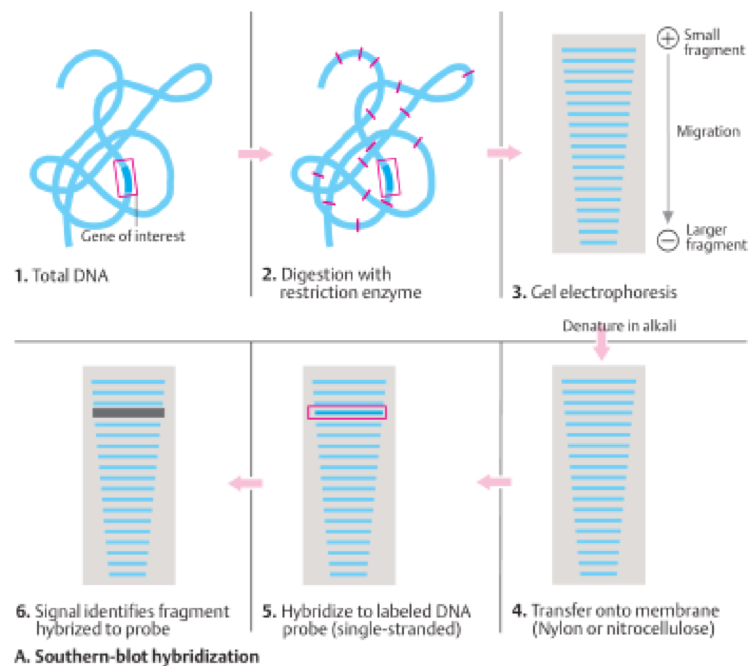
Restriction Analysis by Southern Blot Analysis

Restriction endonucleases are DNA-cleaving enzymes with defined sequences as targets. They are often simply called restriction enzymes. Since each enzyme cleaves DNA only at its specific recognition sequence, the total DNA of an individual present in nucleated cells can be cut into pieces of manageable and defined size in a reproducible way. Individual DNA fragments can then be selected, ligated into suitable vectors, multiplied, and examined. Owing to the uneven distribution of recognition sites, the DNA fragments differ in size. A starting mixture of DNA fragments is sorted according to size. Two procedures detect target DNA or RNA fragments after they have been arranged by size in gel electrophoresis—the Southern blot hybridization for DNA (named after E. Southern who developed this method 1975) and the Northern blot hybridization for RNA (a word play on Southern, not named after a Dr. Northern). Immunoblotting (Western blot) detects proteins by an antibody-based procedure.

Southern blot hybridization

The analysis starts with total DNA (1). The DNA is isolated and cut with restriction enzymes (2). One of the not yet identified fragments contains the gene being sought or part of the gene. The fragments are sorted by size in a gel (usually agarose) in an electric field (electrophoresis) (3). The smaller the fragment, the faster it migrates; the larger, the slower it migrates. Next, the blot is carried out: The fragments contained in the gel are transferred to a nitrocellulose or nylon membrane (4). There the DNA is denatured (made single-stranded) with alkali and fixed to the membrane by moderate heating (~ 80°C) or UV cross-linkage. The sample is incubated with a probe of complementary

singlestranded DNA (genomic DNA or cDNA) from the gene (5). The probe hybridizes solely with the complementary fragment being sought, and not with others (6). Since the probe is labeled with radioactive 32 P, the fragment being sought can be identified by placing an X-ray film on the membrane, where it appears as a black band on the film after development (autoradiogram) (6). The size, corresponding to position, is determined by running DNA fragments of known size in the electrophoresis.

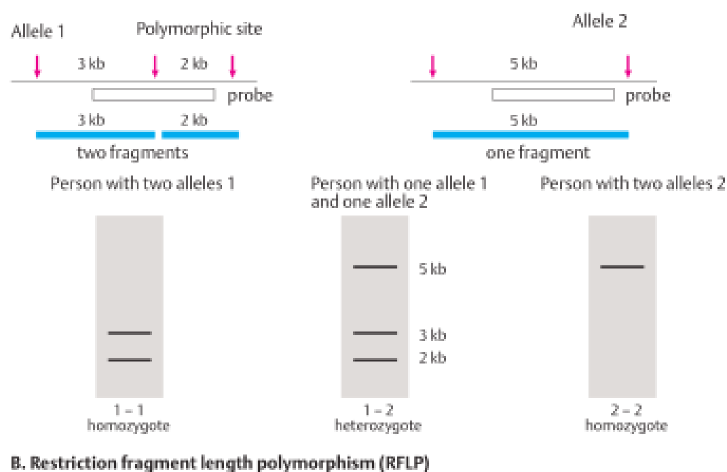


Southern blot hybridization

Restriction fragment length polymorphism (RFLP)

In about every 100 base pairs of a DNA segment, the nucleotide sequence differs in some individuals (DNA polymorphism). As a result, the recognition sequence of a restriction enzyme maybepresent ononechromosome butnotthe other. In this case the restriction fragment sizes differ at this site (restriction fragment length polymorphism, RFLP). An example is shown for two 5 kb (5000 base pair) DNA segments. In one, a restriction site in the middle is present (allele1); in the other

(allele2) it is absent. With a Southern blot, it can be determined whether in this location an individual is homozygous 1–1 (two alleles 1, no 5 kb fragment), heterozygous 1–2 (one allele each, 1 and 2), or homozygous 2–2 (two alleles 2). If the mutation being sought lies on the chromosome carrying the 5 kb fragment , the presence of this fragment indicates presence of the mutation. The absence of this fragment would indicate that the mutation is absent. It is important to understand that the RFLP itself is unrelated to the mutation. It simply distinguishes DNA fragments of different sizes from the same region. These can be used as markers to distinguish alleles in a segregation analysis. In addition to RFLPs, other types of DNA polymorphism can be detected by Southern blot hybridization, although polymerase chain reaction-based analysis of microsatellites is now used more frequently.



Restriction fragment length polymorphism (RFLP)

Automated DNA Sequencing

Large-scale DNA sequencing requires automated procedures based

on fluorescence labeling of DNA and suitable detection systems. In general, a fluorescent label can be used either directly or indirectly. Direct fluorescent labels, as used in automated sequencing, are fluorophores. These are molecules that emit a distinct fluorescent color when exposed to UV light of a specific wavelength. Examples of fluorophores used in sequencing are fluorescein, which fluoresces pale green when exposed to a wavelength of 494 nm; rhodamine, which fluoresces red at 555 nm; and aminomethyl cumarin acetic acid, which fluoresces blue at 399 nm. In addition, a combination of different fluorophores can be used to produce a fourth color. Thus, each of the four bases can be distinctly labeled.

Another approach is to use PCR-amplified products (thermal cycle sequencing). This has the advantage that double-stranded rather than single-stranded DNA can be used as the starting material. And since small amounts of template DNA are sufficient, the DNA to be sequenced does not have to be cloned beforehand.

Thermal cycle sequencing

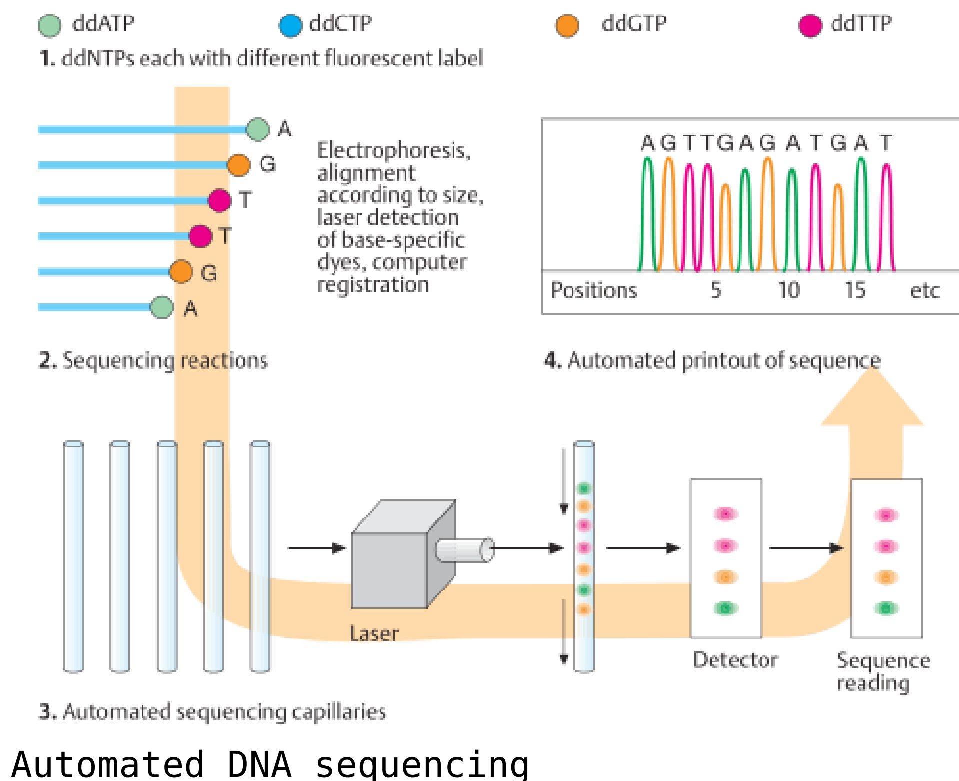
The DNA to be sequenced is contained in vector DNA <fn>Brown, T.A.: Genomes. Bios Scientific Publ., Oxford, 1999.</fn>. The primer, a short oligonucleotide with a sequence complementary to the site of attachment on the single-stranded DNA, is used as a starting point. For sequencing short stretches of DNA, a universal primer is sufficient.This is an oligonucleotide that will bind to vector DNA adjacent to the DNA to be sequenced. However, if the latter is longer than about 750 bp, only part of it will be sequenced. Therefore, additional internal primers are required. These anneal to different sites and amplify the DNA in a series of contiguous, overlapping chain termination experiments <fn>Rosenthal, N.: Fine structure of a gene–DNA sequencing. New Eng. J. Med. 332 :589–591, 1995</fn>. Here, each primer determines which region of the template DNA is being sequenced. In thermal cycle sequencing <fn>Strachan,

T., Read, A.P.: Human Molecular Genetics. 2 nd ed. Bios Scientific Publishers, Oxford, 1999.</fn>, only one primer is used to carry out PCR reactions, each with one dideoxynucleotide (ddA, ddT, ddG, or ddC) in the reaction mixture. This generates a series of different chain-terminated strands, each dependent on the position of the particular nucleotide base where the chain is being terminated <fn>Wilson, R.K., et al.: Development of an automated procedure for fluorescent DNA sequencing. Genomics 6 :626–636, 1990.</fn>. After many cycles and with electrophoresis, the sequence can be read as shown in the previous plate. One advantage of thermal cycle sequencing is that double-stranded DNA can be used as starting material.

Automated DNA sequencing (principle)

Automated DNA sequencing involves four fluorophores, one for each of the four nucleotide bases. The resulting fluorescent signal is recorded at a fixed point when DNA passes through a capillary containing an electrophoretic gel. The base-specific fluorescent labels are attached to appropriate dideoxynucleotide triphosphates (ddNTP). Each ddNTP is labeled with a different color, e.g., ddATP green, ddCTP blue, ddGTP yellow, and ddTTP red <fn>Brown, T.A.: Genomes. Bios Scientific Publ., Oxford, 1999.</fn>. (The actual colors for each nucleotide may be different.) All chains terminated at an adenine (A) will yield a green signal; all chains terminated at a cytosine (C) will yield a blue signal, and so on. The sequencing reactions based on this kind of chain termination at labeled nucleotides <fn>Rosenthal, N.: Fine structure of a gene—DNA sequencing. New Eng. J. Med. 332 :589–591, 1995</fn> are carried out automatically in sequencing capillaries <fn>Strachan, T., Read, A.P.: Human Molecular Genetics. 2 nd ed. Bios Scientific Publishers, Oxford, 1999.</fn>. The electrophoretic migration

of the ddNTP-labeled chains in the gel in the capillary pass in front of a laser beam focused on a fixed position. The laser induces a fluorescent signal that is dependent on the specific label representing one of the four nucleotides. The sequence is electronically read and recorded and is visualized as alternating peaks in one of the four colors, representing the alternating nucleotides in their sequence positions. In practice the peaks do not necessarily show the same maximal intensity as in the schematic diagram shown here. (Illustration based on Brown, 1999, and Strachan and Read, 1999).



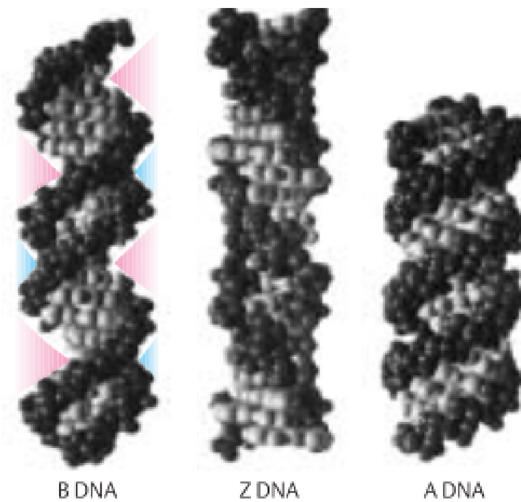
Automated DNA sequencing

Alternative DNA Structures

Gene expression and transcription can be influenced by changes of DNA topology. However, this type of control of gene expression is relatively universal and non specific.Thus, it

is more suitable for permanent suppression of transcription, e.g., in genes that are expressed only in certain tissues or are active only during the embroyonic period and later become permanently inactive.

A. Three forms of DNA



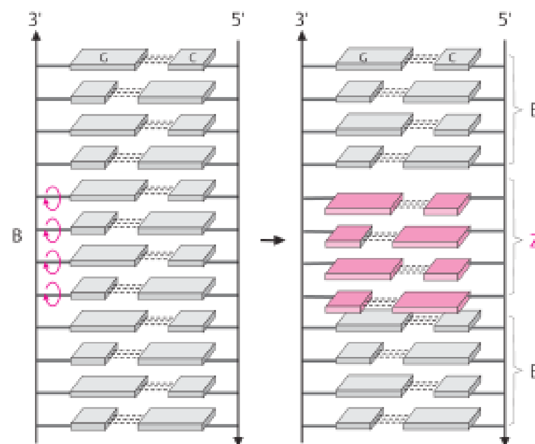
Three forms of DNA

The DNA double helix does not occur as a single structure, but rather represents a structural family of different types. The original classic form, determined by Watson and Crick in 1953, is B-DNA. The essential structural characteristic of B-DNA is the formation of two grooves, one large (major groove) and one small (minor groove). There are at least two further, alternative forms of the DNA double helix, Z-DNA and the rare form A-DNA. While B-DNA forms a right-handed helix, Z-DNA shows a left-handed conformation. This leads to a greater distance (0.77nm) between the base pairs than in B-DNA and a zig zag form(thus the designation Z-DNA). A-DNA is rare. It exists only in the dehydrated state and differs from the B form by a 20-degree rotation of the perpendicular axis of the helix. A-DNA has a deep major groove and a flat minor groove (Figures from Watson et al, 1987).

B. Major and minor grooves in B-DNA

The base pairing in DNA (adenine–thymine and guanine–cytosine) leads to the formation of a large and a small groove because the glycosidic bonds to deoxyribose (dRib) are not diametrically opposed. In B-DNA, the purine and pyrimidine rings lie 0.34 nm apart. DNA has ten base pairs per turn of the double helix. The distance from one complete turn to the next is 3.4 nm. In this way, localized curves arise in the double helix. The result is a somewhat larger and a somewhat smaller groove. <fn>Stryer, L.: Biochemistry, 4 th ed. W.H. Freeman & Co., New York, 1995.</fn>

C. Transition from B-DNA to Z-DNA



Transition from B-DNA to Z-DNA

B-DNA is a perfect regular double helix except that the base pairs opposite each other do not lie exactly at the same level. They are twisted in a propeller-like manner. In this way, DNA can easily be bent without causing essential changes in the local structures. In Z-DNA the sugar–phosphate skeleton has a zigzag pattern; the single Z-DNA groove has a greater density of negatively charged molecules. Z-DNA may occur in limited segments in vivo. A segment of B-DNA consisting of GC pairs can be converted into Z-DNA when the bases are rotated

180 degrees. Normally, Z-DNA is thermodynamically relatively unstable. However, transition to Z-DNA is facilitated when cytosine is methylated in position 5 (C5). The modification of DNA by methylation of cytosine is frequent in certain regions of DNA of eukaryotes.Therearespecificproteinsthatbind to Z-DNA, but their significance for the regulation of transcription is not clear. <fn>Watson, J.D. et al.: Molecular Biology of the Gene. 3rd ed. Benjamin/Cummings Publishing Co., Menlo Park, California, 1987.</fn>

Eukaryotic gene structure

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

Exons and introns

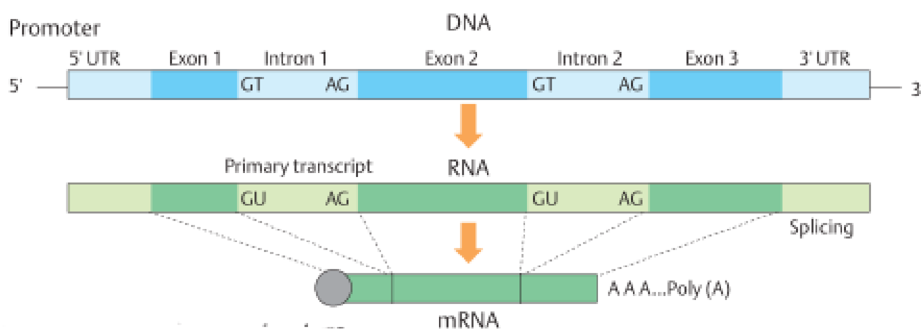
In 1977, it was unexpectedly found that the DNA of a eukaryotic gene is longer than its corresponding mRNA. The reason is that certain sections of the initially formed primary RNA transcript are removed before translation occurs. Electron micrographs show that DNA and its corresponding transcript (RNA) are of different lengths (1). When mRNA and its complementary single-stranded DNA are hybridized, loops of single-stranded DNA arise because mRNA hybridizes only with certain sections of the single stranded DNA. In (2), seven loops (A to G) and eight hybridizing sections are shown (1 to

7 and the leading section L). Of the total 7700 DNA base pairs of this gene (3), only 1825 hybridize with mRNA. A hybridizing segment is called an exon. An initially transcribed DNA section that is subsequently removed from the primary transcript is an intron. The size and arrangement of exons and introns are characteristic for every eukaryotic gene (exon/intron structure). (Electron micrograph from Watson et al., 1987).

Intervening DNA sequences (introns)

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

Basic eukaryotic gene structure

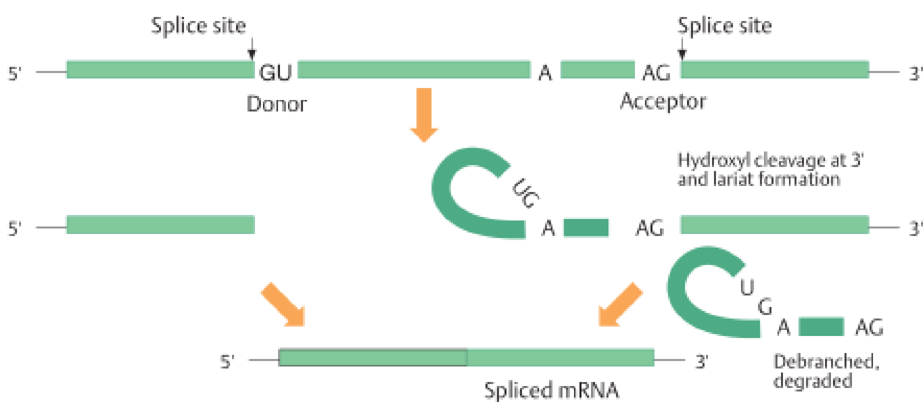


Basic eukaryotic gene structure

Exons and introns are numbered in the 5′ to 3′ direction of the coding strand. Both exons and introns are transcribed into a precursor RNA (primary transcript).The first and the last exons usually contain sequences that are not translated. These are called the 5′ untranslated region (5′ UTR) of exon 1 and the 3′ UTR at the 3′ end of the last exon. The non coding segments (introns) are removed from the primary transcript and the exons on either side are connected by a process called splicing. Splicing must be very precise to avoid an

undesirable change of the correct reading frame. Introns almost always start with the nucleotides GT in the 5′ to 3′ strand (GU in RNA) and end with AG. The sequences at the 5′ end of the intron beginning with GT are called splice donor site and at the 3′ end, ending with AG,are called the splice acceptor site. Mature mRNA is modified at the 5? end by adding a stabilizing structure called a “cap” and by adding many adenines at the 3’end (polyadenylation).

Splicing pathway in GU–AG introns



Splicing pathway in GU – AG introns

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

Strachan and Read, 1999)

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cDNA cloning

cDNA is a single-stranded segment of DNA that is complementary to the mRNA of a coding DNA segment or of a whole gene. It can be used as a probe (cDNA probe as opposed to a genomic probe) for the corresponding gene because it is complementary to coding sections (exons) of the gene. If the gene has been altered by structural rearrangement at a corresponding site, e.g., by deletion, the normal and mutated DNA can be differentiated. Thus, the preparation and cloning of cDNA is of great importance. From the cDNA sequence, essential inferences can be made about a gene and its gene product. <fn>Watson, J.D., et al.: Molecular Biology of the Gene, 3rd ed. Benjamin/Cummings Publishing Co., Menlo Park, California, 1987.</fn>

Preparation of cDNA

cDNA is prepared from mRNA. Therefore, a tissue is required in which the respective gene is transcribed and mRNA is produced in sufficient quantities. First, mRNA is isolated. Then a primer is attached so that the enzyme reverse transcriptase can form complementary DNA (cDNA) from the mRNA. Since mRNA contains poly(A) at its 3′ end, a primer of poly(T) can be attached. From here, the enzyme reverse transcriptase can start forming cDNA in the 5′ to 3′ direction. The RNA is then removed by ribonuclease. The cDNA serves as a template for the formation of a new strand of DNA. This requires the enzyme DNA polymerase. The result is a double strand of DNA, one strand of which is complementary to the original mRNA. To this DNA, single sequences (linkers) are attached that are complementary

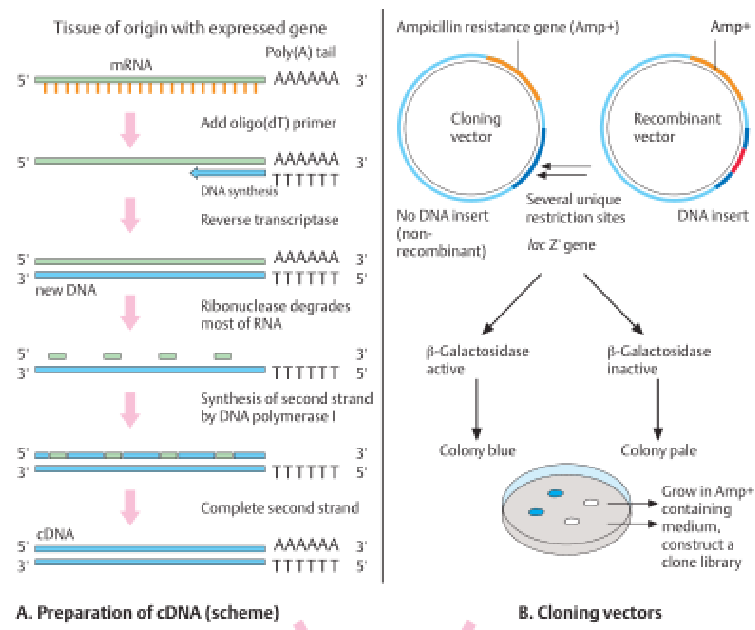
to the single-stranded ends produced by the restriction enzyme to be used. The same enzyme is used to cut the vector, e.g., a plasmid, so that the cDNA can be incorporated for cloning.

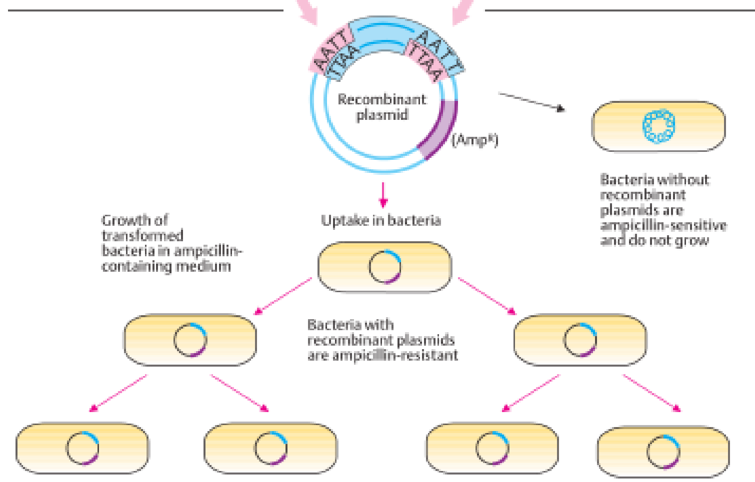
Cloning vectors

The cell-based cloning of DNA fragments of different sizes is facilitated by a wide variety of vector systems. Plasmid vectors are used to clone small DNA fragments in bacteria. Their main disadvantage is that only 5–10 kb of foreign DNA can be cloned. A plasmid cloning vector that has taken up a DNA fragment (recombinant vector), e.g., pUC8 with 2.7 kb of DNA, must be distinguished from one that has not. In addition, an ampicillin resistance gene (Amp +) serves to distinguish bacteria that have taken up plasmids from those that have not. Several unique restriction sites in the plasmid DNA segment where a DNA fragment might be inserted serve as markers along with a marker gene, such as the lacZ gene. The uptake of a DNA fragment by a plasmid vector disrupts the plasmid's marker gene. Thus, in the recombinant plasmid the enzyme ! -galactosidase will not be produced by the disrupted lagZ gene, whereas in the plasmid without a DNA insert (non recombinant) the enzyme is produced by the still intact lacZ gene. The activity of the gene and the presence or absence of the enzyme are determined by observing a difference in color of the colonies in the presence of an artificial substrate sugar. Beta-Glactosidase splits an artificial sugar (5-bromo-4-chloro-3-indolyl! D -galactopyranoside) that is similar to lactose, the natural substrate for this enzyme, into two sugar components, one of which is blue. Thus, bacterial colonies containing non recombinant plasmids with an intact lacZ gene are blue. In contrast,coloniesthathavetakenuparecombinant vector remain pale white. The latter are grown in a medium containing ampicillin (the selectable marker for the uptake of plasmid vectors). Subsequently, a clone library can be constructed. (Figure adapted from Brown, 1999).

cDNA cloning

Only those bacteria become ampicillin resistant that have incorporated a recombinant plasmid. Recombinant plasmids, which contain the gene for ampicillin resistance, transform ampicillinsensitive bacteria into ampicillin-resistant bacteria. In an ampicillin-containing medium, only those bacteria grow that contain the recombinant plasmid with the desired DNA fragment. By further replication in these bacteria, the fragment can be cloned until there is enough material to be studied. (Figures after Watson et al., 1987).





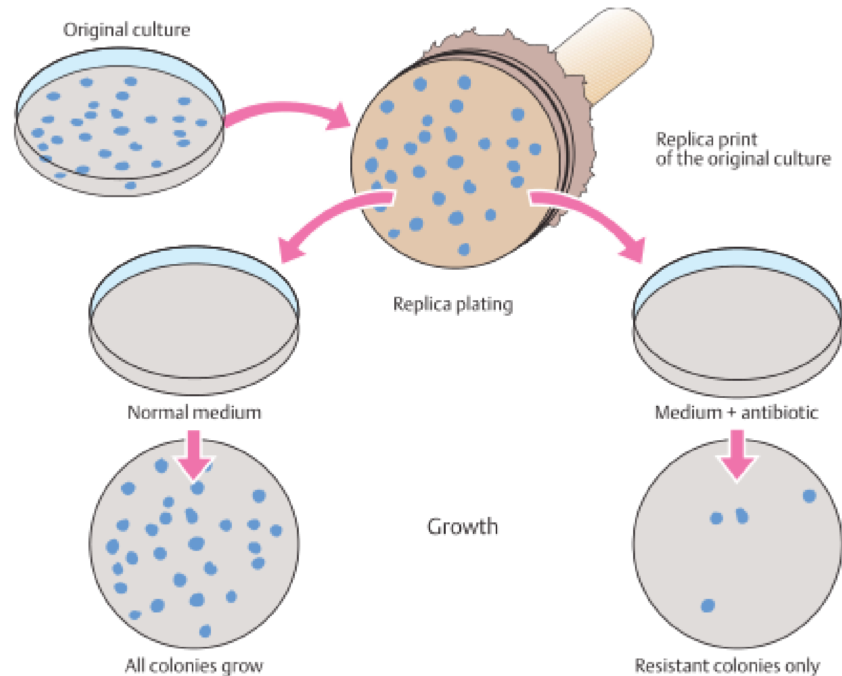
cDNA cloning principle

Isolation of Mutant Bacteria

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

A. Replica plating to recognize mutants

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

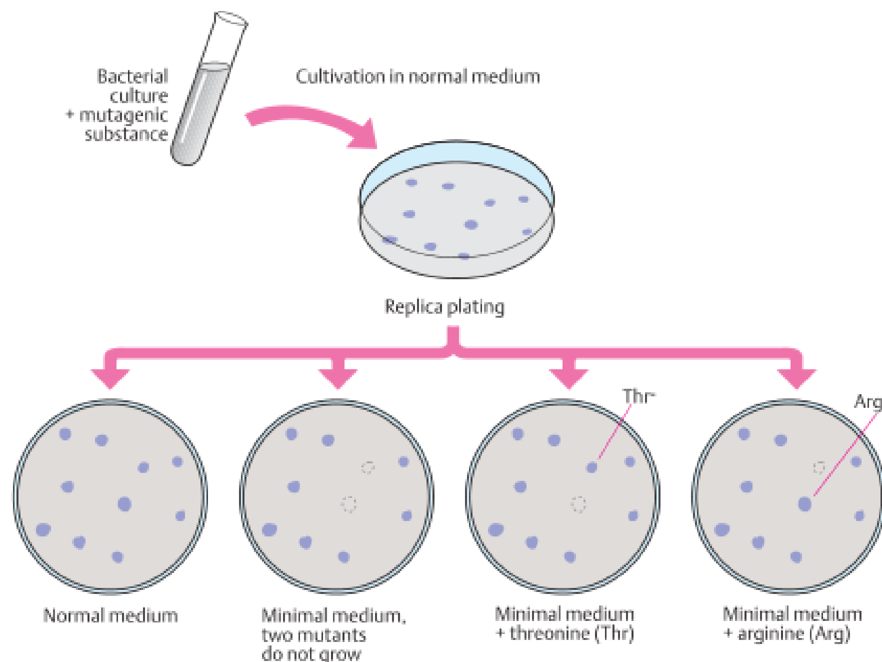


Replica plating to recognize mutants

B. Mutant bacteria identified through an auxotrophic medium

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

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



Mutant bacteria identified through an auxotrophic medium

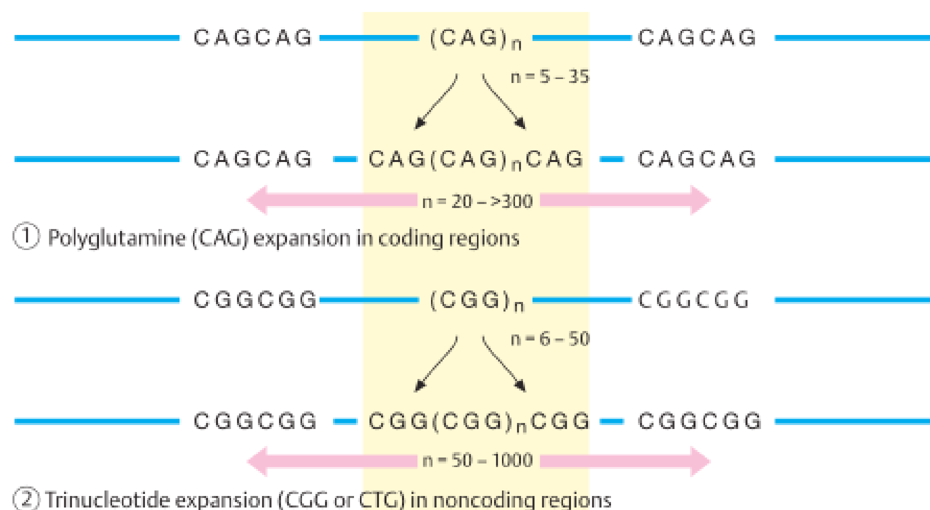
Trinucleotide Repeat Expansion

The human genome contains tandem repeats of trinucleotides. Normally they occur in groups of 5–35 repeats. When their number exceeds a certain threshold and they occur in a gene or close to it, they cause diseases. Once the normal, variable length has expanded, the increased number of repeats tends to

increase even further when passed through the germline or during mitosis. Thus, trinucleotide expansions form a class of unstable mutations, to date observed in humans only.

A. Different types of trinucleotide repeats and their expansions

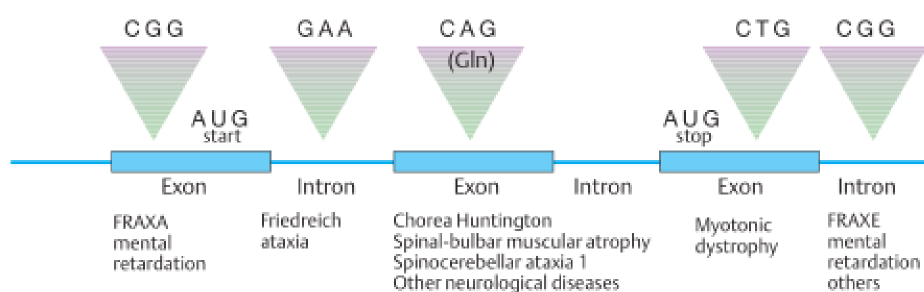
Trinucleotide repeats can be distinguished according to their localization with respect to a gene. Expansions are greater outside genes and more moderate within coding regions. In several severe neurological diseases, abnormally expanded CAG repeats are part of the gene. CAG repeats encode a series of glutamines (polyglutamine tracts). Within a normal number of repeats, which varies according to the gene involved, the gene functions normally. However, an expanded number of repeats leads to an abnormal gene product with altered function. Trinucleotide repeats also occur in noncoding regions of a gene. Fairly common types are CGG and GCC repeats. The increase in the number of these repeats can be drastic, up to 1000 or more repeats. The first stages of expansion usually do not lead to clinical signs of a disease, but they do predispose to increased expansion of the repeat in the off spring of a carrier (premutation).



Different types of trinucleotide repeat expression

B. Unstable trinucleotide repeats in different diseases

Disorders due to expansion of trinucleotide repeats can be distinguished according to the type of trinucleotide repeat,i.e., the sequence of the three nucleotides, their location with respect to the gene involved, and their clinical features. All involve the central or the peripheral nervous system. Type I trinucleotide diseases are characterized by CAG trinucleotide expansion within the coding region of different genes. The triplet CAG codes for glutamine. About 20 CAG repeats occur normally in these genes, so that about 20 glutamines occur in the gene product. In the disease state the number of glutamines is greatly increased in the protein. Hence, they are collectively referred to as polyglutamine disorders. Type II trinucleotide diseases are characterized by expansion of CTG, GAA, GCC, or CGG trinucleotides within a noncoding region of the gene involved, either at the 5′ end (GCC in fragile X syndrome type A, FRAXA), at the 3′ end (CGG in FRAXE; CTG in myotonic dystrophy), or in an intron (GAA in Friedreich ataxia).

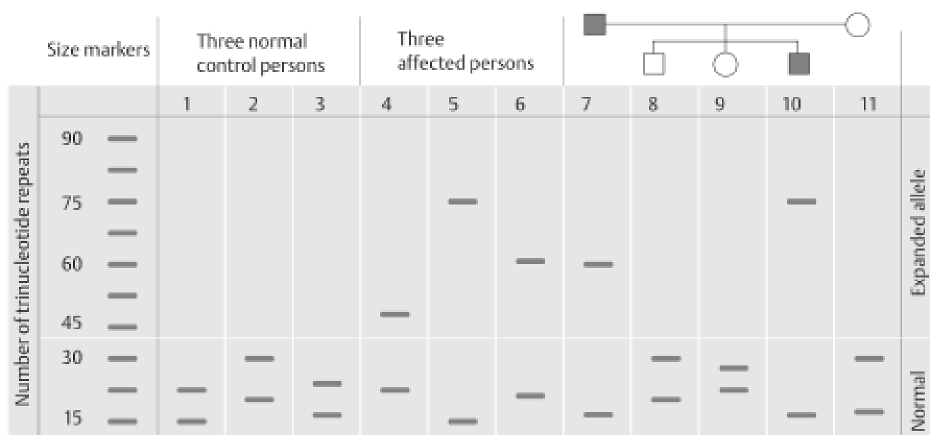


Unstable trinucleotide repeats in different diseases

C. Principle of laboratory

diagnosis of unstable trinucleotide repeats

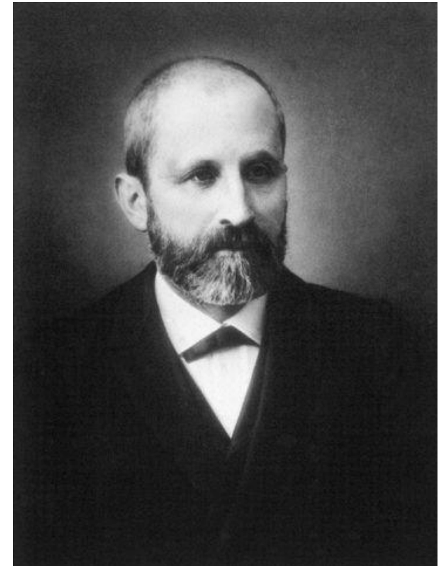
The laboratory diagnosis compares the sizes of the trinucleotide repeats in the two alleles of the gene examined. One can distinguish very large expansions of repeats outside coding sequences (50 to more than 1000 repeats) and moderate expansion within coding sequences (20 to 100–200). The figure shows 11 lanes, each representing one person: normal controls in lanes 1–3; confirmed patients in lanes 4–6; and a family with an affected father (lane 7), an affected son (lane 10), the unaffected mother (lane 11), and two unaffected children: a son (lane 8) and a daughter (lane 9). Size markers are shown at the left. Each lane represents a polyacrylamide gel and the (CAG) n repeat of the Huntington locus amplified by polymerase chain reaction shown as a band of defined size. Each person shows the two alleles. In the affected persons the band representing one allele lies above the threshold in the expanded region (in practice the bands are somewhat blurred because the exact repeat size varies in DNA from different cells).



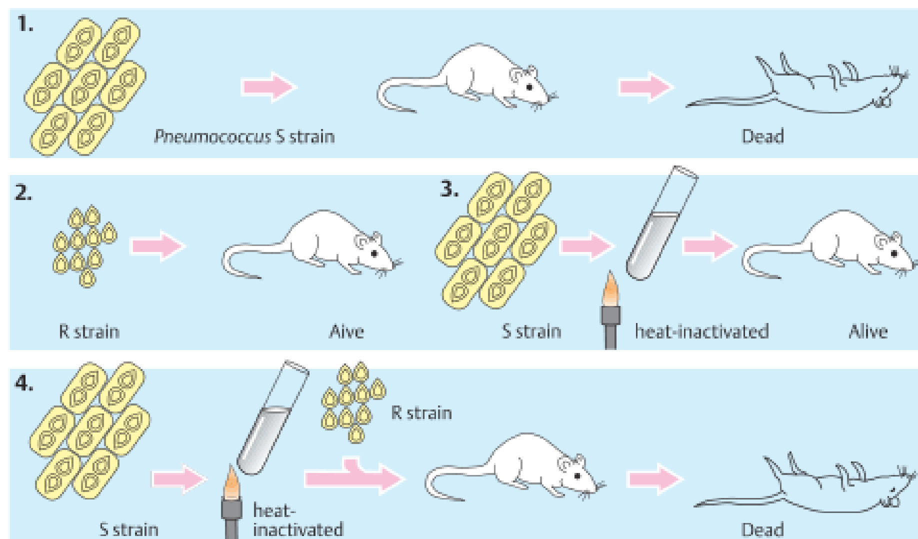
Principle of laboratory diagnosis of unstable trinucleotide repeats leading to expansion

DNA as Carrier of Genetic Information

Although DNA was discovered in 1869 by Friedrich Miescher as a new, acidic, phosphorus containing substance made up of very large molecules that he named "nuclein", its biological role was not recognized. In 1889 Richard Altmann introduced the term "nucleic acid". By 1900 the purine and pyrimidine bases were known. Twenty years later, the two kinds of nucleic acids, RNA and DNA, were distinguished. An incidental but precise observation (1928) and relevant investigations (1944) indicated that DNA could be the carrier of genetic information.



A. The observation of Griffith

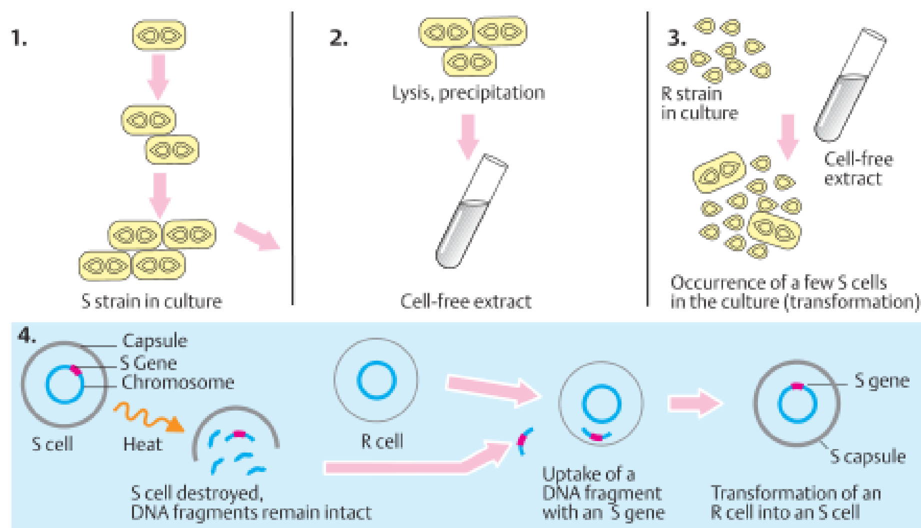


The observation of Griffith

In 1928 the English microbiologist Fred Griffith made a remarkable observation. While investigating various strains of Pneumococcus, he determined that mice injected with strain S

(smooth) died (1). On the other hand, animals injected with strain R (rough) lived (2). When he inactivated the lethal S strain by heat, there were no sequelae, and the animal survived (3). Surprisingly, a mixture of the nonlethal R strain and the heat-inactivated S strain had a lethal effect like the S strain (4). And he found normal living pneumococci of the S strain in the animal’s blood. Apparently, cells of the R strain were changed into cells of the S strain (transformed). For a time, this surprising result could not be explained and was met with skepticism. Its relevance for genetics was not apparent.

B. The transforming principle is DNA



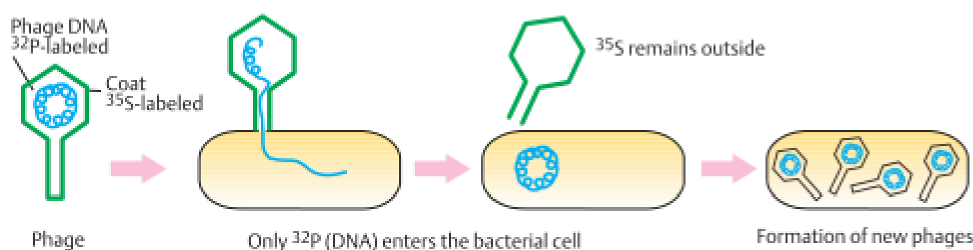
The transforming principle is DNA

Griffith’s findings formed the basis for investigations by Avery, MacLeod, and McCarty (1944). Avery and co-workers at the Rockefeller Institute in New York elucidated the chemical basis of the transforming principle. From cultures of an S strain (1) they produced an extract of lysed cells (cell-free extract) (2). After all its proteins, lipids, and polysaccharides had been removed, the extract still retained the ability to transform pneumococci of the R strain to pneumococci of the S strain (transforming principle) (3). With

further studies, Avery and co-workers determined that this was attributed to the DNA alone. Thus, the DNA must contain the corresponding genetic information. This explained Griffith's observation. Heat inactivation had left the DNA of the bacterial chromosomes intact. The section of the chromosome with the gene responsible for capsule formation (S gene) could be released from the destroyed S cells and be taken up by some R cells in subsequent cultures. After the S gene was incorporated into its DNA, an R cell was transformed into an S cell(4).

C. Genetic information is transmitted by DNA alone

The final evidence that DNA, and no other molecule, transmits genetic information was provided by Hershey and Chase in 1952.They labeled the capsular protein of bacteriophages (see p. 88) with radioactive sulfur (35 S) and the DNA with radioactive phosphorus (32 P). When bacteria were infected with the labeled bacteriophage, only 32 P (DNA) entered the cells, and not the 35 S (capsular protein). The subsequent formation of new, complete phage particles in the cell proved that DNA was the exclusive carrier of the genetic information needed to form new phage particles, including their capsular protein. Next, the structure and function of DNA needed to be clarified. The genes of all cells and some viruses consist of DNA, a long-chained threadlike molecule.



DNA Amplification by Polymerase Chain Reaction (PCR)

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

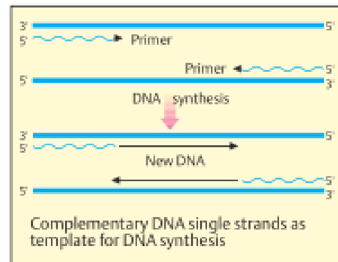
Polymerase chain reaction (PCR)

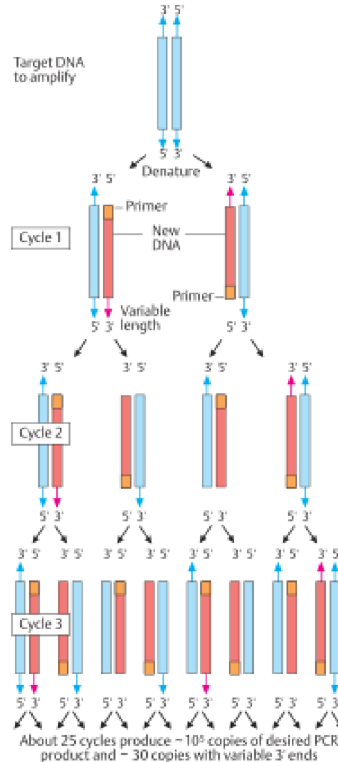
PCR is a cell-free, rapid, and sensitive method for cloning DNA fragments. A standard reaction and a wide variety of PCR-based methods have been developed to assay for polymorphisms and mutations. Standard PCR is an in vitro procedure for amplifying defined target DNA sequences, even from very small amounts of material or material of ancient origin. Selective amplification requires some prior information about DNA sequences flanking the target DNA. Based on this information, two oligonucleotide primers of about 15–25 base pairs length are designed. The primers are complementary to sequences outside the 3′ ends of the target site and bind specifically to these. PCR is a chain reaction because newly synthesized DNA strands act as templates for further DNA synthesis for about 25–35 subsequent cycles. Theoretically each cycle doubles the amount of DNA amplified. At the end, at least 10^5 copies of the specific target sequence are present. This can be visualized as a distinct band of a specific size after gel electrophoresis. Each cycle, involving three precisely time-

controlled and temperature-controlled reactions in automated thermal cyclers, takes about 1–5min. The three steps in each cycle are (1) denaturation of double-stranded DNA, at about 93–95°C for human DNA, (2) primer annealing at about 50–70°C depending on the expected melting temperature of the duplex DNA, and (3) DNA synthesis using heat-stable DNA polymerase (from microorganisms living in hot springs, such as Thermophilus aquaticus, Taq polymerase), typically at about 70–75°C. At each subsequent cycle the template (shown in blue) and the DNA newly synthesized during the preceding cycle (shown in red) act as templates for another round of synthesis. The first cycle results in newly synthesized DNA of varied lengths (shown with an arrow) at the 3′ ends because synthesis is continued beyond the target sequences. The same happens during subsequent cycles, but the variable strands are rapidly outnumbered by new DNA of fixed length at both ends because synthesis cannot proceed past the terminus of the primer at the opposite template DNA.

cDNA amplification and RT-PCR

A partially known amino acid sequence of a polypeptide can be used to obtain the sequence information required for PCR. From its mRNA one can derive cDNA, and determine the sequence of the sense and the antisense strand to prepare appropriate oligonucleotide primers (1). When different RNAs are available in small amounts, rapid PCR based methods are employed to amplify cDNA from different exons of a gene. cDNA is obtained by reverse transcriptase from mRNA, which is then removed by alkaline hydrolysis (2). After a complementary new DNA strand has been synthesized, the DNA can be amplified by PCR (3). Reverse transcriptase PCR (RT-PCR) can be used when the known exon sequences are widely separated within a gene. With rapid amplification of cDNA ends (RACE-PCR), the 5? and 3? end sequences can be isolated from cDNA.





Polymerase chain reaction (PCR)

Bacteriophages

The discovery of bacterial viruses (bacteriophages or phages) in 1941 opened a new era in the study of the genetics of prokaryotic organisms. Although they were disappointing in the original hope that they could be used to fight bacterial infections, phages served during the 1950s as vehicles for genetic analysis of bacteria. Unlike viruses that infect plant or animal cells, phages can relatively easily be analyzed in their host cells. Names associated with phage analysis are Max

Delbrück, Salvador Luria, and Alfred D. Hershey (the “phage group,” see: Cairns et al., 1966).

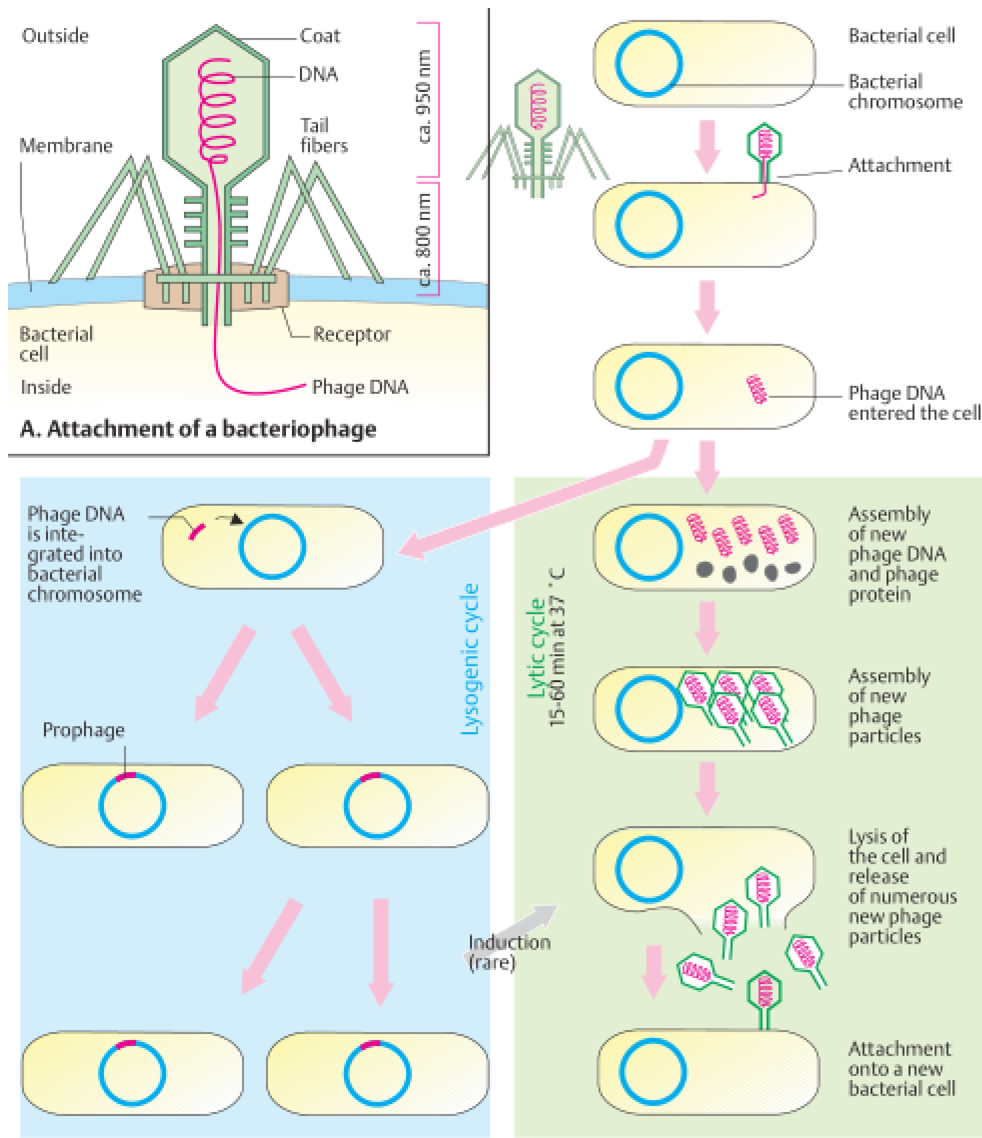
A. Attachment of a bacteriophage

Phages consist of DNA, a coat (coat protein) for protection, and a means of attachment (terminal filaments). Like other viruses, phages are basically nothing more than packaged DNA. One or more bacteriophages attach to a receptor on the surface of the outer cell membrane of a bacterium. The figure shows how an attached phage inserts its DNA into a bacterium. Numerous different phages are known,e.g., for Escherichia coli and Salmonella (phages T1, T2, P1, F1, lambda, T4, T7, phiX174 and others).

B. Lytic and lysogenic cycles of a bacteriophage

Phages do not reproduce by cell division like bacteria, but by intracellular formation and assembly of the different components. This begins with the attachment of a phage particle to a specific receptor on the surface of a sensitive bacterium. Different phages use different receptors, thus giving rise to specificity of interaction (restriction). The invading phage DNA contains the information for production of coat proteins for new phages and factors for DNA replication and transcription. Translation is provided for by cell enzymes. The phage DNA and phage protein synthesized in the cell are assembled into new phage particles. Finally, the cell disintegrates (lysis) and hundreds of phage particles are released. With attachment of a new phage to a new cell, the procedure is repeated (lytic cycle). Phage reproduction does not always occur after invasion of the cell. Occasionally, phage DNA is integrated into the bacterial chromosome and replicated with it (lysogenic cycle). Phage DNA that has been integrated into the bacterial chromosome is designated a

prophage. Bacteria containing prophages are designated lysogenic bacteria; the corresponding phages are termed lysogenic phages. The change from a lysogenic to a lytic cycle is rare. It requires induction by external influences and complex genetic mechanisms.

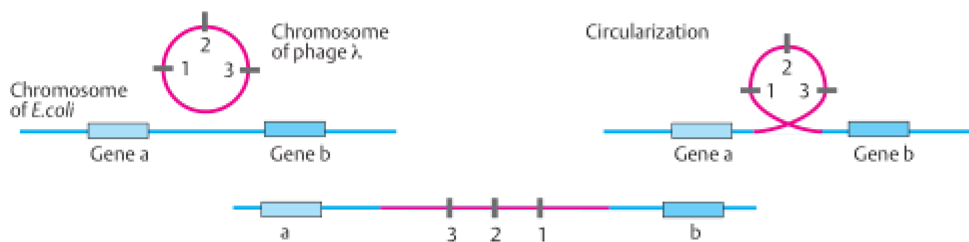


B. Lytic and lysogenic cycle of a bacteriophage

C. Insertion of a lambda phage into the bacterial chromosome by crossing-over

A phage can be inserted into a bacterial chromosome by different mechanisms. With the lambda phage (λ), insertion

results from crossing-over between the E. coli chromosome and the lambda chromosome. First, the lambda chromosome forms a ring. Then it attaches to a homologous section of the bacterial chromosome. Both the bacterial and the lambda chromosome are opened by a break and attach to each other. Since the homologies between the two chromosomes are limited to very small regions, phage DNA is seldom integrated. The phage is released (and the lytic cycle is induced) by there verse procedure.(Figures adapted from Watson et al., 1987).



C. Insertion of phage lambda into the bacterial chromosome by crossing-over