indicator. Dr. Bruce Ames developed the testing method that uses *Salmonella*; however, the name is also used in a broader sense for the method that uses *E. coli* (12).

1.1.2. S9 Liver Extract and Metabolic Activation

S. typhimurium and *E. coli*, used in Ames test, are prokaryotes and therefore do not exactly reflect the conditions in humans. In mammalian organisms chemical molecules are often metabolized in the liver. This can lead to the generation of mutagenic metabolites. S9 is a liver extract (e.g. rat, hamster) that contains active liver enzymes (P450 activity) simulating the hepatic metabolism in *in vitro* assays. The P450 activity in S9 can be increased through induction with pure chemicals like **Phenobarbital**. Metabolic activation systems based on chemically induced S9 have been widely applied in *in vitro* studies; e.g. the *Salmonella* mutagenicity test and the micronucleus test.

S9 is the supernatant of homogenized liver that has been centrifuged at 9000g for 10min. For gaining the liver mircrosomesn, the homogenized liver is centrifuged at 100000g for 1h. For use in Ames test, the S9 standard mixture is: **4-10% S9 liver extract, 4mM NADP, 5mM Glucose-6-phosphat, 10mM Na/K Phosphate buffer (pH7.4), 8mM Mg-Aspartat, 33mM KCl (12)**

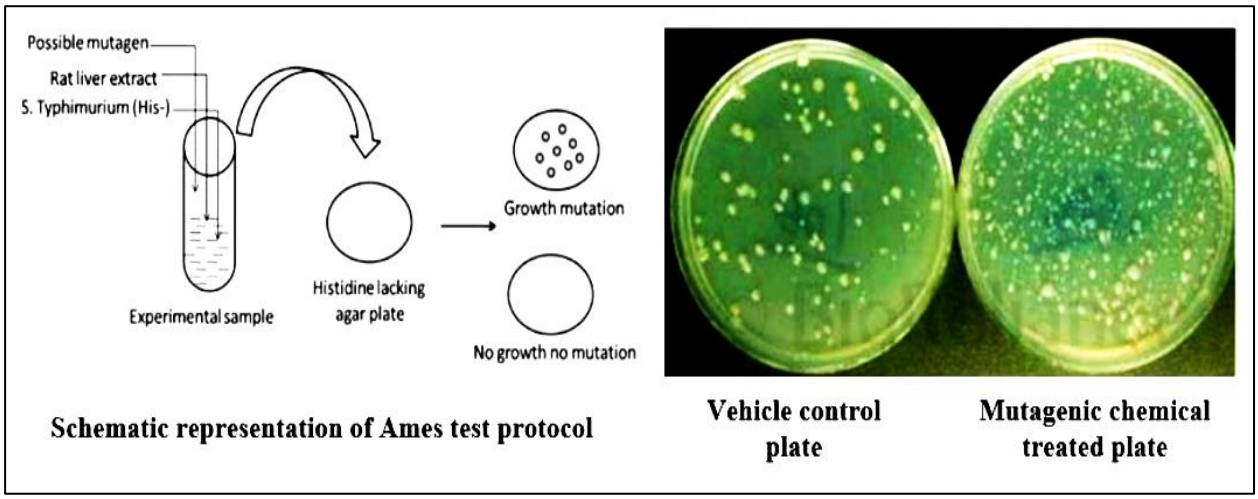

Figure 11: Schematic representation of Ames test protocol [7]

1.2. Gene mutation tests using mammalian cells

Gene mutation tests using cultured mammalian cells mainly involve the use of a recessive mutation of an endogenous drug-resistance marker gene (HPRT or TK) as an indicator. In

contrast with the Ames test, which is a reverse mutation test, the mutation test involving these genes is termed a forward mutation test. This test aids the detection of various types of mutations that lead to the loss or inactivation of proteins encoded by these genes. HPRT, located on the X chromosome, encodes **hypoxanthine phosphoribosyltransferase (HPRT)** (figure 12), an enzyme which regulates the salvage cycle of purine bases. The loss of activity of this enzyme in cells results in the development of resistance to 6-thioguanine (6-TG), a DNA synthesis inhibitor that exerts a toxic effect via nucleic acid metabolism. The test involves the subjection of cells to treatment with a test chemical and the subsequent culture of cells on a medium containing 6-TG. The evaluation of mutagenicity of the chemical is based on the number of cell colonies that present with 6-TG resistance development due to the mutation. Not only established cell lines but also primary cultured cells, including human lymphocytes, may be used for conducting this test, only if the cells are diploid cells with an active copy of the X chromosome. Usually, Chinese hamster cell lines, such as CHO, CHL, and V79, are used (13).

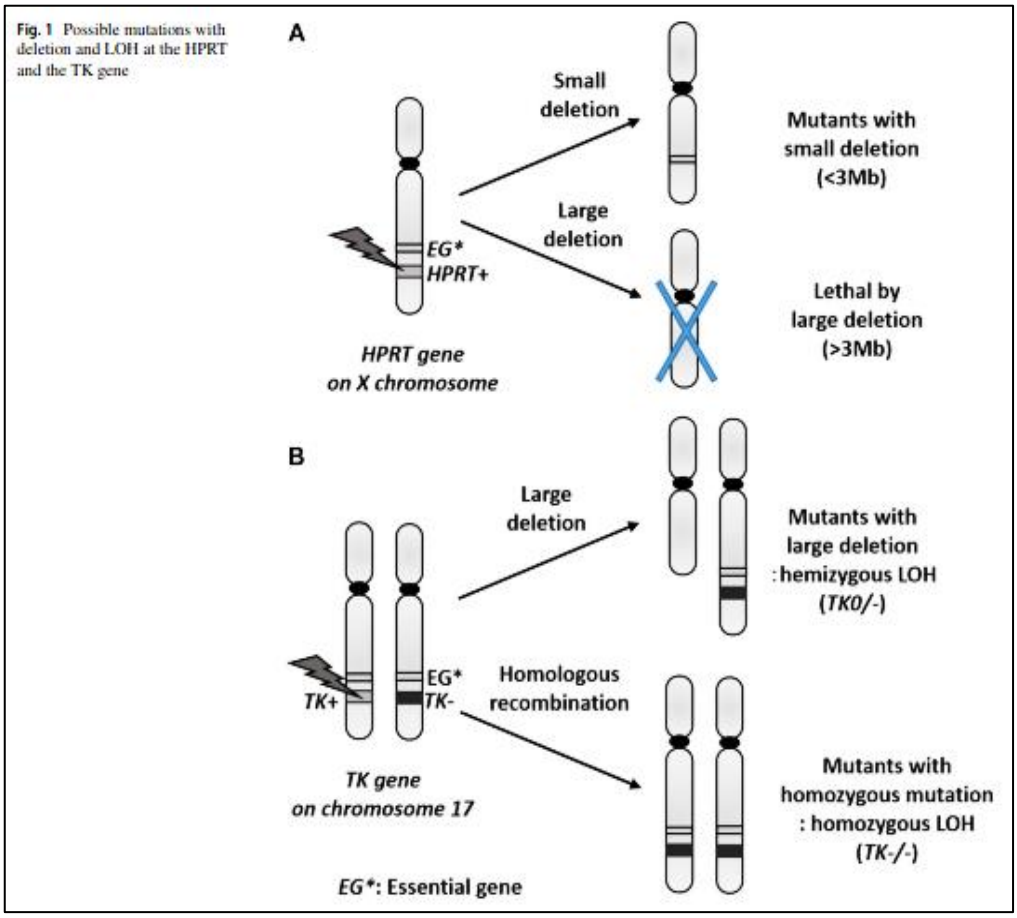

Figure 12: possible mutations with deletion and LOH at the HPRT and the TK gene [8]

1.3. Chromosome aberration tests using mammalian cells

Chromosome aberration testing, a technique used to explore structural and numerical (polyploidy) aberrations of chromosomes via optical microscopy, involves the subjection of cultured mammalian cells to treatment with a test substance and the preparation of chromosome specimens of cells in metaphase (using colchicine) after a set period. This test enables the detection of transient chromosomal aberrations, which differ from stable chromosomal aberrations observed in hereditary diseases and/or cancer tissues (12).

1.3.1. Types of Chromosome Aberrations

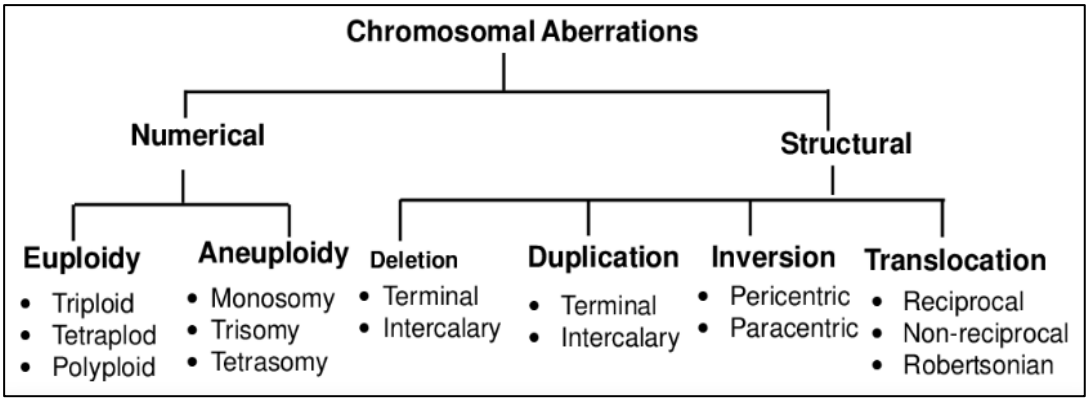

Figure 13: Classification of chromosome aberrations 9

1.3.1.1.Structural Aberrations: These occur when there is an abnormal number of chromosomes in a cell:

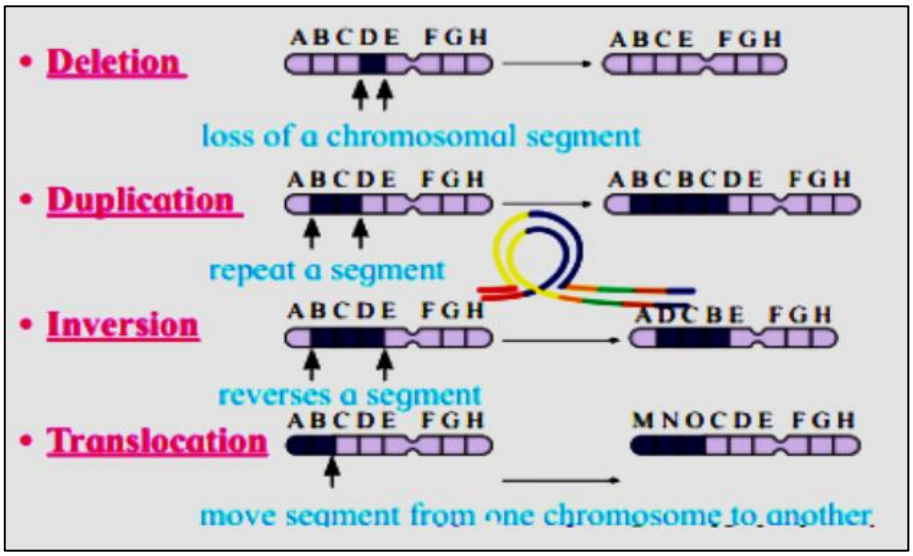

Figure 14: Structural Aberrations (10)

✓ **Aneuploidy**: This is the presence of an abnormal number of chromosomes in a cell. It results from nondisjunction during cell division. Examples include **Monosomy** (Missing one

chromosome); and **Trisomy** (An extra chromosome e.g., Down syndrome with three copies of chromosome 21, 47,XY,+21).

- ✓ **Polyploidy**: This is the presence of more than two complete sets of chromosomes. It's less common in humans but seen in some plants and animals. Examples includeN**Triploidy** (hree sets of chromosomes (69 chromosomes total), and **Tetraploidy** (Four sets of chromosomes 92 chromosomes total) (13).

1.3.1.2.Numerical Aberrations

These involve changes in the structure of chromosomes and can affect their function (13).

- ✓ **Deletions**: A segment of the chromosome is missing. For example:
- ✓ **Inversions**: A segment of the chromosome is reversed end to end. This can be paracentric (within one arm of the chromosome) or pericentric (involving the centromere).
- ✓ **Translocations**: A segment of one chromosome is transferred to another chromosome.
- ✓ **Ring Chromosomes**: A chromosome forms a ring shape due to the ends joining together. This can lead to loss of genetic material.
- ✓ **Isodicentric Chromosomes**: A chromosome with two identical arms due to a duplication.

1.3.2. Methods of Chromosome Aberration Detection

1.3.2.1. Conventional Methods

Conventional methods for detecting chromosome aberrations typically include karyotyping and some cytogenetic techniques (12, 13):

- ✓ **Karyotyping**: it is the process of staining and arranging chromosomes into a standard format to analyze their number and structure. It detects numerical abnormalities (e.g., trisomy 21 for Down syndrome) and identifies large structural abnormalities (e.g., deletions, duplications, translocations). It is also useful in prenatal screening, cancer diagnosis, and fertility investigations.
- ✓ **Fluorescence In Situ Hybridization (FISH)**: FISH uses fluorescently labeled DNA probes that bind to specific chromosomal regions to visualize particular sequences. It detects specific genetic abnormalities such as submicroscopic deletions, duplications, and translocations and can identify chromosomal abnormalities that are not apparent in conventional karyotyping.It is useful in prenatal diagnosis, cancer cytogenetics, and detecting specific genetic syndromes.

- ✓ **G-banding**: it is a staining technique used during karyotyping to create a distinct banding pattern on chromosomes. The banding pattern is used to identify chromosomes and detect large structural abnormalities.
- ✓ **Spectral Karyotyping (SKY) and Multicolor FISH (M-FISH):** these advanced FISH techniques use multiple fluorescent probes to label different chromosomes or chromosomal regions with distinct colors. It allows for the simultaneous visualization of all chromosomes or specific chromosomal regions and it is useful for complex cases where conventional karyotyping may not provide enough information.

1.3.2.2. Molecular Methods

Molecular methods for detecting chromosome aberrations are more advanced and precise compared to conventional cytogenetic techniques. They often provide greater detail about genetic variations and are especially useful for detecting submicroscopic changes that may not be visible with traditional methods.

- ✓ **Chromosomal Microarray Analysis (CMA):** also known as array comparative genomic hybridization (aCGH), is a high-resolution technique that detects copy number variations (CNVs) across the entire genome. The hybridized microarray is scanned to measure the intensity of fluorescence, which reflects the relative copy number of specific chromosomal regions. It detects submicroscopic deletions and duplications that are too small to be seen with traditional karyotyping. It is useful for diagnosing developmental delays, congenital anomalies, and some cancers and provides a genome-wide view of CNVs (12, 13).
- ✓ **Next-Generation Sequencing (NGS):** is a powerful technique that sequences large amounts of DNA quickly and provides comprehensive information about genetic variations. Sequencing data are analyzed using bioinformatics tools to identify genetic variants, including CNVs and structural variations. It detects a wide range of genetic abnormalities, including point mutations, CNVs, and structural variations. It is useful for comprehensive genetic analysis, cancer genomics, and research into genetic disorders, and it can also be used for whole-genome, whole-exome, or targeted sequencing (12, 13).
- ✓ **Quantitative PCR (qPCR):** measures the quantity of specific DNA sequences through amplification and detection of fluorescence. It detects specific numerical aberrations or gene amplifications and it is useful for targeted analysis of known genetic variants or abnormalities (12, 13).
- ✓ **Multiplex Ligation-dependent Probe Amplification (MLPA):** is a technique used to detect copy number changes and specific mutations in multiple genes simultaneously. It detects

deletions, duplications, and other copy number changes in multiple genes or regions simultaneously. It is useful for genetic testing of inherited disorders and certain cancers (12, 13).

- ✓ **Digital Droplet PCR (ddPCR):** is an advanced form of PCR that provides highly sensitive and precise quantification of DNA. It detects rare genetic mutations, copy number variations, and low-abundance targets with high precision. It is also useful in cancer research, liquid biopsies, and monitoring minimal residual disease (12, 13).

1.4. Micronucleus test using mammalian cells

The micronucleus test continues to gain increasing importance as a simple cytogenetic technique, because the analysis of chromosomes in the metaphase is time-consuming and the observation of chromosomes requires technical expertise (12, 13). The micronucleus is a corpuscle composed of chromatin that exhibits staining in a similar manner as the main nucleus and contains a chromosomal fragment or a whole chromosome separate from the main nucleus. A chromosomal fragment or a chromosome that is not bound by spindle fibres is produced due to an abnormality in the chromosomal mitotic apparatus, which induces a structural or numerical chromosomal aberration. This fragment or whole chromosome does not migrate to the poles at the time of cell division, and as a result it is not incorporated into the main nucleus, thus remaining instead as a micronucleus (Figure 15)(12, 13).

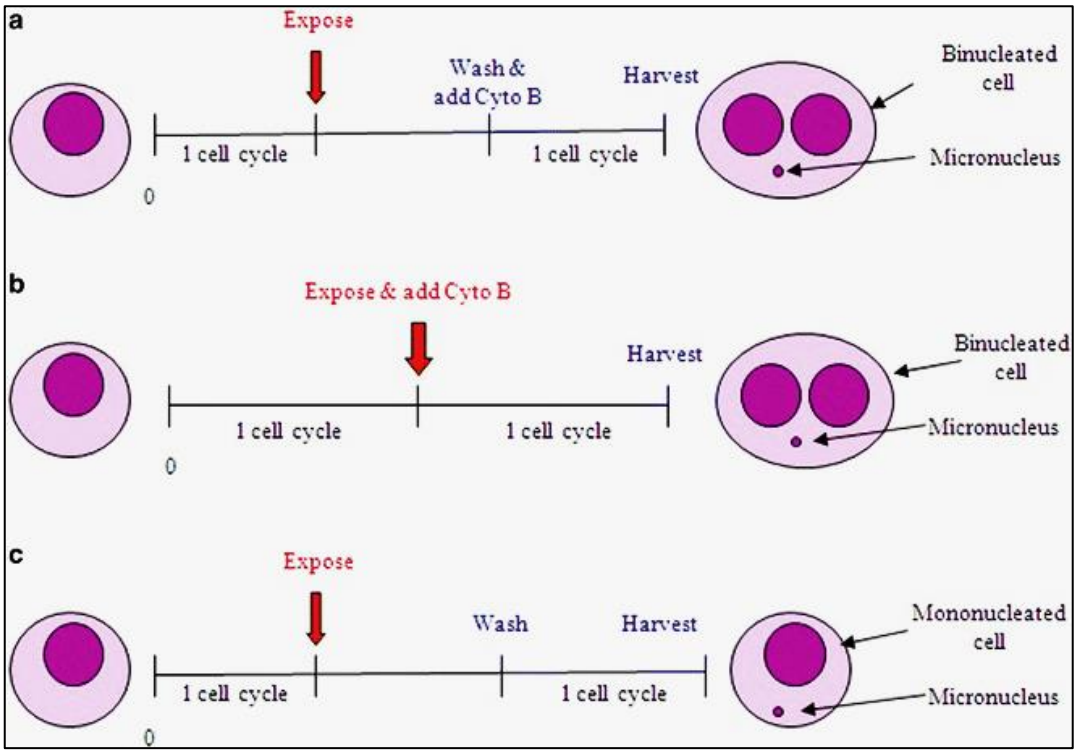

Figure 15: *in vitro* Micronucleus Assay [11]

When coupled with fluorescence *in situ* hybridization (FISH), micronucleus assay is able to reveal the capability to induce structural chromosome aberrations (clastogenic activity) and/or numerical chromosome changes (aneuploidogenic activity) (Figure 16).

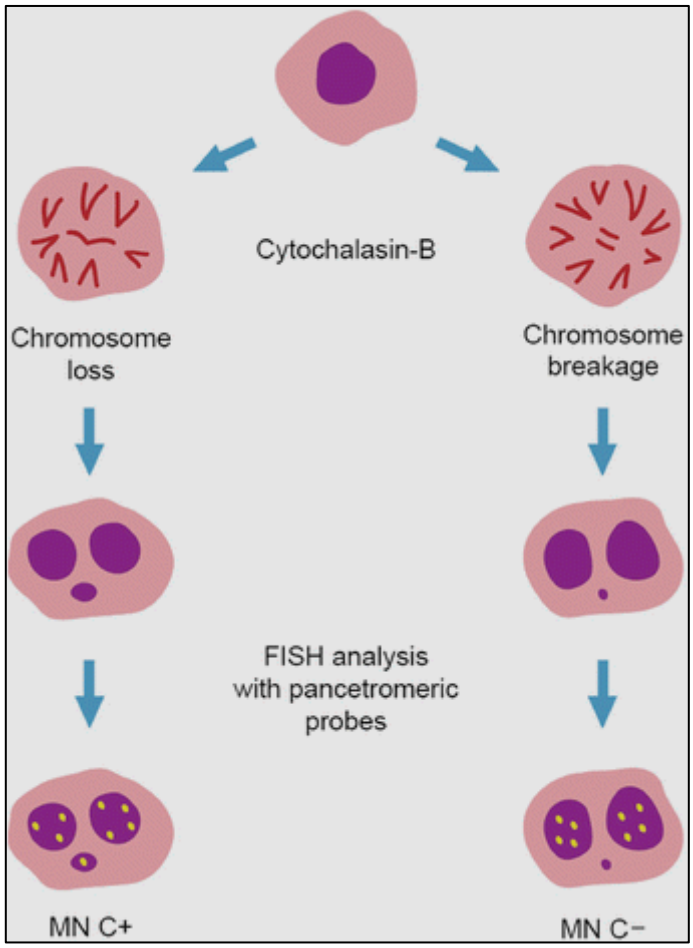

Figure 16: *in vitro* Micronucleus Assay and FISH Analysis [12]

2.*In vivo* tests

2.1.Erythrocyte micronucleus test

The mammalian erythrocyte micronucleus test is an *in vivo* test that is conducted to measure the potency of chemicals that induce chromosomal aberrations; the test involves the enumeration of the number of immature (polychromatic) erythrocytes containing micronuclei in animal bone marrow or peripheral blood. Rodents are usually included for this test; however, the possibility of using other animal species (dogs, primates, and humans) has also been investigated. When a bone marrow erythroblast undergoes differentiation into an immature erythrocyte (also known as a polychromatic erythrocyte or reticulocyte) and moves into peripheral blood, the cell assumes an anucleate characteristic due to enucleation of the main nucleus. However, any micronucleus formed therein remains in the cytoplasm. At this stage, these cells lack a main nucleus, enabling comparatively easy visualisation and detection of their micronuclei. As micronuclei are

generated from an acentric chromosome, late-segregating chromosome fragment, or a whole chromosome in certain cases, this test enables the detection of substances that induce chromosomal aberrations as well as substances that induce aneuploidy (13).

2.2. Gene mutation tests using transgenic animals

This test mainly uses transgenic mice and rats. Multiple copies of a reporter gene are transferred to the genomes of these animals using λ phage DNA as the vector to facilitate the detection of mutations. The reporter gene is derived from a λ phage or a bacterium and is considered genetically neutral because it is not expressed in the animals. Therefore, there is no selection pressure due to its genetic function. Usually, the animals are exposed to a chemical, following which DNA derived from various organs (e.g. the liver, stomach, large intestine, and bone marrow) undergoes *in vitro* packaging into phage particles. The collected λ phages are then transferred to their host E. coli, enabling the detection of any mutation that has occurred *in vivo* in the animals, because such mutations are reflected in mutant *E. coli*. The greatest advantage of this test is that it may be used to detect a mutation in any organ, including germ cells, and may help ascertain whether the test substance induces cancer through its genotoxicity, via observation of the mutation in the carcinogenesis-affected target organ(13,14).

2.3. Comet assay

Comet assay, also known as single cell gel electrophoresis, is a sensitive technique for measuring DNA damage in single cells and has become well known in the field of toxicology and drug discovery. Comet assay is based upon the ability of denatured cleaved DNA fragments, or damaged DNA, to migrate out of the cell under electrophoresis creating a "comet tail", while the undamaged DNA remains within the cell membrane creating the "comet head." (Figure 17). Comet assay is most commonly run under alkaline conditions to detect single- and double-stranded DNA breaks but can also be run under neutral conditions to detect only double-stranded DNA breaks.

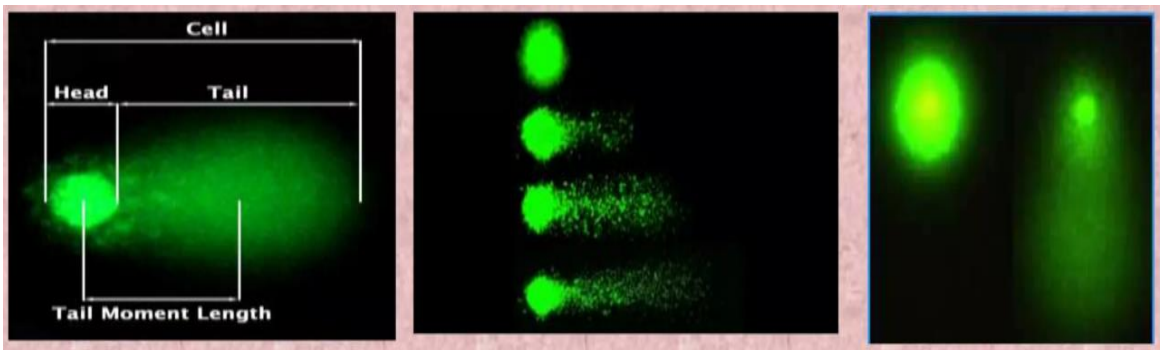

Figure 17: comet assay [13]

The quantification of DNA damage using the computer software gives the tail moment that is defined as the product of the tail length and the fraction of total DNA in the tail (Tail moment=tail length x % of DNA in the tail). This is calculated automatically by the computer software system as an average for the 50 cells selected for measurement (14).

2.4. Sister Chromatid Exchange Assay

The sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The test may be performed *in vitro*, using continuous cell lines, or *in vivo* using animal models such as mice, rats, and hamsters. For *in vitro* assays, cell cultures are exposed to test chemicals, and allowed to replicate in the presence of bromodeoxyuridine. The cells are then treated with colchicine or colcemid to arrest cells in a metaphase-like stage of mitosis, and following harvesting chromosome preparations made. Preparations are stained and metaphase cells analyzed for SCEs using a microscope (13, 14).

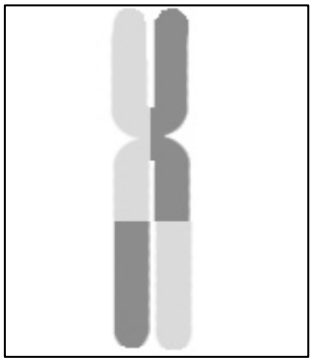

Figure 18: Scheme of a sister chromatid exchange. The ends of the chromatids are reversed in the lower area [13].

2.5. Unscheduled DNA synthesis (UDS) test

The unscheduled DNA synthesis test is a short-term genotoxicity assay that allows identification of substances that induce DNA repair in liver cells of treated animals. This endpoint can be assessed by determining the uptake of radiolabelled nucleotide after excision-repair in cells that are not undergoing scheduled (S-phase) DNA synthesis (14).

3. Evaluation and interpretation of genotoxicity test results

In vitro test systems that involve the use of mammalian cells often yield false-positive results for carcinogenicity prediction in rodents. Though the cause of such false-positive results is often unknown, caution must be exercised because strong cytotoxicity, high osmotic pressure, precipitation, and non-physiological pH, among others, may lead to the achievement of a positive result via nonspecific effects Guidelines that provide information regarding the highest

concentrations of the substance and the cytotoxicity levels that should be considered during testing must be rendered available to prevent the achievement of such false-positive results. Positive in vitro results may indicate whether a chemical exhibits genotoxic characteristics. However, the biological signifcance of such positive *in vitro* results must be verified via accurate follow-up tests in many cases. Positive in vitro test results using mammalian cells are subjected to follow-up by selecting an appropriate in vivo test. Generally, an *in vivo* test with the same end point is selected. Additionally, as outlined in the guidelines pertaining to the pharmaceuticals mentioned previously, when a micronucleus test using a hematopoietic tissue has already been conducted, it is recommended that testing should be performed using a different tissue specimen.

CHAPTER IV
Mutagenesis, Genotoxicity and Carcinogenesis Relationship

Cancer is a genetic disease due to the accumulation of numerous mutations rendering the tumour cell insensitive to control by the local cellular environment and by the whole organism. During at least one step in the carcinogenic process, a mutator phenotype in target cells may occur due to mutations controlling the fidelity of DNA replication or DNA repair, the apoptosis pathways or the cell cycle checkpoint regulations. Among the multiple mutations found in human cancers such as gene amplification, chromosome alterations and translocations, point mutations are very important and the molecular mechanisms of their production are well documented. Various mechanisms that a cell can use to produce point mutations due to lower fidelity in the DNA polymerisation step or to inefficient repair pathways are the origin of cancer.

1. Evidence for a Mutational Origin of Cancer

Several lines of evidence in favor of the somatic mutation hypothesis for cancer are given below (15):

a. Cancer property is an irreversible and transmissable cellular characteristic in accordance with a genetically determined trait.

b. Tumors have been shown by immunological methods to be monoclonal, i.e., they emanate from one transformed cell. If the reverse had been true, that tumors are formed from several transformed cells, a mutational origin would have been difficult to maintain.

c. Known carcinogenic and mutagenic chemicals share the property of being electrophilic and bind to nucleophilic centers of macromolecules, including DNA.

d. Experimental evidence, taking into consideration mammalian biotransformation, has shown that most carcinogens are also mutagenic.

e. In the human disease Xeroderma pigmentosum increase of tumors is associated with a genetically-determined lack of excision repair of DNA. In another system using fish, tumor induction by UV was counteracted by visible light, which cleaves UV-induced thymine dimers through photoreactivation, suggesting the involvement of DNA repair in tumorigenesis.

f. Specific cellular and homologous viral genes, oncogenes, have been shown to be involved in tumor formation. Recent investigations have given direct evidence that certain cancer forms are attributable to defined alterations in DNA in or in the vicinity of oncogenes.

2. Mutagenic events in carcinogenesis

2.1. Activation of a proto-oncogene

2.1.1. Definition

Proto-oncogene is a gene involved in normal cell growth. Mutations (changes) in a proto-oncogene may cause it to become a transforming gene, i.e an oncogene, which can cause the growth of cancer cells. They can be activated into oncogenes by mutation on their coding part or by gene amplification resulting from a translocation (15).

2.1.2. Oncogenes classes

Oncogenes are divided into 6 major classes:

- ✓ growth factors
- ✓ transmembrane receptors for growth factors
- ✓ G-proteins or membrane proteins binding GTP
- ✓ membrane tyrosine protein kinases
- ✓ cytosolic protein kinases
- ✓ nuclearly active proteins: control the transcription of target genes by interacting with DNA.

2.1.3.Mechanisms of oncogene activation

The activation of oncogenes involves genetic changes to cellular protooncogenes. The consequence of these genetic alterations is to confer a growth advantage to the cell. Three genetic mechanisms activate oncogenes in human neoplasms: **(1) mutation**, **(2) gene amplification**, and **(3) chromosome rearrangements**. These mechanisms result in either an alteration of protooncogene structure or an increase in protooncogene expression (Figure 19). Because neoplasia is a multistep process, more than one of these mechanisms often contribute to the genesis of human tumors by altering a number of cancer-associated genes. Full expression of the neoplastic phenotype, including the capacity for metastasis, usually involves a combination of protooncogene activation and tumor suppressor gene loss or inactivation. The figure 19 summarizes the different mutagenic events involved in carcinogenesis (16, 17).

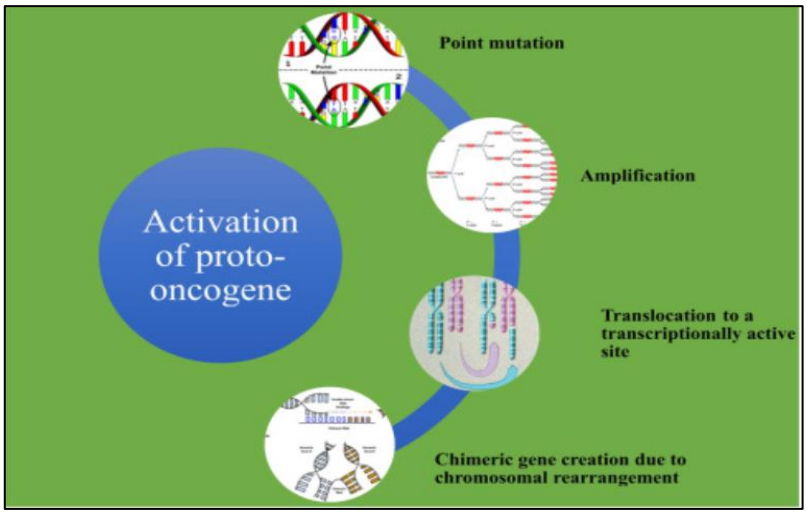

Figure 19: Proto-oncogene activation [14]

a- Point mutation in a coding sequence for a proto-oncogene

Mutations activate protooncogenes through structural alterations in their encoded proteins. These alterations, which usually involve critical protein regulatory regions, often lead to the

uncontrolled, continuous activity of the mutated protein. Various types of mutations, such as base substitutions, deletions, and insertions, are capable of activating proto-oncogenes. Missense mutations resulting in the substitution of one amino acid by another are capable of activating proto-oncogenes into oncogenes by affecting a catalytic site. Deletions, which most often result in a loss of function, can sometimes lead to abnormal activation if they affect a regulatory region Mutation can also appear via **v**iral DNA integration into the host cell genome. The viral DNA interfere with the host cell's tumor suppressor genes or gene products, while the RNA tumor viruses carry their own oncogene via the reverse transcriptase, integrate into the host cell genome, and over-stimulate the cells' proliferation apparatus (18).

b- **Gene amplification**

It refers to the expansion in copy number of a gene within the genome of a cell. Gene amplification was first discovered as a mechanism by which some tumor cell lines can acquire resistance to growth-inhibiting drugs. The process of gene amplification occurs through redundant replication of genomic DNA, often giving rise to karyotypic abnormalities called double-minute chromosomes (DMs) and homogeneous staining regions (HSRs). DMs are characteristic minichromosome structures without centromeres. HSRs are segments of chromosomes that lack the normal alternating pattern of light- and dark-staining bands. Both DMs and HSRs represent large regions of amplified genomic DNA containing up to several hundred copies of a gene. Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth (16, 17).

c- Chromosomal Rearrangements

Recurring chromosomal rearrangements are often detected in hematologic malignancies as well as in some solid tumors. These rearrangements consist mainly of chromosomal translocations and, less frequently, chromosomal inversions. Chromosomal rearrangements can lead to hematologic malignancy via two different mechanisms:

(1) the transcriptional activation of proto-oncogenes: Transcriptional activation, sometimes referred to as gene activation, results from chromosomal rearrangements that move a proto-oncogene close to an immunoglobulin or T-cell receptor gene Transcription of the protooncogene then falls under control of regulatory elements from the immunoglobulin or T-cell receptor locus. This circumstance causes deregulation of protooncogene expression, which can then lead to neoplastic transformation of the cell.

(2) the creation of fusion genes: Fusion genes can be created by chromosomal rearrangements when the chromosomal breakpoints fall within the loci of two different genes. The resultant

juxtaposition of segments from two different genes gives rise to a composite structure consisting of the head of one gene and the tail of another. Fusion genes encode chimeric proteins with transforming activity. In general, both genes involved in the fusion contribute to the transforming potential of the chimeric oncoprotein. Mistakes in the physiologic rearrangement of immunoglobulin or T-cell receptor genes are thought to give rise to many of the recurring chromosomal rearrangements found in hematologic malignancy (18).

2.2. Inactivation of a tumor suppressor gene

Tumor suppressor genes are associated with cell cycle arrest, apoptosis, and DNA damage repair. They can be activated in the physiological state after DNA damage, but are rendered inactive by mutation in the coding regions, by inhibition of transcription, by deletion, or by aneugenesis. They play an indirect role in initiating the tumor process (16).

2.3. Activation of genes encoding telomerase

Telomerase plays a major role against cellular senescence by allowing, through 5' resynthesis, the restoration of telomeres. The level of cellular activity of telomerase is increased in cancer cells. It is one of the factors that contribute to the proliferation and immortalization of cancer cells. The increase in telomerase activity is one of the three most important phases of cancer. The development of molecules directed against telomerase thus offers a perspective for the treatment of cancers (16).

3. Multi-Step Process of Cancer

A compound that reacts with DNA and somehow changes the genetic makeup of the cell is called a mutagen. The mutagens that predispose cells to develop tumors are called initiators and the non-reactive compounds that stimulate tumor development are called promoters. Approximately 70% of known mutagens are also carcinogens--cancer-causing compounds. A compound that acts as both an initiator and a promoter is referred to as a 'complete carcinogen' because tumor development can occur without the application of another compound (18).

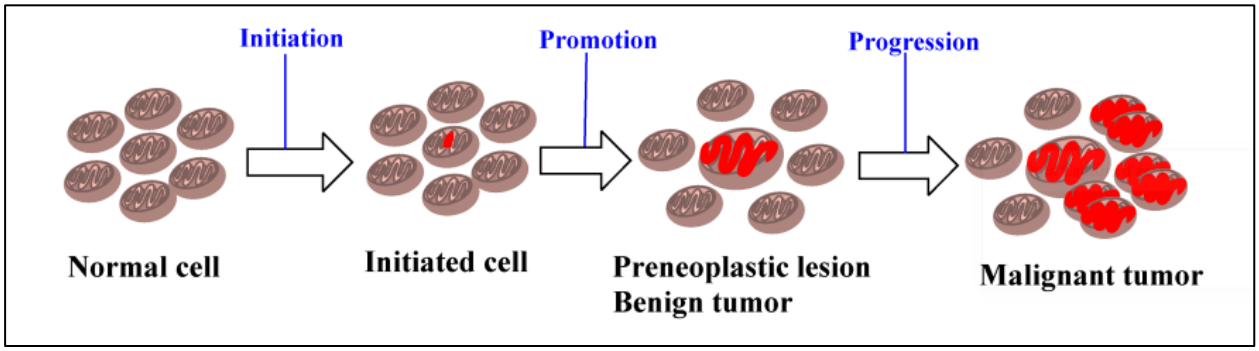

Figure 20. A brief depiction of initiation, promotion, and progression in the process of carcinogenesis [15].

3.1.Initiation

Initiation is the first step in the two-stage model of cancer development. Initiators, if not already reactive with DNA, are altered (frequently they are made electrophilic) via drug-metabolizing enzymes in the body and are then able to cause changes in DNA (mutations). Since many initiators must be metabolized before becoming active, initiators are often specific to particular tissue types or species. The effects of initiators are irreversible; once a particular cell has been affected by an initiator it is susceptible to promotion until its death. Since initiation is the result of permanent genetic change, any daughter cells produced from the division of the mutated cell will also carry the mutation (18).

3.2.Promotion

Once a cell has been mutated by an initiator, it is susceptible to the effects of promoters. These compounds promote the proliferation of the cell, giving rise to a large number of daughter cells containing the mutation created by the initiator. 6Promoters have no effect when the organism in question has not been previously treated with an initiator.

Unlike initiators, promoters do not covalently bind to DNA or macromolecules within the cell. Many bind to receptors on the cell surface in order to affect intracellular pathways that lead to increased cell proliferation. There are two general categories of promoters: specific promoters that interact with receptors on or in target cells of defined tissues and nonspecific promoters that alter gene expression without the presence of a known receptor. Promoters are often specific for a particular tissue or species due to their interaction with receptors that are present in different amounts in different tissue types (18).

3.3.Progression

The term progression refers to the stepwise transformation of a benign tumor to a neoplasm and to malignancy. Progression is associated with a karyotypic change since virtually all tumors that advance are aneuploid (have the wrong number of chromosomes). This karyotypic change is coupled with an increased growth rate, invasiveness, metastasis and an alteration in biochemistry and morphology (18).

4. Can Cancer be Initiated by Non-Genetic Mechanisms?

Although much experimental data strongly suggest that mutational changes of DNA are involved in cancer initiation, this does not rule out the possibility that some cancer forms may be caused by epigenetic mechanisms. It is reasonable to assume that the origin of cancer is related to the process of differentiation in multicellular organisms and this process is probably an epigenetic

event. Initiation of cancer could, in accordance with this view, occur as a result of an alteration of an epigenetic control of differentiation. Some support for such a mechanism has been suggested by transplantation experiments in both animals and plants. Transplantation of nucleic from frog renal carcinomas into enucleated frog eggs has thus given rise to normal tadpoles. Reversion of malignancy to non-malignancy has also been shown after transplantation of several types of tumors (17).

5.Genotoxicity and carcinogenic risk assessment

As genotoxicity testing usually involves the qualitative test to detect the presence or absence of genotoxicity and/ or mutagenicity, the result is considered either “positive” or “negative.” Such qualitative results of genotoxicity tests are considered for health risk assessments. In several cases, toxic chemical health risk assessments involve the use of a dose–response model based on theoretical and empirical studies, which include a threshold below which no health effects are detected.

This allows the prescription of an acceptable daily intake (ADI). However, determination of an ADI poses challenges when a chemical has been found to be both carcinogenic and genotoxic. Unlike other toxicities, genotoxicity is not based on thresholds. Therefore, no ADI can be set because, theoretically, the health risk does not become zero unless intake becomes zero. This helps specify risk assessments for genotoxic carcinogens. Mutagens are a type of genotoxicant that exhibit reactions with DNA to eventually cause gene mutation. Mutation rates never reach a value of zero because mutations are stochastic events. Additionally, even a single mutation in a gene that is important for cellular oncogenesis, such as an oncogene or a tumour suppressor gene, leads to the development of an original cancer cell, which may be sufcient to cause carcinogenesis (17).

Therefore, the probability of oncogenesis cannot be completely eliminated either. Consequently, it is valid to consider that no threshold can be set for mutagens that cause irreversible mutations. However, a few researchers have suggested that a threshold is applicable to such mutagens. This concept is based on the contention that the induction of a mutation can be reduced to virtually zero because biological defence mechanisms, such as DNA repair, metabolic reactions, and scavengers, function efficiently at an extremely low dose range. However, this concept cannot be applied to risk assessment, because a technique demonstrating utilisation in the evaluation of such biological defence mechanisms, with applicability to test results, has not been developed. Carcinogens are currently classifed into two groups, i.e. **mutagenic carcinogens and non-mutagenic (or sometimes called non-genotoxic) carcinogens** (17).

The target of the former is DNA while that of the latter is non-DNA such as proteins. It is generally accepted in regulatory sciences that there is no threshold for the risk of mutagenic carcinogens because even one base change in DNA may lead to cancer. In contrast, damage in protein does not necessarily accelerate carcinogenesis because there are multiple copies of the protein in the cell. Therefore, it is theoretically possible to set a threshold for non-mutagenic carcinogens . CA and MN tests detect both mutagenic and non-mutagenic carcinogens while Ames test detects only DNA-interacting mutagenic chemicals (16, 18).

This **type of mutagenic carcinogen** is usually detected in a bacterial mutation (mutagenicity) assay. Other types of genotoxicants that are non-mutagenic typically have threshold mechanisms and usually do not pose carcinogenic risk in humans at the level ordinarily present as impurities". This principle is likely to exert a substantial impact on future risk assessments of these substances because it is applicable to chemicals and other substances that are present at trace levels in food, such as additives and residual agricultural chemicals.

Compared to the determination of a threshold that is associated with zero risk, an alternate method has been proposed for the assessment/management of the risk posed by genotoxicants, which is based on the concept that “even if a substance exhibits mutagenicity and/or genotoxicity, it may be considered virtually safe as long as the exposure dose is sufciently low to result in extremely low carcinogenicity with a socially acceptable level of risk (Figure 21) (18).

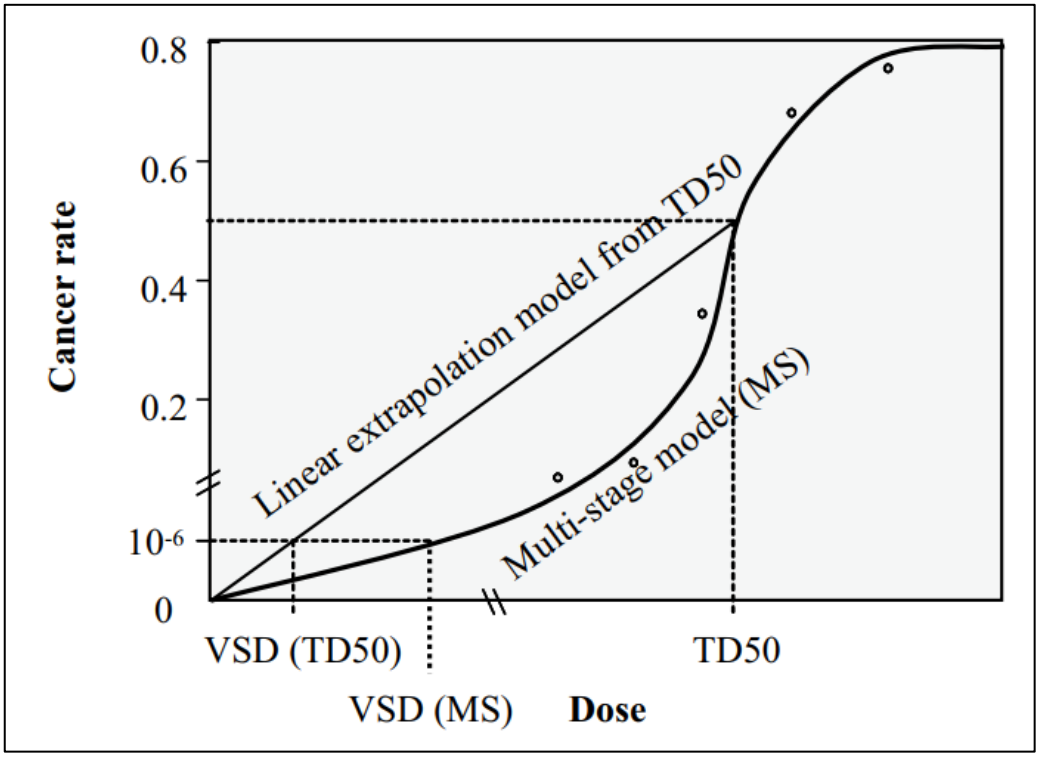

Figure 21: Calculation of virtually safe dose [16]

Based on carcinogen databases, it is possible to conclude that a majority of chemicals may pose only a minor virtual health hazard, even if they are mutagenic carcinogens, as long as their daily intake is below 1.5 μg/ person/day. Such a comprehensive threshold is known as the "Threshold of Toxicological Concern (TTC)." (16).

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