Structure of DNA

Two separate long **polymer chains** wound around each other in the form of a **double helix**

The polymer chains are formed from sugar units and phosphate units that alternate with each other to make up the strands.

The two strands are wound around each other in opposite polarity and are held together by means of hydrogen bonding which occurs between pairs of bases that are attached to the sugar units of each strand.

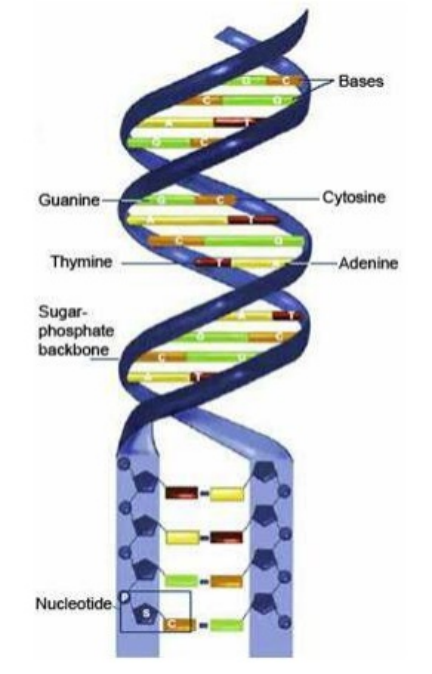
Adenine on one strand can only pair with **thymine** on the opposite strand, and**guanine** on one strand can only pair with **cytosine** on the opposite strand.

The two sugar- phosphate strands are wound around each other, making one full revolution every 3.4 nm (1nm = 10−9 m) in a right-handed spiral, which is wound around a central axis, such that a major grove and a minor grove are formed.

The base pairs are spaced at intervals of 0.34 nm along the chain.

The result is an extremely long, thin molecule which reaches up to 1 mm in length and diameter of 2 nm.

DNA ladder or molecule



Base Sequence and the Genetic Code

The precise sequence of bases along the DNA molecule forms the code that carries the genetic information.

The complementary base pairing endows the molecule with the ability to provide an exact copy of itself.

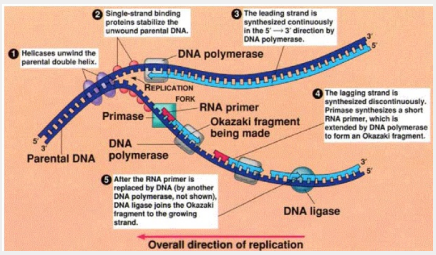
DNA Replication

Replication is not continuous but occurs at a definite part or phase of the cell division cycle, **the S phase**.

The two (old) strands of the DNA molecule are separated and two (new) strands are made with the exact complimentary base pairing along the two old strands so that two new DNA double helix molecules are formed each made up of one old and one new strand.

The two new DNA molecules eventually separate to two daughter cells so that the process of replication copies the genetic information in the mother cell and permits its transmission to two identical daughter cells.

DNA replication



Target for Radiation Damage

In simplistic terms, **the living cell consists of** an outer cell membrane, a cytoplasm, a nuclear envelope or membrane and a nucleus. The nucleus contains the deoxyribonucleic acid (DNA), which is the backbone of the chromosomes and carries all the information that determines the nature of the cell and regulates its operation.

By **Molly**
cheatography.com/molly/

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Target for Radiation Damage (cont)

The sensitive site for radiation-induced cell death is believed to be located in the nucleus as opposed to the cytoplasm.

Single Strand Breaks

The number is linearly related to the dose over a wide range (<0.2Gy to 60,000Gy).

Most are induced via the OH* radicals of water.

The number induced in oxygenated cells is three to four times that found in cells irradiated under hypoxic conditions.

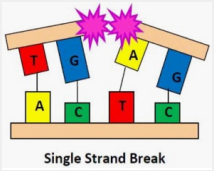
Repair is very rapid and efficient and involves the excision of the strand containing the defective piece of DNA.

The complementary (undamaged) single strand is used as the template for the resynthesis of a new length of DNA.

SSBs are much less important than DSBs in determining cell death.

Non-repaired SSBs can take part in the formation of DSBs

Single Strand Breaks



Double Strand Breaks

Double strand breaks of DNA, if not accurately repaired, **can lead to cell death** or the birth of an **abnormal (cancerous) cell.**

The relationship between the number induced and radiation dose is believed to be 'linear quadratic' ($P = \alpha D + \beta D2$).

They are produced by the passage of one ionising event (αD) or as a result of two independent SSBs ($\beta D2$).

They were initially thought to be unrepairable and this idea formed the basis for the long-standing view that DSBs were the lethal radiation-induced lesions.

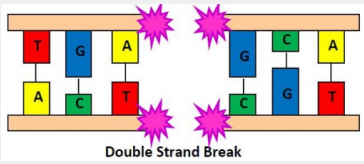
Recent measurements show that many can be repaired or at least rejoined.

These methods can only detect whether the free ends of a broken DNA molecule have joined together; they cannot indicate whether the original base pairing of the genetic code has been re-established.

The techniques cannot detect the presence of 'mis-repair' or 'error prone' repair which might be a cause of significant genetic damage.

In the absence of a perfect template, it is difficult to see how repair can be achieved without some erroneous base-pair acquisition or loss.

Double strand break



DNA Base Damage

Damage to bases of DNA was first recognised in bacteria.

Highly sensitive tests can now measure such lesions, especially thymine damage.

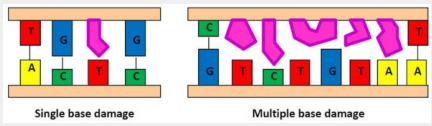
The number is linearly related to dose.

They arise via the OH* radicals of water.

Thymine damage is more frequent in mammalian cells than SSBs however, there is no direct evidence showing that this forms a biologically important lesion.

Excision repair mechanism is responsible for rapid and efficient removal of damaged bases.

DNA Base Damage



Other Mechanisms of DNA Damage

Normal metabolic processes:

Free radical generation during metabolism

DNA replication may be associated with transcription errors, repaired through a number of pathways

External sources:

Other Mechanisms of DNA Damage (cont)

Non-ionising radiation, such as UV electromagnetic radiation, can cause crosslinks between adjacent bases

Chemicals may react with DNA, causing adducts (additions to the DNA molecule that disrupt its structure) or crosslinks

Heat can cause breakage of DNA molecules

Base mismatch, which often occurs during DNA replication, is repaired with **mismatch repair proteins (MMR)**. **Bulky DNA lesions or adducts** are repaired by **nucleotide excision repair (NER)**. Other processes to remove specific adducts are also present.

Summary of Assays for DNA Damage and Repair

DNA damage is assessed by: Comet Assay, Pulsed Field Gel Electrophoresis (PFGE), or Micronucleus Assay.

Cells that are more sensitive to radiation will show increased fragmentation of DNA, detectable with pulsed field gel electrophoresis or the comet assay. The micronucleus assay is capable of detecting DNA breaks by measuring the formation of micronuclei, which occur when DNA fragments are not aligned on the mitotic spindle.

Summary of Assays for DNA Damage and Repair (cont)

DNA repair can also be assessed using these methods. By allowing cells to survive for some time after radiation exposure, and comparing the fragmentation of DNA with cells immediately sacrificed, the amount of repair that occurs is quantifiable.

Comet Assay for DNA Damage/Repair

Single-cell electrophoresis

Single cells are placed on a glass slide, held in suspension by an agarose gel. They are then exposed to radiation (or some other stimulus) before being lysed by an aqueous solution. The DNA is unable to escape the agarose gel, whereas the remainder of the cell is removed by the solution. The DNA occupies this space (the **nucleoid**).

The slide is then immersed in an electrophoresis solution and has a current applied. Undamaged DNA remains trapped in the nucleoid, whereas damaged DNA is small enough to move through the agarose gel. Once the current has been applied for a specified time, the slide is stained for DNA molecules and visualised under a specialised microscope, often with image analysis software to calculate the presence of DNA damage.

Comet Assay for DNA Damage/Repair (cont)

The term **comet assay** is derived from the appearance of the nucleoid after electrophoresis has taken place. The undamaged DNA remains in the nucleoid in a sphere, the 'head' of the comet. The damaged DNA travels towards the anode, forming the 'tail' of the comet.

Detects differences in DNA damage (and repair) at a single cell level and is commonly used for biopsy specimens from tumours.

Pulsed Field Gel Electrophoresis (Assay)

Gel Electrophoresis works because fragments of DNA have a negative charge, causing them to migrate towards the anode if a charge is run through the gel containing the DNA molecules.

This approach is limited due to poor sensitivity to large (over 50 kbp: kilo-base pairs) fragments of DNA, which tend to move at the same rate through the gel.

Pulsed field gel electrophoresis involves three pairs of electrodes, aligned at 0°, 120°, and -120° with respect to the direction of travel. Charge is run through the sample for 10 - 60 seconds between a pair of electrodes, with an equal time spent on each group for a net forward migration (see Figure 2.10). Larger fragments take longer to realign themselves to the changing voltage, and therefore there is increased separation of DNA fragments.

Micronucleus Assay

A **micronucleus** is the aberrant formation of a third, small nucleus during a mitotic division.

These micronuclei form when there is a piece of DNA not attached to the mitotic spindle (due to double strand breaks).

For this experiment, dividing cells are exposed to a stimuli that causes double strand breaks. Cytokinesis is inhibited by cytochalasin-B. The cells are then stained and examined under a microscope; the number of cells that contain micronuclei are counted. About 1,000 cells need to be counted for an accurate result.

DNA Repair

Sensing of DNA Damage: Several genes are involved in the response to ionising radiation. The actual genes involved depend on the damage inflicted as well as the stage in the cell cycle.

Basics of DNA Repair: For therapeutic radiation, the repair of double strand breaks (DSBs) is the most important as these seem to be the lesions that lead to cell death.

DNA Repair (cont)

DSBs are difficult problems for the cell to repair. The two ends may dissociate, although the histone molecules may provide some structural support. If several breaks are formed in a cell, then the cell may unite the strands incorrectly. The final problem is that there may not be an appropriate template to repair the damage, particularly in G1 and early S phases.

DSB repair is performed by two cellular processes: **Homologous Recombination (HR)** and **Non-Homologous End Joining (NHEJ)**

Repair by **NHEJ** operates throughout the cell cycle but dominates in G1/S-phases. The process is error prone because it does not rely on sequence homology.

HR utilises sequence homology with an undamaged copy of the broken region and hence can only operate in late S- or G2-phases of the cell cycle.

Other DNA repair mechanisms such as base excision repair (BER), mismatch repair (MR) and nucleotide excision repair (NER) respond to damage such a base oxidation, alkylation, and strand intercalation.

Homologous Recombination (HR)

The ideal repair pathway, but it requires an undamaged copy of the DNA to function (and replace the damaged section).

This means that homologous recombination **only occurs after duplication of the DNA has occurred in preparation for mitosis.**

The first step in HR is the detection of the DSB. This is performed by the ATM/ATR gene products, as well as the MRN complex. When activated, these proteins signal numerous other molecules (including p53), inducing a cell cycle arrest. The ends of the damaged DNA strand are processed and damaged bases are removed.

The resulting repair process attracts the sister chromatid, unwinds it and uses the undamaged DNA strand of the sister chromatid to fill in gap left by the double strand break. Then there is DNA synthesis of the missing nucleotides on the undamaged templates and ligation. This creates a complex strand crossover between the damaged and undamaged strands known as a **Holliday junction**, which is finally resolved before the repair process is complete.

By **Molly**
cheatography.com/molly/

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Non-Homologous End Joining (NHEJ)

Used in all phases of the cell cycle, as it does not require a sister chromatid to function.

Not as accurate as HR as it does not use a template for repair. It is still a relatively accurate repair pathway, as it is responsible for DNA damage repair in most cells at some time.

NHEJ also starts with recognition of the strand break and signaling to the cell that damage has occurred. PRKDC plays an important role in attracting repair proteins as well as preventing the ends from dissociating.

As a first step, each end of the double strand break must be 'processed', removing damaged bases and adding bases if necessary (this is where the error comes in).

The second step involves the ligation of the two ends.

Each side of the double strand break is recognised by XRCC5 and XRCC6 (Ku-70 / Ku-80). These attract PRKDC to the break, which bridges the gap and notifies the cell that damage has occurred through phosphorylation of numerous signalling molecules.

A collection of NHEJ related proteins then processes each end before ligating the ends together.

NHEJ and HR

BER / SSBR

Base Excision Repair (BER) / Single Strand Break Repair (SSBR)

Perhaps the most straightforward repair pathway.

If a base is altered by any means, it causes an abnormality in the shape of the DNA helix; this can be detected by glycosylase which removes (excises) the damaged base.

The DNA is then 'nicked' by the AP endonuclease enzyme; the sugar/phosphate backbone of the affected base is also removed by APE1, leaving a single strand break.

The alternative method of arriving at this situation is when radiation induces a SSB, which is then recognised by the PARP protein. The ends are processed (cleaned by PNK) to leave a 'clean' single strand break.

The end process of both methods is a gap in one strand of the DNA helix, which must be repaired. Short patching is performed by POLB (polymerase beta), which inserts the correct base (replaces damaged base), and LIG3 which unites the strand.

Long patching is more complicated, involving the removal of a section of the DNA around the single break and reconstruction of the region by polymerase and ligation of the ends.

Nucleotide Excision Repair (NER)

Used when a stretch of DNA has been damaged.

Particularly important in the response to ultraviolet radiation, which can cause bulky DNA adducts (not typical of ionising radiation).

NER is carried out by an array of proteins. The damaged strand is detected, and incisions made up and downstream of the lesion by 5 - 10 bases. The entire section is removed and the gap in the DNA is then copied from the undamaged side of the DNA strand and ligated onto the free ends.

Mismatch Repair (MMR)

Occurs during DNA replication, and ensures highly accurate translation of DNA (important in carcinogenesis).

If an incorrect base is inserted by DNA polymerase, the MMR proteins are able to detect the abnormal shape of the DNA helix (incorrect pairing of bases) and excise the incorrect base.

These abnormal bases are excised with a small margin of bases on either side. The gap is then filled by DNA polymerase and the ends ligated - repair of the lesion.

By **Molly**
cheatography.com/molly/

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DNA in Chromosomes

Chromosomes are thread-like structures of DNA and protein.

The basic DNA molecule is associated with beads or discs of proteins (**histones**) around which the DNA is wrapped and the protein discs are packed to form a fibre that can be seen under the electron microscope. This fibre is looped, folded and branched in an irregular fashion to form the chromosomes that are visible under the light microscope during metaphase.

A single continuous DNA molecule extends from one end of the chromosome to the other. Somewhere along the length of chromosomes is a region that does not stain, called the **centromere.**

The chromosome therefore comprises two strands (the chromatids) held together at the centromere region.

In cell division, the centromere divides the chromosome and each daughter cell receives one chromatid from each chromosome. The presence of a centromere is essential, therefore, for the migration of chromosomal pieces to the poles of the cell.

Chromosome Damage

The arms of chromosomes are subject to breakage.

Exposure to ionising radiation increases the frequency of breaks, these occur when the radiation passes through the chromosomal thread. The thread breaks into parts, but broken ends of chromosomes are 'sticky', and because of this, the parts frequently stick together again. This process of healing, called **restitution**, occurs following most chromosome breaks, probably in more than 90 percent of them.

Restituted chromosomes either lose no genetic matter or so little that cells bearing them function normally enough to escape detection. Occasionally, however, restitution does not follow a break resulting in chromosome aberrations. Breakage of chromosomes without restitution is lethal if cell division occurs after the chromosome break. Cell death, as a result of chromosome breakage, is known as **'mitotic death'**. It is a principal mechanism of cell killing in radiotherapy.

Chromosome damage arises when a single chromosome is broken before the material has been duplicated in S phase.

Chromosome Damage (cont)

When the chromatin generates an identical strand, it replicates the break caused by the radiation. Hence, a chromosome aberration is visible at mitosis, as there are identical breaks in a pair of strands.

Chromatid damage results from damage to one arm of the duplicated chromosome, with no damage to the other.

The radiation dose is given later in interphase, after DNA has been doubled, the arms are separated, and the radiation only breaks one chromatid. This leads to chromatid abberations.

Dicentric Chromosome

An example of a lethal aberration

Produced when breaks occur in two chromosomes within the same nucleus.

If the 'broken ends' are within close proximity, they may rejoin to produce a dicentric chromosome with an accompanying fragment.

A **ring** is formed when both ends are lost from the same chromosome. The chromosome then attaches its new ends together, leading to formation of a ring.

An **anaphase bridge** is formed when a duplicated chromosome loses both ends of a paired arm. The arms then unite, and when the cell tries to divide at mitosis it is unable to separate the fused arms.

By **Molly**
cheatography.com/molly/

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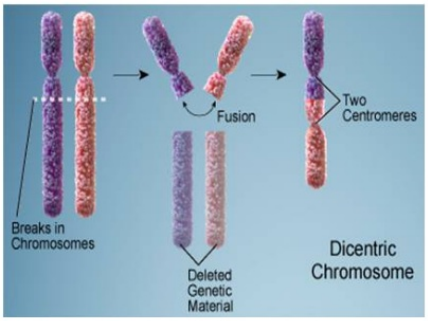
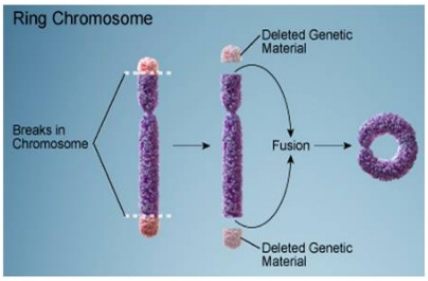
Dicentric Chromosome





Anaphase bridge

Assays for Chromosome Damage

Conventional Smear: the cells must be specially prepared to view the chromosomes:

Ideally a highly mitotic population is cultured
Cells are arrested in metaphase
The cells are treated to cause swelling of the nucleus and spreading out of the chromosomes
The cells are plated on a slide and left to dry
The slide is stained for DNA molecules

This method allows chromosomes to be visualised under light microscopy, where they can be counted and observed for **abnormalities**. Abnormalities such as translocations are often difficult to visualise with this method, however lethal chromosome abnormalities are typically visible.

In Vivo Lymphocyte Assay: Peripheral lymphocytes can be harvested after radiation exposure. They can be simulated to divide in culture (eg. with phytohaemagluttinin), and then arrested in metaphase. Chromosome smears can then be performed to judge the number of abnormalities present.

In-Situ Hybridisation: involves a a variety of techniques that have a similar process:

A probe is used to bind to a specific sequence of DNA, RNA or protein

Assays for Chromosome Damage (cont)

If required, an antibody directed against the probe is added to the cell. This antibody is capable of creating a visible effect when bound to the probe.

Silver In-Situ Hybridisation (SISH) is more commonly used for gene number counting.

The antibody used in SISH causes silver atoms to collect in the region of the gene. The number of genes can then be counted using a normal light microscope (the silver appears as a dark spot).

Fluorescense In-Situ Hybridisation (FISH): DNA strands can be targeted by specific probes. These can either be 'chromosome painting', which cause each chromosome to fluoresce a difference colour; or can be directed against specific genes.

Chromosome painting is particularly useful at detecting translocations, as the translocated arm will be a different colour to the host chromosome. FISH requires a specific microscope which can cause the molecules to fluoresce.

Cytogenetic Dosimetry

The detection of the presence or absence of dicentric chromosomes in cells, particularly in lymphocytes, is a method routinely used to identify or exclude people who are suspected of being irradiated.

Situations can occur when either:

Cytogenetic Dosimetry (cont)

1. the exposure registered on the personal monitor, such as a thermoluminescent dosimeter (TLD), does not appear to reflect dose received by the wearer

2. the person having suspected overexposure was not wearing any type of physical dosimeter.

The dicentric chromosome is a sensitive and reliable indicator of dose in persons having recent radiation exposures because it:

1. is easily identified;

2.occurs with a low background frequency;

3. is rarely observed following exposure to chemicals.

The Lymphocyte Culture System: To calibrate dose and effect, aliquots of whole blood from normal adults are exposed to 60Co γ radiation (or x-radiation) to doses 0.25 to 5.0 Gy (25 to 500 rads).

The lymphocytes are incubated in culture medium at 37°C for sufficient time to allow a large proportion of the lymphocytes to complete one round of DNA synthesis (approximately 48 hours).

The cells are then arrested (halted in the cell cycle) after division by the addition of an inhibitor and are then harvested, stained and examined under a microscope to determine the frequency of dicentric induction.

Cytogenetic Dosimetry (cont)

Approximately 500 cells are examined although the actual number depends on the level of exposure and statistical certainty required.

It has been found that the dose dependency for yield of dicentrics is adequately described by the linear-quadratic model, $Y = \alpha D + \beta D2$ where Y is dicentric yield (dicentrics per cell), D is radiation dose, and α and β determine the relative importance of single and two hit events

Radiobiological Definition of Cell Death

Cells are generally regarded as having been “killed” by radiation if they have **lost reproductive integrity**, not by whether they physically survive in the population.

Loss of reproductive integrity can occur by apoptosis, necrosis, mitotic catastrophe or by induced senescence. Although all but the last of these mechanisms ultimately results in physical loss of the cell this may take a significant time to occur, e.g mitotic death may not happen until several divisions have taken place.

Apoptosis or programmed cell death (previously called interphase cell death) is a strong feature in embryological development and in lymphocyte turnover.

Radiobiological Definition of Cell Death (cont)

Apoptosis (which is non-inflammatory) can be identified by microscopy: shrinkage of cellular morphology, condensation of chromatin, nucleosome laddering indicating chromatin degradation, and cell membrane blebbing.

Apoptosis occurs in particular cell types after low doses of irradiation e.g. lymphocytes, serous salivary gland cells, and certain cells in the stem cell zone in testis and intestinal crypts.

Mitotic Death occurs if a cell proceeds through mitosis without proper alignment of chromosomes on the metaphase plate, the division of the cell may lead to aneuploidy in both daughter cells. The cells die due to loss (or gain) of significant genetic material

This may be due to loss of genes that allow mitosis to occur or due to inability of the cell to pass on genetic material once the catastrophe has occurred.

Mitotic death is the principal mechanism of cell killing in radiotherapy

A rapid fall of cell numbers after irradiation is likely to be due to apoptosis but may also occur by mitotic death in rapidly proliferating populations.

Radiobiological Definition of Cell Death (cont)

Whether apoptosis reflects overall cell killing in tumour cell inactivation by radiation is currently unresolved and may only be the case for certain types of tumour cells.

Necrosis is typified by cell edema, poor staining of nuclei, increase of membrane permeability, shut down of cell metabolism, and an accompanying inflammatory response. Cellular necrosis generally occurs after high radiation doses.

Bystander Effect: The induction of biologic effects in cells that are not directly traversed by a charged particle, but are in proximity to cells that are.

Heritable biologic effects do not require direct damage to DNA!

Experiments indicate that irradiated cells secrete a molecule (capable of killing cells) into the medium that can transferred onto unirradiated cells.

Senescence or replicative senesence (RS) is a programmed cellular stress response to the accumulation of damage to a cell.

Radiobiological Definition of Cell Death (cont)

It is observed when cells stop dividing, and this differs from the behaviour of stem cells and tumour cells which do not show these limitations. Senescent cells are somewhat edematous and show poor cell-cell contact, increased polyploidy, decreased ability to express heat shock proteins, and shortening of **telomeres** (caps at the end of chromosomes).

It silences genes necessary for the transition from G1 to S phase of the cell cycle.

Autophagic Cell Death: the cell consumes itself. It is thought to be induced by radiotherapy and chemotherapy.

By **Molly**
cheatography.com/molly/

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