Introduction

The Genetic Material

Today, we all know that DNA is the genetic material that carries information from generation to generation. But, have you ever wondered how and when DNA was discovered? What experiments and observations led to the discovery of DNA? Let's explore this fun journey of the discovery of the genetic material here.

Discovery of DNA

Scientists had narrowed down that the genetic material was on chromosomes in the nucleus of a cell. However, the exact molecule was discovered only much later. Let's take a look at the series of experiments that scientists undertook that brought us closer to the discovery of DNA.

Frederick Griffith

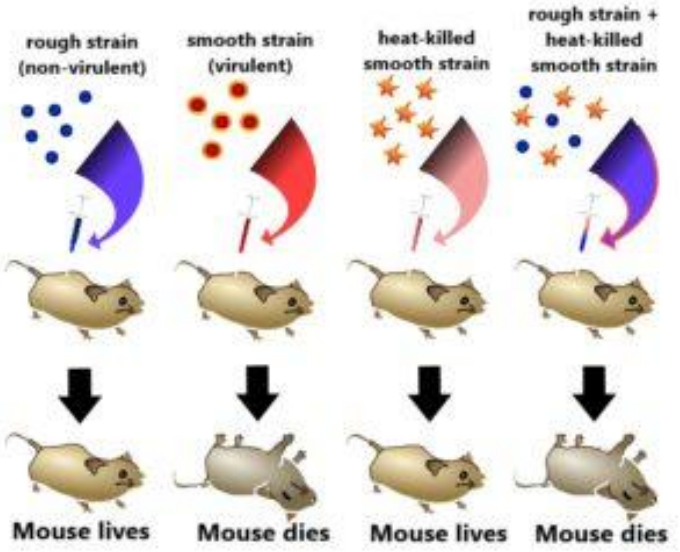
While working with *Streptococcus pneumoniae* (the bacterium that causes pneumonia) in 1928, Frederick Griffith observed a miraculous transformation in this bacterium. When you grow this bacterium on a culture plate, some produce shiny colonies (denoted as 'S') and some produce rough colonies (denoted as 'R').

The S strain bacteria have a polysaccharide coat which gives rise to smooth, shiny colonies. The R strain lacks this coat and hence, it gives rough colonies. Also, the S strain is virulent and causes pneumonia; while the R strain is non-virulent. He performed the following experiment with these strains and saw different observations.

1. S strain → Inject into mice → Mice develop pneumonia and die.
2. R strain → Inject into mice → Mice live.

3. Heat-killed S strain → Inject into mice → Mice live. (Griffith found that heating kills the bacteria).

4. Heat-killed S strain + R strain → Inject into mice → Mice die.



Frederick Griffith's experiment [Source: Wikimedia Commons]

- **Observations** – Not only did the mice injected with the heat-killed S strain + R strain die, but Griffith also recovered live S strain bacteria from these dead mice.

- **Conclusions** – He concluded that this was because the R strain had somehow been 'transformed' by the heat-killed S strain. This he argued was due to the transfer of a '**transforming principle**' from the S strain to the R strain, which made the R strain virulent. Although significant, his observations did not identify the biochemical nature of the transforming principle.

Oswald Avery, Colin MacLeod & Maclym McCarty

Avery, MacLeod, and McCarty, together set out to determine the biochemical nature of the 'transforming principle' identified by Griffith. These people purified DNA, RNA, and proteins from the heat-killed S strain and determined which macromolecule converted the R strain into the S strain.

- **Experiment** – They first treated the heat-killed S strain with proteases to break down proteins. Subsequently, they treated it with RNAses and then DNAses to break down RNA and DNA, respectively.

- **Observations** – Both protease and RNAse treatments did not affect the transformation of the R strain into the virulent one. Finally, treatment with DNAses inhibited the transformation of the R strain.

- **Conclusions** – They concluded that the genetic material is not protein or RNA, but it is **DNA**. However, this discovery was not accepted by all biologists.

Alfred Hershey & Martha Chase

Much earlier, scientists believed that the genetic material was protein. In 1952, Hershey & Chase were the ones to conclusively prove that DNA is the genetic material. They worked with bacteriophages – viruses that infect bacteria. A bacteriophage attaches and delivers its genetic material into a bacterial cell, where it generates more virus particles. Hersey & Chase used bacteriophages to experiment as follows:

- **Labelling** – Some viruses were grown on a medium containing radioactive phosphorus and some on a medium with radioactive sulfur.

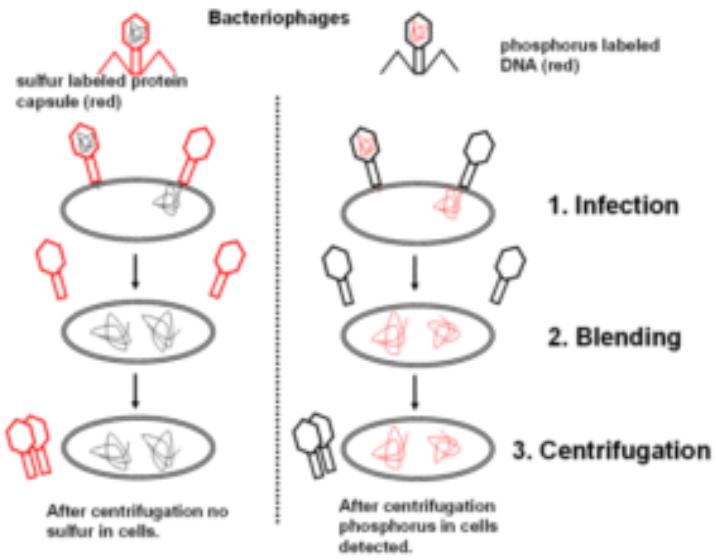
- **Viruses** – grown on radioactive phosphorus have radioactive DNA but not protein since DNA contains phosphorus but protein does not. Contrarily, viruses grown on radioactive sulfur have radioactive protein but not DNA since DNA does not contain sulfur.

- **Infection** – The radioactive phages were then allowed to infect the bacteria – *E. coli.*

- **Blending** and **Centrifugation** – As the infection progressed, the viral coats were removed from the bacteria by blending. Then, centrifugation was used to separate the viral particles from the bacteria.

Observations – Bacteria infected with viruses that have radioactive DNA, were radioactive, while bacteria infected with viruses that have radioactive protein, were not radioactive.

Conclusions – This experiment conclusively showed that DNA is the genetic material transferred from virus to bacteria, and not protein.



Hershey and Chase experiment [Source: Wikimedia Commons]

Properties Of Genetic Material

For a molecule to act as the genetic material, it should have the following characteristics:

- Be capable of replication i.e. create its own replica.
- It should be stable, structurally and chemically.
- It must have the scope for slow changes (mutations) to evolve.
- Be expressed in the form of 'Mendelian Characters'.

Although DNA is the genetic material in most organisms, in some viruses, RNA is the genetic material. In fact, according to studies, RNA was the first genetic material. But, since RNA is unstable, DNA evolved from RNA with chemical modifications, making it more stable and more fit to carry genetic information.

Gene: Concept and Units

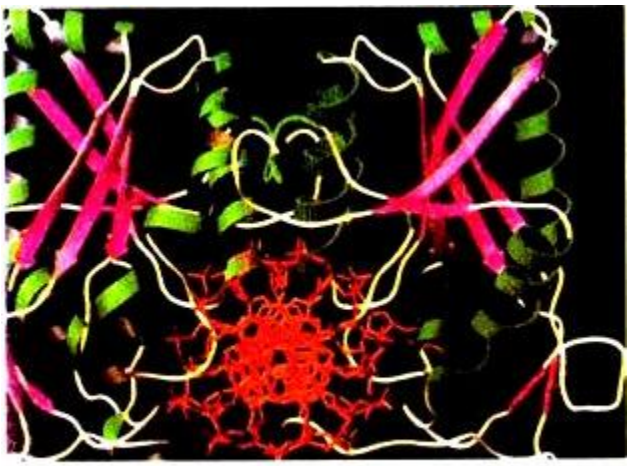
Concept of Gene:

Although the role of hereditary units (factors) in transfer of genetic characters over several generations in organisms was advocated by Gregor John Mendel, yet the mystery of the 'hereditary units' was un-ravelled during early 1900s. In 1909, W. Johanson coined the term 'gene' that acts as hereditary units. However, early work done by several workers proposes various hypotheses to explain the exact nature of genes.

In 1906, W. Bateson and R.C. Punnet reported the first case of linkage in sweet pea and proposed the presence or absence theory. According to them the dominant character has a determiner, and the recessive character lacks determiner. In 1926, T.H. Morgan discarded all the previous existing theories and put forth the particulate gene theory.

He thought that genes are arranged in a linear order on the chromosome and look like beads on a string. In 1928, Belling proposed that the chromosome that appeared as granules would be the gene. This theory of gene was well accepted by the cytologists.

In 1933, Morgan was awarded Nobel Prize for advocating the theory of genes. After the discovery of DNA as carrier of genetic information's, the Morgan's theory was discarded. Therefore, it is necessary to understand both, the classical and modern concepts of gene.



First experimental proof for the role of DNA as a genetic material was given by Avery, Mc Cleod and Mc Carty.

According to the classical concepts a gene is a unit of:

(i) Physiological functions,

(ii) Transmission or segregation of characters, and

(iii) Mutation.

In 1969, Shapiro and co-workers published the first picture of isolated genes. They purified the lac operon of DNA and took photographs through electron microscope.

Avery, McCleod and Mc Carty (1944) gave the first experimental proof for the role of DNA as genetic material. Therefore, the presence of genes was supposed on DNA. However, in some viruses like TMV, retroviruses, revoviruses, etc. the chemical nature of genes is RNA but not DNA.

In 1908, the British physician Sir E.R. Garrod first proposed one-gene-one product hypothesis. In 1941, G.W. Beadle and E.L. Tatum working at St. Standford university clearly demonstrated one-gene-one enzyme hypothesis based on experiments on Neurospora crassa. They made it clear that genes are the functional units and transmitted to progenies over generations; also they undergo mutations.

They treated N. crassa with X-rays and selected for X-ray induced mutations that would have been lethal. Their selection would have been possible when N. crassa was allowed to grow on nutrient medium containing vitamin B_6.

This explains that X-rays mutated vitamin B_6. synthesizing genes. They concluded that a gene codes for the synthesis of one enzyme. In 19.58, Beadle and Tatum with Lederberg received a Nobel Prize for their contribution to physiological genetics.

Units of a Gene:

After much extensive work done by the molecular biologists the nature of gene became clear. A gene can be defined as a polynucleotide chain that consists of segments each controlling a

particular trait. Now, genes are considered as a unit of function (cistron), a unit of mutation (mutant) and a unit of recombination (recon).

1. Cistron:

One-gene-one enzyme hypothesis of Beadle and Tatum was redefined by several workers in coming years. A single mRNA is transcribed by a single gene. Therefore, one-gene-one raRNA hypothesis was put forth. Exceptionally, a single mRNA is also transcribed by more than one gene and it is said to be polycistronic.

Therefore, the concept has been given as one-gene-one protein hypothesis. The proteins are the polypeptide chain of amino acids translated by mRNA. Therefore, it has been correctly used as one-gene-one polypeptide hypothesis.

Moreover, genes are present within the chromosome and their cis-trans effect govern the function. Therefore, S. Benzer termed the functional gene as cistron (Fig. 6.1 A). Crossing over within the functional genes or cistron is possible.

The cis and Trans arrangement of alleles may be written as below:

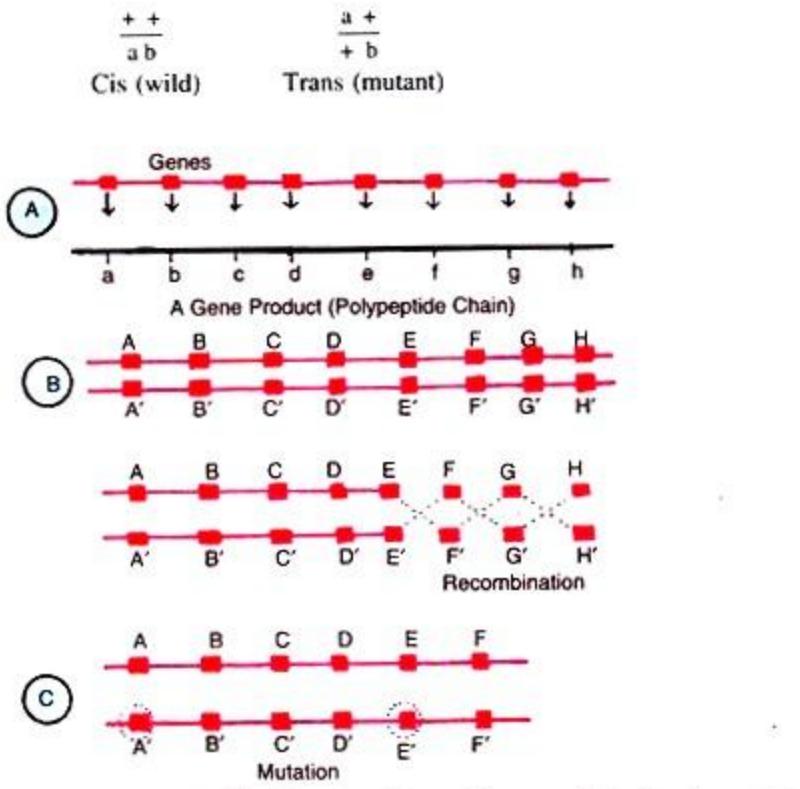


Fig. 6.1 : The genes as a unit of function i.e. cistron (A), recombination *i.e.* recon (B) and mutation i.e. muton (C)

2. Recon

Earlier, it was thought that crossing over occurs between two genes. In 1962, Benzer demonstrated that the crossing over or recombination occurs within a functional gene or cistron. In a cistron the recombinational units may be more than one. Thus, the smallest unit capable of undergoing recombination is known as recon (Fig. 6.1B).

Benzer (1955) found that the cultures of T4 bacteriophage formed plaques on agar plates of Escherichia coli. Normally T4 formed small plaques of smooth edges, whereas the mutant T4 phage formed the larger plaques of rough edges (Fig. 6.2).

The DNA molecule of T4 phage consists of several genes one of which is called rll region. Formation of rough edged plaques was governed by two adjacent genes (cistrons rllA and rllB) in mutant bacteriophage (Fig. 6.3 A).

Both the regions function independently and consist of 2,500 and 1,500 nucleotides, respectively. In rllA gene over 500 mutational sites are present where crossing over may occur. Through crossing over exchange of two segments of DNA occurs. If crossing over takes place within the gene by mating two rll mutant of T4 phage, a normal wild type phage can be produced.

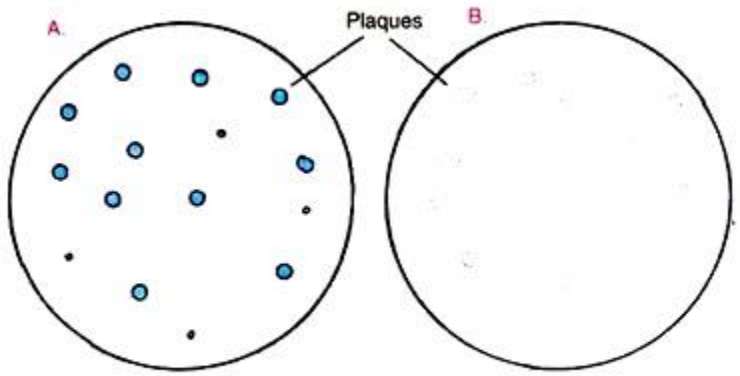


Fig. 6.2 : Formation of normal and smooth edged plaques (A) and rough edged plaques (B) by T4 bacteriophage.

Similar was the result of Benzer. Thus, the work of Benzer lends support that crossing over within a gene occurs (Fig. 6.3B) which explains that the recombinational unit (recon) is much smaller than the functional unit i.e. cistron.

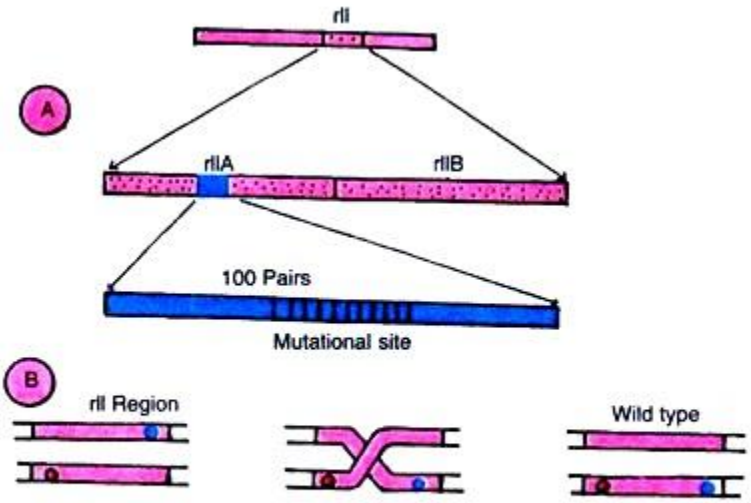


Fig. 6.3 : Diagrammatic presentation of rll region of T4 bacteriophage (A), and demonstration of crossing over within the genes (B).

3. Muton:

Benzer (1962) coined the term muton to denote the smallest unit of chromosome that under goes mutational changes. Hence, muton may be defined as 'the smallest unit of DNA which may be changed is the nucleotide'. Thus, changes at nucleotide level are possible (Fig. 6.1C). The smallest unit of muton is the nucleotide.

Therefore, cistron is the largest unit in size followed by recon and muton. This can be explained that a gene consists of several cistron, a cistron contains many recon, and a recon a number of mutons. However, if the size of a recon is equal to muton, there would be no possibility in recon for consisting of several mutons.

Molecular basis of Mutation

INTRODUCTION •

Term mutation was given by Devries in 1901 while studying evening primerose Oenothera lamarckiana • Most of these were chromosomal variations • Some were point variations • Originally the term mutation was given to both chromosomal as well as point mutations. • Recently chromosomal mutations are studied separately. • The term mutation is now given only to point mutations.

DEFINITION • Any sudden change occurring in hereditary material is called as mutation. • They may be harmful, beneficial or neutral. • A mutation is defined as an inherited change in genetic information.

TYPES OF GENES MUTATIONS • Number of ways to classify gene mutations: – On the basis of the molecular nature of the defect. – On the nature of the phenotypic effect-- amino acid sequence of the protein is altered or not. – On the basis of the causative agent of the mutation.

TYPES OF MUTATIONS TWO TYPES OF MUTATION OCCURS IN NATURE • SOMATIC MUTATION • GERM LINE MUTATION Somatic mutations • Arise in the somatic cells. • Passed on to other cells through the process of mitosis. • Effect of these mutations depends on the type of the cell in which they occur & the developmental stage of the organism. • If occurs early in development, larger the clone of the mutated cells.

GERM LINE MUTATION- • They occur in the cells that produce gametes • Passed on to future generations • In multicellular organisms, the term mutation is generally used for germ line mutations

MUTAGENS RESPONSIBLE FOR MUTATION θThe physical and chemical agent that caused mutation is known as mutagen. θChemical mutagen ⎫ Alkylating agent ⎫ Base analogs ⎫ Methylating agent ⎫ DNA intercalating agent ⎫ DNA crosslink agent ⎫ Reactive oxygen species (ROS)

θPhysical mutagen:-⏐Uv radiation⏐Ionising radiation

CHEMICAL MUTAGEN

On the basis of causative agent types of mutations Spontaneous mutations: • Mutations that result from natural changes in DNA. •

Spontaneous mutation contain depurination and deamination for a particular base are two common chemical event that produces spontaneous mutation. Induced mutations: • Results from changes caused by environmental chemicals & radiations. • Any environmental agent that increases the rate of mutation above the spontaneous is called a mutagen such as chemicals & radiations.

Nitrous Acid Causes Oxidative Deamination of Bases

Induced Mutation

Base modifying agents θThese are chemicals that act as mutagens by modifying the chemical structure and properties of bases. θThree types of mutagens that work in this way: 1)deaminating agent 2)hydroxylating agent 3)an alkylating agent

Alkylating agent⏐ These are naturally occuring and human made highly reactive chemicals that alter the structure of DNA and cause mutations.⏐ e.g. Ethylmethane sulphonate(EMS) and mustard gases, MMS.⏐ They donate an alkyl group to amino and keto groups in nucleotides.⏐ EMS alkylates the keto group in no 6 position of guanine and in number 4 position of thymine.

e.g. 6-ethylguanine acts as an analog of adenine and pairs with thymine

Hydroxylating agent⏐ Hydroxylamine is a mutagen that reacts specifically with cytosine ,modifying it by adding a hydroxyl group (OH) so that it pair with adenine instead of guanine.

Base analogs • Base analogs are bases that are similar to those normally found in DNA . • The base analogs pair with a different base in DNA. • One base analog mutagen is 5- bromouracil, which has a bromine residue instead of methyl group of thymine. • In normal states,5BU resembles thymine and pairs with adenine in DNA.

In rare states, it pairs with guanine • Not all base analogs are mutagens. • For e.g. AZT(azidothymidine) ,an approved drug given to patients with AIDS, is an analog of thymidine. • It is not a mutagen because it does not base pair changes.

Mutagenic effects of the base analog 5-bromouracil (5BU

Intercalating agent • Intercalating agent inserts itself between adjacent base pairs of the DNA strand that is template for new DNA synthesis. • An extra base is inserted into the new DNA strand opposite the intercalating agent. • After one more round of replication ,during which the intercalating agent is lost ,the overall result is a base pair addition mutation.

If the intercalating agent inserts itself into the new DNA strand in place of a base ,then when that DNA helix replicates after the intercalating agent is lost. • The result is a base pair deletion mutation. • If a base pair addition or base pair deletion point mutation occurs in a protein coding gene, the result is a frameshift mutation. • E.g. proflavin,acridine and ethidium bromide

Intercalating mutations

Radiation • Radiation occurs in non-ionizing or ionizing forms. • Ionization occur when energy is sufficient to knock an electron out of an atomic shell and break covalent bonds. • Except for UV light ,non ionizing radiation does not induce mutations. • But all forms ionizing radiation ,such as X rays, cosmic rays and radon can induce mutation.

Effect of UV radiation • UV radiation promotes the formation of covalent bonds between adjacent thymine residue in a DNA strand ,creating a cyclobutyl ring. • They form abnormal chemical bond between adjacent pyrimidine molecule (mainly thymine) in the same strand of the double helix.

Ionizing radiation penetrates tissue, colliding with molecules and knocking electrons out of orbits ,creating ions. • The ion can result in the breakage of covalent bonds, including those in the sugar phosphate back bone of DNA. • Ionizing radiation is the leading causes of gross mutation in humans. • High dosages of ionizing radiation kill cells so use in treating some form of cancer.

Spontaneous generation of addition and deletion mutants by DNA looping-out errors during replication

1. Tautomeric shift and mutation
2. . Tautomeric Shifts: --chemical fluctuations, --conformation states (stable==========unstable) Pyrimidine Purine
3. . Tautomeric Shifts Affect Base-Pairing C:T T:G
4. . Mutation Caused by Tautomeric Shifts
5. . DNA damaged by free radicals θA free radical is any species capable of independent existence that contains one or more unpaired electrons. θThey are unstable, very reactive and short-lived as they tend to catch an electron from other molecules.
6. DNA Modification • Free radicals induce several types of DNA damage including – strand breaks, – DNA-protein cross-links – and a large range of base and sugar modifications. • Of the free radicals the highly reactive hydroxyl radical (.OH) is the most prominent in the development of base and sugar modifications. • DNA damage also occurs through reactive nitrogen species undergoing mainly nitration and deamination of purines
7. Mutations on the basis of the Phenotypic effects of mutations: ϖMost common phenotype in natural populations of the organism is called as wild type phenotype. ϖThe effect of mutation is considered with reference to wild type phenotype
8. Forward mutation: ⎫ a mutation that alters the wild type phenotype ⎫ Reverse mutation (reversion): a mutation that changes a mutant phenotype back in to the wild type
9. Missense mutation: a base is substituted that alters a codon in the mRNA resulting in a different amino acid in the protein product TCA AGT UCA TTA AAT UUA Ser Leu LLeeuu

10. Silent mutation: alters a codon but due to degeneracy of the codon, same amino acid is specified. TCA AGT UCA TCG AGC UCG Ser Ser
11. Nonsense mutation: changes a sense codon into a nonsense codon. Nonsense mutation early in the mRNA sequence produces a greatly shortened & usually nonfunctional protein TCA AGT UCA TGA ACT UGA Ser Stop codon
12. Neutral mutation: mutation that alters the amino acid sequence of the protein but does not change its function as replaced amino acid is chemically similar or the affected aa has little influence on protein function. CTT GAA CUU ATT TAA AUU Leu Ile
13. Loss of function mutations: Structure of protein is so altered that it no longer works correctly. ϖ Mutation can occur in regulatory region that affects transcription , translation or spilicing of the protein. ϖ Frequently recessive. ϖ Complete or partial loss of the normal function.
14. Gain of function mutations: ϖProduces an entirely new trait ϖCauses a trait to appear in inappropriate tissues or at inappropriate times in development ϖFrequently dominant Conditional mutations: ϖExpressed only under certain conditions Lethal mutations: ϖCause the death of the organism
15. Suppressor mutation: ϖSuppresses the effect of other mutation ϖ Occurs at a site different from the site of original mutation ϖ Organism with a suppressor mutation is a double mutant but exhibits the phenotype of un mutated wild type ϖ Different from reverse mutation in which mutated site is reverted back into the wild type sequence

or

Spontaneous and Induced Mutations

In general, the appearance of a new mutation is a rare event. Most mutations that were originally studied occurred spontaneously. This class of mutation is termed **spontaneous mutations**. Historically, geneticists recognized these in nature. The mutations were collected, and the inheritance of these mutations were analyzed. But these mutations clearly represent only a small number of all possible mutations. To genetically dissect a biological system further, new mutations were created by scient ists by treating an organism with a mutagenizing agent. These mutations are called **induced mutations**.

The spontaneous mutation rate varies. Large gene provide a large target and tend to mutate more frequently. A study of the five coat color loci in mice showed that the rate of mutation ranged from 2×10^{-6} to 40×10^{-6} mutations per gamete per gene. Data from several studies on eukaryotic organisms shows that in general the spontaneous mutation rate is $2\text{-}12 \times 10^{-6}$ mutations per gamete per gene. Given that the human genome contains 100,000 genes, we can conclude that we would predict that 1-5 human gametes would contain a mutation in some gene.

Mutations can be induced by several methods. The three general approaches used to generate mutations are **radiation**, **chemical** and **transposon insertion**. The first induced mutations were created by treating Drosophila with X-rays. Using this a pproach Mueller to induce lethal mutations. In addition to X-rays, other types of radiation treatments that have proven useful include gamma rays and fast neutron bombardment. These treatments can induce point mutations (changes in a single nucleotide) or deletions (loss of a chromosomal segment).

Chemical mutagens work mostly by inducing **point mutations**. Point mutations occur when a single base pair of a gene is changed. These changes are classified as **transitions** or **transversions**. Transitions occur when a purine is convert ed to a purine (A to G or G to A) or a pyrimide is converted to a pyrimidine (T to C or C to T). A transversion results when a purine is converted to a pyrimidine or a pyrimidine is converted to a purine. A transversion example is adenine being converte d to a cytosine. You can determine other examples.

Two major classes of chemical mutagens are routinely used. These are **alkylating agents** and **base analogs**. Each has a specific effect on DNA. Alkylating agents [such as ethyl methane sulphonate (EMS), ethyl ethane sulphonate (EES) and musta rd gas] can mutate both replicating and non-replicating DNA. By contrast, a base analog (5-bromouracil and 2-aminopurine) only mutate DNA when the analog is incorporated into replicating DNA. Each class of chemical mutagen has specific effects that can lead to transitions, transversions or deletions.

Scientists are now using the power of transposable elements to create new mutations. Transposable elements are mobile pieces of DNA that can move from one location in a geneome

to another. Often when they move to a new location, the result is a new mutan t. The mutant arises because the presence of a piece of DNA in a wild type gene disrupts the normal function of that gene. As more and more is being learned about genes and genomes, it is becoming apparent that transposable elements are a power source f or creating insertional mutants.

The detailed knowledge of the structure and funciton of transposable elements is now being applied in the pursuit of new mutations. Stocks are created in which a specific type of element is present. This stock is then crossed to a genetic stock that doe s not contain the element. Once that element enters the virgin stock, it can begin to move around that genome. By carefully observing the offspring, new mutants can be discovered. This approach to developing mutants is termed insertional mutagenesis.

Isolation Of Bacterial Mutants

Mutations are induced in microorganisms for various research studies. Once induced, there is a need to isolate the mutants from those organisms that have not mutated. Mutants can be easily detected and efficiently isolated from non-mutated parents, if certain characteristics of the organisms are known. Some commonly used methods for the isolation of mutants are: replica plating technique, resistance selection method, substrate utilization method, and Ames test

Replica plating method

This technique is used to isolate auxotrophic mutants. In this procedure, mutagenized culture (which is a mixture of both mutated and non-mutated bacteria) is spread on an agar plate which acts as a medium of growth for the bacteria. After incubation for some time, both the parent and mutant bacteria begin to grow. This is called the **master plate**. Using a sterile velvet pad, colonies are transferred from the master plate on to secondary plates (replica plating) that contain media that are capable of sustaining only mutants, and not the non-mutants. After further incubation, replica plates are compared with the master plates, which allows for the identification and isolation of mutant bacteria from the replica plate. In some cases, it may so happen that only non-mutants are able to grow on the replica plate, and not the mutants. This too helps in the identification of mutants.

Resistance selection method

This method is useful to isolate antibiotic resistant mutants. Usually, wild type bacteria are not resistant to antibiotics. So, once mutation has been induced, the bacteria are grown in the presence of antibiotics. All bacteria that are now able to grow on antibiotic containing media, are able to do so because they have been able to acquire antibiotic sensitivity due to mutations in their genes. These are then isolated and used for further studies.

Substrate utilization method

In this method, mutants are isolated using chromogenic substrate. A synthetic analog of a natural substrate (food source) which has a dye linked to it is used in the growth medium. For example, the enzyme β-galactosidase, catalyzes the hydrolysis of lactose into its constituent sugars, glucose and galactose. A commonly used chromogenic substrate for β-galactosidase is a special kind of colorless galactose called X-gal, which is a synthetic analogue containing a dye linked to galactose. The color of the dye is only observed when X-gal is hydrolyzed by β-galactosidase. Bacteria with a functional β-galactosidase gene turn blue on a growth medium containing X-gal, and bacteria that do not produce β-galactosidase stay white.

Ames test

This test uses bacteria to test whether a particular chemical is mutagen. A strain of *Salmonella typhimurium* bacterium carrying a defective gene causes it to lose its ability to synthesize histidine. However, this mutation may be reversed due to a back mutation, which allows the bacteria to regain function, and the ability to grow on a medium, lacking histidine.

OR

ISOLATION OF MUTANTS

1. ISOLATION OF MUTANTS: ⎫ Mutation occurring in microorganism can be detected and efficiently isolated from the parent organism of other mutants. ⎫ While studying we must be aware of wild type characters of an organism ,so the mutants can easily detected. ⎫ In bacteria and other haploid microorganism, the detection system are straight forward because any new allele should be observed immediately.

2. • In albino mutation, the detection is very simple. It requires only change in colour of bacterial colony. • The other detection systems are rather complex.

3. SOME DETECTION METHODS ′ Replica plating technique. ′ Resistance selection method. ′ Substrate utilization method. ′ Ames method

4. 1. REPLICA PLATING TECHNIQUE: ′ Joshua and Esther Ledgerberg (1952) developed a new technique called replica plating . ′ This technique is used to detect auxotrophic mutants and wild type strains on the basis of ability to grow in the absence of amino acids. ′ Also this test is used to demonstrate the presence of antibiotic resistance in bacterial cultures prior to exposure of antibiotic

5. STEPS INVOLVED θ Generate the mutants by treating a culture with a mutagen e.g.nitrosoguanidine . θ Inoculate a plate containing complete growth medium and incubate it at proper temperature. Both wild type and mutant survivors will from complete medium. θ This plate containing complete medium is called master plate.

6. ′ Prepare a piece of sterile velvet and gently on the upper surface of the master plate to pick up bacterial cell from each colony. ′ As pressed the master plate, again gently press the velvet on the replica plates containing complete medium in one set and lacking cine in only leucine in the other set. ′ Thus, the bacterial cells are transferred in replica plates in the same position as in master plate. ′ Incubate the plates and compare the replica plate with master plate for bacterial colony not growing on replica plate..

7. 2.RESISTANCE SELECTION METHOD ′ This is another method used for isolation of mutants. ′ Generally the wild the wild type cells not resistant either to antibiotics or bacteriophage. ′ Therefore, it is possible to grow the bacterium in the presence of agent. ′ This method is applied for isolation of mutants resistant to chemical compounds that can be amended in agar, phage resistant mutants.

8. 3.SUBSTRATE UTILIZATION METHOD : ′ This method is employed in the selection of bacteria. Several bacteria utilize only a few carbon sources. ′ The cultures are plated on to medium containing alternate carbon sources. ′ Any colony that grows on medium can use the substrate and are possibly mutants. These can be isolated. ′ Sugar utilization mutants are also isolated by means of color indicator plates. ′ EMB medium is used for this purpose. ′ This medium contain lactose sugar as carbon source and complete mixture of amino acids.

9. ′ Therefore both lactose wild type and lactose mutant cells can grow and form colonies on EMB agar plates. ′ The lac+ cells catabolize lactose and secrete acids,therefore the pH of the medium decreases. This will result in staining of colony to dark purple. ′ On the other hand, Lac- cells are unable to utilize lactose and use some of the amino acids as carbon source. ′ After utilization of amino acid, ammonia is produced that increases the pH and de colorize the dye resulting in white colony.

10. 4. AMES TEST : ′ 4. Ames test In 1974 Bruce Ames developed a method for evaluating the potential of chemical to cause cancer, known as Ames test . ′ Ames test is based on the principle that both cancer and mutations results from the damage of DNA, and results of experiments have demonstrated that 90% of known carcinogen are also mutagens. ′ Several species of salmonella typhimurium are employed. Each strain contains a different mutation in the operon histidine biosynthesis.

11. STEPS OF AMES TEST:- ′ Prepare the culture of Salmonella histidine auxotrophs (His-). ′ Mix the bacterial cells and test substance(mutagen) in dilute molten top agar with a small amount of histidine in one set, and control with cmplete medium plus large amount of histidine . ′ Pour the molten mix on the top of minimal agar plates and incubate at 37°C for 2-3 days.

12. ′ Until histidine is depleted all the His- cells will grow in the presence of test mutagen. ′ When the histidine is completely exhausted only the revertants will grow on the plate. ′ The number of spontaneous revertants is low, whereas the number of revertant induced by carcinogen is quite high. ′ High number of colonies represent the greater mutagenicity.

13. o A mammalian liver extract is added to the above molten top agar before plating. o The extract converts the carcinogen in to electrophilic derivatives which will soon react with DNA molecule. o In natural way it is occurs in mammalian system when foreign particle are metabolized in the liver. o Bacteria does not have metabolizing capacity, therefore, the liver extract is added to this test, to promote transformation.

Isolation and Analysis of Mutants

Many different types of mutants have been identified in organisms ranging from bacteria to humans. Mutants can differ from their normal counterparts in a variety of ways. Some mutations

cause only subtle changes; for example, certain mutations in the fruit fly, *Drosophila melanogaster*, result in failure of a single type of neuronal cell to develop, but mutant flies otherwise are normal. Other mutations lead to significant changes in development, cellular function, appearance (Figure 8-8), and behavior of an individual. Many mutations are nonlethal, but some result in organismal death.

The procedures used to identify and isolate mutants, referred to as *genetic screens*, depend on whether the experimental organism is haploid or diploid and, if the latter, whether the mutation is recessive or dominant. Usually, mutations are induced by treatment with a mutagen, and the mutagenized population subjected to a genetic screen designed to identify and isolate individuals with mutations affecting a particular process of interest. Genes that encode proteins essential for life are among the most interesting and important ones to study. Since phenotypic expression of mutations in essential genes leads to death of the individual, ingenious screens are needed to isolate and maintain organisms with a lethal mutation.

Characterization of mutants in a variety of experimental organisms has been used to investigate many different fundamental biological processes. Genetic analyses of mutants defective in a particular process can reveal: (a) the number of genes required for the process to occur; (b) the order in which gene products act in the process; and (c) whether the proteins encoded by different genes interact with one another. Genetic studies of this type have helped unravel various metabolic pathways, regulatory mechanisms, and developmental processes.

Temperature-Sensitive Screens Can Isolate Lethal Mutations in Haploids

In haploid organisms (e.g., prokaryotes and yeast), all mutations are in effect dominant so that the mutant phenotype is exhibited immediately in the progeny of the mutagenized population. For instance, mutations that disrupt arginine synthesis lead to cells that require arginine for growth. Such mutations are easily detected by growing mutagenized populations in the presence and absence of arginine.

In prokaryotes and haploid eukaryotes such as yeast, essential genes can be studied through the use of *conditional* mutations. For instance, a mutant protein may be fully functional at 30 °C but completely inactive at 37 °C, whereas the normal protein would be fully functional at both

temperatures. A temperature at which the mutant phenotype is observed is called *nonpermissive;* a *permissive* temperature is one at which the phenotype is not observed. Mutant strains can be maintained at a permissive temperature; then, for analysis, a subculture can be set up at a nonpermissive temperature. Such **temperature-sensitive (ts) mutants** can also be generated in *Drosophila* and *C. elegans,* but cannot be isolated in warm-blooded animals.

An example of a particularly important temperaturesensitive screen in the yeast *S. cerevisiae* comes from the studies of L. H. Hartwell and colleagues in the late 1960s and early 1970s. They set out to identify genes important in regulation of the cell cycle. Cell division in this yeast occurs through a budding process, and the size of the bud, which is easily visualized by light microscopy, is an indication of the cell's position in the cell cycle. In these studies, the researchers first identified mutagenized yeast cells that did not grow at 36 °C (Figure 8-9a). Then they used video microscopy to analyze the identified mutants for cell-division defects at the nonpermissive temperature (Figure 8- 9b). These yeast mutants were not simply slow growing as they might be if they carried a mutation affecting general cellular metabolism; rather, they grew normally but showed a stage-specific block in growth at the nonpermissive temperature. The cell-cycle stage at which cell growth was arrested at the nonpermissive temperature indicated when the protein encoded by the mutated gene was required. Cloning and analysis of various genes defined by cell-cycle mutations are described in detail in Chapter 13. This work has provided important insights about the regulation of cell division in organisms ranging from yeast to humans.

Recessive Lethal Mutations in Diploids Can Be Screened by Use of Visible Markers

In diploid organisms, phenotypes resulting from recessive mutations can be observed only in individuals homozygous for the mutant alleles. Figure 8-10 outlines a procedure for inducing, identifying, and maintaining recessive lethal mutations in *Drosophila,* a diploid organism. Male fruit flies are treated with a mutagen and then mated with females, yielding F_1 progeny that are heterozygous for any induced mutations. Because these mutations are recessive, the mutant phenotype is not observed in the F_1 generation, and two additional crosses are needed to reveal the mutant phenotype. By using fly strains carrying known mutations (called *markers*) that give rise to visible phenotypes, researchers can distinguish heterozygous F_2 progeny carrying one

mutagenized chromosome and one normal chromosome from siblings with other genotypes. Mating of these F_2 heterozygous siblings produces an F_3 generation in which one-fourth of the flies will be homozygous for any mutation induced on the mutagenized chromosome, and if the mutation is in a gene essential for viability, they will not survive; one-fourth will be homozygous for the normal allele; and half will be heterozygous. The effects of the mutation can then be assessed in the homozygous class that does not survive, and the mutation can be maintained in the flies that are heterozygous for the mutation.

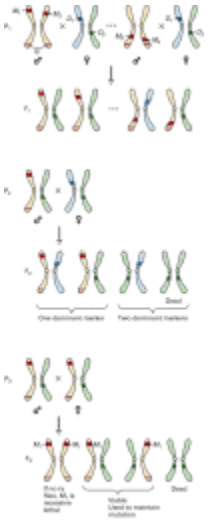


Figure 8-10

Procedure used to identify and maintain recessive lethal mutations on chromosome 3 (an autosome) in *Drosophila*, a diploid organism. This approach requires three sequential crosses. First, many males are treated with a mutagen (e.g., EMS), producing flies (more...)

Current understanding of the molecular mechanisms regulating development of multicellular organisms is based, in large part, on this type of genetic screen in *Drosophila*. C. Nüsslein-Volhard, E. Wiechaus, and their colleagues systematically screened for recessive lethal mutations affecting embryogenesis in *Drosophila* using a scheme similar to that shown in Figure 8-10. Dead homozygous embryos carrying lethal recessive mutations identified by this screen were analyzed for specific defects in the cuticular structures on the embryo surface (Chapter 14). A detailed picture of embryonic development has emerged from the characterization of these defects and the analysis of both the structure of the encoded proteins and their patterns

of expression during embryogenesis. We will discuss some of the fundamental discoveries based on these genetic studies in Chapters 14 and 23.

Complementation Analysis Determines If Different Mutations Are in the Same Gene

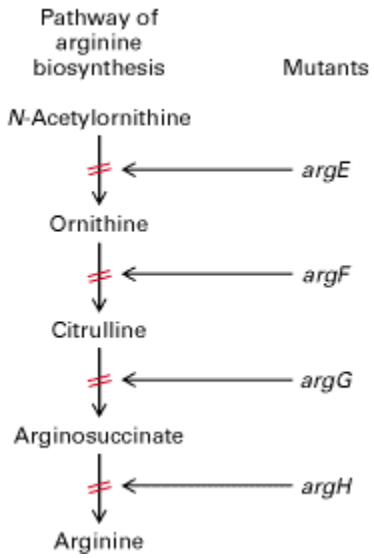
A common type of genetic analysis can reveal whether different recessive mutations associated with the same phenotype are in the same gene or in different genes. This analysis depends on the phenomenon of genetic complementation, that is, the restoration of the wild-type phenotype by mating of two different mutants. If two mutations, *A* and *B*, are in the *same* gene, then a diploid organism heterozygous for both mutations (i.e., carrying one *A* allele and one *B* allele) will exhibit the mutant phenotype. In contrast, if mutation *A* and *B* are in *separate* genes, then heterozygotes carrying a single copy of each mutant allele will exhibit the wild-type (normal) phenotype. In this case, the mutations are said to *complement* each other.

Complementation analysis of a set of mutants exhibiting the same phenotype can distinguish the individual genes in a set of functionally related genes, all of which must function to produce a given phenotypic trait. In the yeast *S. cerevisiae*, for example, four enzymes are required for growth on galactose (Figure 8-11a). If any one of these enzymes is absent or defective, yeast cells cannot grow on galactose. Because haploid yeast cells exist in one of two different mating types, **a** or α, which can be mated to yield **a**/α diploids, yeast can be subjected to complementation analysis like other diploid organisms. Figure 8-11b illustrates complementation analysis of Gal^- yeast strains defective for growth on galactose. When Gal^- strains with mutations in different *GAL* genes are mated, the resulting diploid cells will grow on galactose, because the wild-type gene in each strain will compensate for the genetic defect in the other. In contrast, diploids formed from Gal^- strains that are mutated in the same gene will not grow on galactose.

Metabolic and Other Pathways Can Be Genetically Dissected

Various types of analysis can order the genes involved in biochemical pathways and other cellular processes. A fairly straightforward example involves the genetic dissection of the biochemical pathway for synthesis of arginine in the bread mold *Neurospora crassa*. Four different mutant strains that are unable to synthesize arginine and require arginine for growth

(called *arginine* auxotrophs) were identified years ago. Each of the steps in biosynthesis of arginine is catalyzed by an enzyme encoded by a separate gene. The order of action of the different genes, hence the order of the biochemical reactions in the pathway, was determined by assessing which mutants could grow on different intermediates (Figure 8-12). Numerous biochemical pathways have been dissected by this type of study.



Other types of cellular processes also are amenable to genetic analysis. For example, the maturation pathway for secretory proteins in yeast has been dissected and ordered by analysis of a set of conditional temperature-sensitive secretion-defective *(sec)* mutants. In these mutant strains, the secretion of all proteins is blocked at the higher (nonpermissive) temperature but is normal at the lower (permissive) temperature. At the higher temperature, *sec* mutants accumulate proteins in the rough endoplasmic reticulum (ER), Golgi complex, or secretory vesicles (see Figure 17-14). At least 60 gene products are required to complete the maturation pathway as defined by the number of genes in which mutations give rise to a secretion defect. The genes can be ordered in a pathway by analyzing double-mutant combinations of *sec* genes. For instance, when ER and Golgi accumulating mutants are combined, proteins accumulate in the ER. These types of studies have shown that the pathway must be ordered in the following sequence: rough ER → Golgi → secretory vesicles. This maturation pathway is believed to apply to all secretory proteins in all eukaryotic organisms, including plants.

Suppressor Mutations Can Identify Genes Encoding Interacting Proteins

The phenomenon of genetic suppression can be used to identify proteins that specifically interact with one another in the living cell. The underlying logic is as follows: point mutations may lead to structural changes in protein A that disrupt its ability to associate with another protein (protein B) involved in the same cellular process. Similarly, mutations in protein B might lead to small structural changes that would inhibit its ability to interact with protein A. In rare cases small structural changes in protein A may be suppressed by compensatory changes in protein B. In these rare cases, strains carrying a specific mutant allele of protein A or B would be mutant, but strains carrying both would be normal. This is analogous to changes made in a lock and key.

Identification of such suppressor mutations has been elegantly applied in studies of the cytoskeletal protein actin in yeast (Figure 8-13). A strain of yeast that was temperature-sensitive for growth and carried a mutant actin allele called *act1-1* was plated at the nonpermissive temperature. A few cells were capable of growth at this temperature; these *revertants* were shown to have a second mutation in another gene, called *SAC6,* that allowed the *act1-1* mutants to grow. This *sac6* mutation acted as a dominant suppressor of the *act1-1* mutation, so that the double mutants *(act1-1 sac6)* exhibited the wild-type phenotype. This suppression was found to be allele-specific; that is, the *sac6* mutation suppressed the *act1-1* mutation but not other *act1* mutations. Single mutants carrying any one of several different *sac6* mutations were, like *act1-1* mutants, temperature-sensitive for growth. Remarkably, some *act1* mutations were found to be dominant suppressors of the recessive temperaturesensitive lethality of various *sac6* mutations.