RECOMBINANT DNA TECHNOLOGY

BIOMEDICAL IMPORTANCE

- The development of recombinant DNA techniques, high-throughput screening ,low cost genome-scale DNA and RNA sequencing has revolutionized biology and is having increased impact on clinical medicine

- Manipulation of a DNA sequence and construction of chimeric molecule provides a means of studying how a specific segment of DNA controls function
  - Continue……

- **Understanding molecular genetics technology is important for several reasons:**

  1. it offers a rational approach to understand the **molecular basis of disease**. For example, **familial hypercholestrolemia**, **sickle cell disease**, **the thalassemias**, **muscular dystrophy** **as well as more complex multifactorial diseases like vascular and heart disease**, **Alzheimer disease,cancer,obesity and diabetes**

  2. **Human proteins** can be produced in abundance for therapy

- 3. Proteins for **preparation of vaccine** and for **diagnostic testing** can be easily obtained
- 4. This technology is used both to **diagnose existing diseases as well as to predict the risk of developing a given disease** and individual response to pharmacologic therapeutics-so called **personalized medicine**
- 5. Special techniques have led to remarkable advances in forensic medicine, which have allowed for the **molecular diagnostic analysis of DNA from single cells**.
- 6. Finally, in extremely well understood disease, potentially **curative gene therapy** for disease cause by **single gene deficiency**

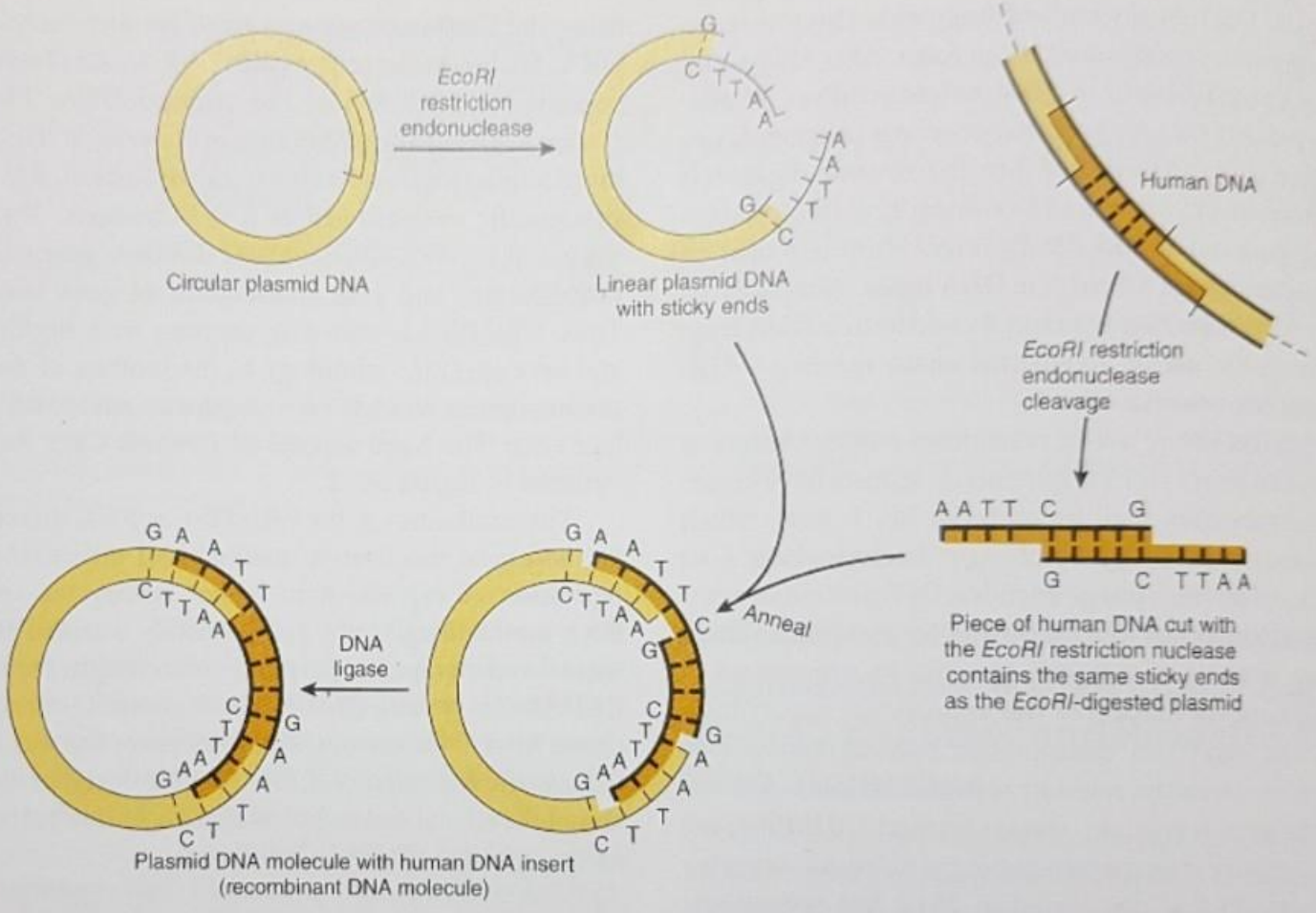


FIGURE 39–3 Use of restriction endonucleases to make new recombinant or chimeric DNA molecules. When inserted back into a bacterial cell (by the process called DNA-mediated transformation), typically only a single plasmid is taken up by a single cell, and the plasmid DNA replicates clonally, not only itself, but also the physically linked new DNA insert. Since recombining the sticky ends, as indicated, typically regenerates the same DNA sequence recognized by the original restriction enzyme, the cloned DNA insert can be cleanly cut back out of the recombinant plasmid circle with this endonuclease. Alternatively, the insert sequences can be specifically amplified from the purified chimeric plasmid DNA by PCR (Figure 39–7). If a mixture of all of the DNA pieces created by treatment of total human DNA with a single restriction nuclease is used as the source of human DNA, a million or so different types of recombinant DNA molecules can be obtained, each pure in its own bacterial clone. (Modified and reproduced, with permission, from Cohen SN: The manipulation of genes. Sci Am [July] 1975;233:25. Copyright © The Estate of Bunji Tagawa.)

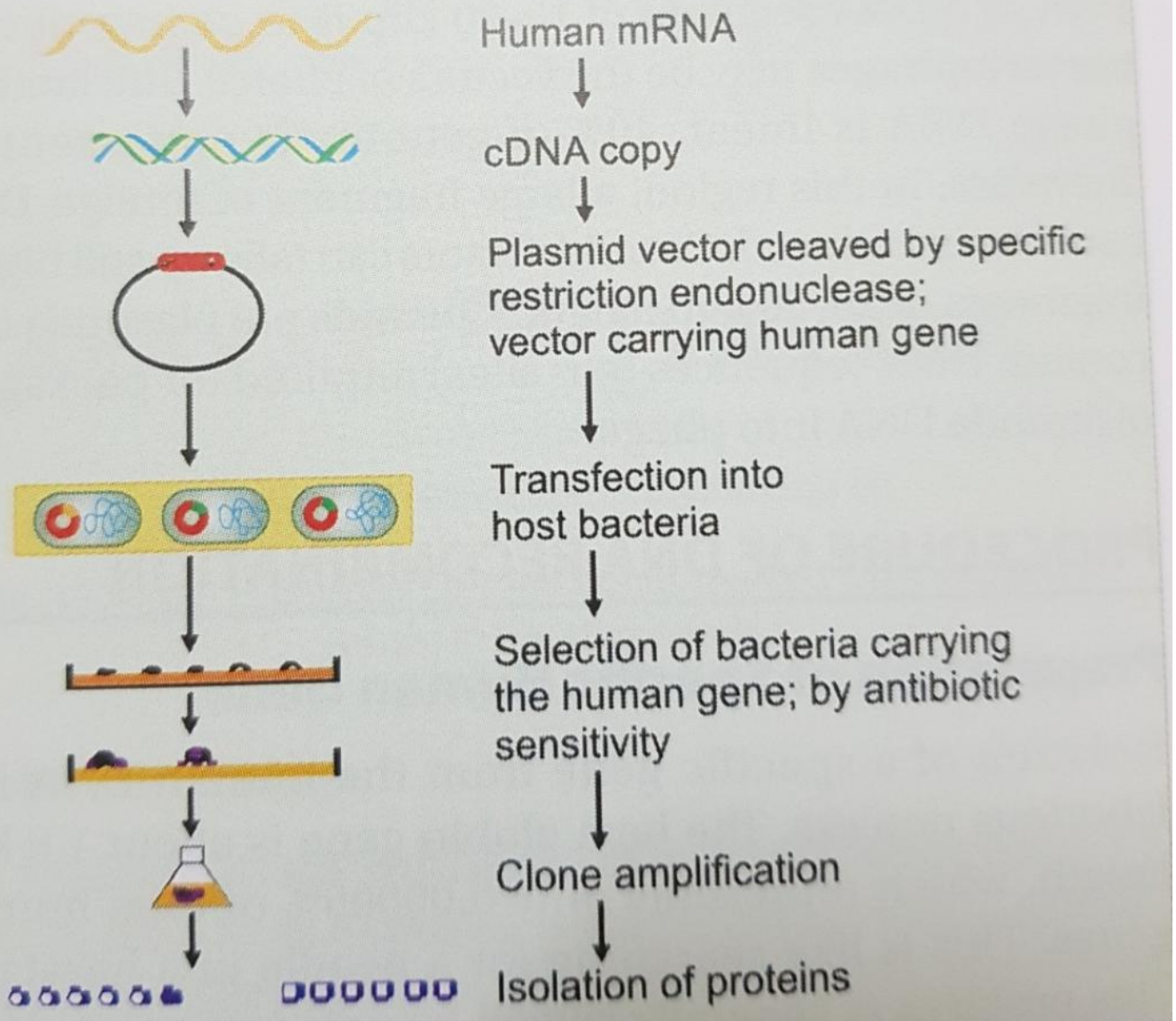


Fig. 37.10: DNA recombinant technology.

RDT IS USED IN MOLECULAR ANALYSIS OF DISEASE

Normal gene Variations

- There is normal variation of DNA sequence just as it is true of more obvious aspect of human structure
- Polymorphisms, occur approximately once in every 500 to 1000 nucleotides
- There are also genomic deletions and insertions of DNA as well as single base substitutions.
- In healthy people, these alterations in noncoding regions of DNA or sites that cause no change in function of encoded protein
- This heritable polymorphism of DNA structure can be associated with certain disease within a large kindred

Gene Variations Causing Disease

- Classic genetics taught that genetic diseases were due to point mutation that lead to an impaired protein
- Genetic disease could result from derangement of any of the steps leading from replication to transcription to RNA processing/transport and protein synthesis,PTMs etc
- This point is again nicely illustrated by examination of beta-globin gene
- Defective production of beta globin results in variety of diseases and is due to many different lesion in and around Beta globin gene

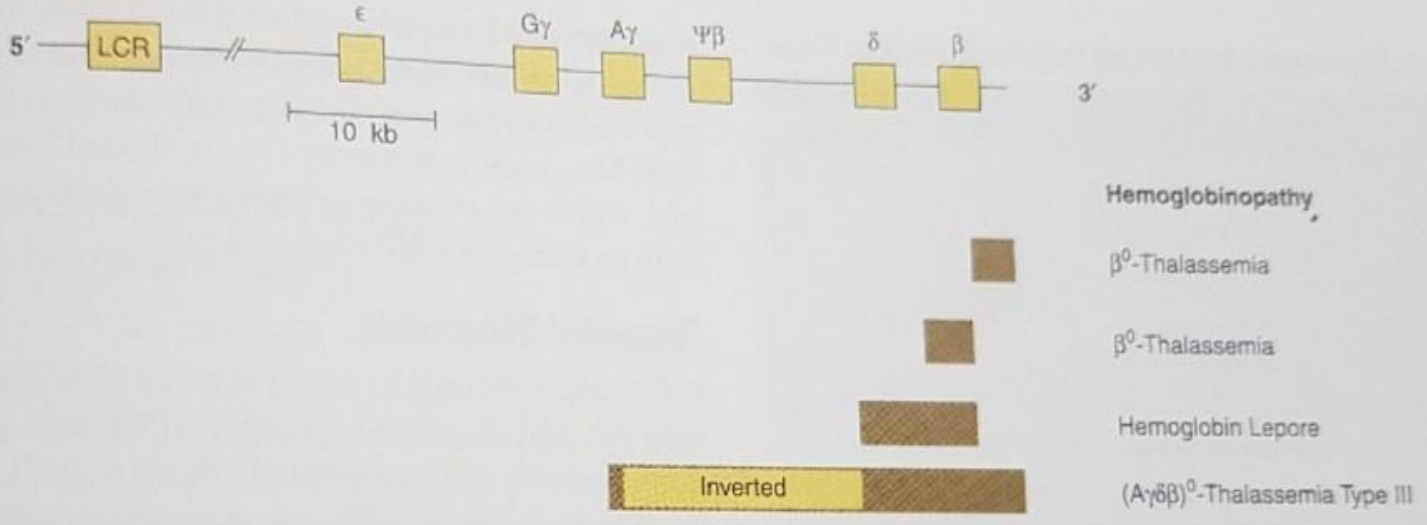


FIGURE 39–8 Schematic representation of the β-globin gene cluster and of the lesions in some genetic disorders. The β-globin gene is located on chromosome 11 in close association with the two γ-globin genes and the δ-globin gene. The β-gene family is arranged in the order 5′-ε-Gγ-Aγ-ψβ-δ-β-3′. The ε locus is expressed in early embryonic life (as $\alpha_2\varepsilon_2$). The γ–genes are expressed in fetal life, making fetal hemoglobin (HbF, $\alpha_2\gamma_2$). Adult hemoglobin consists of HbA ($\alpha_2\beta_2$) or HbA 2 ($\alpha_2\delta_2$). The ψβ is a pseudogene that has sequence homology with β but contains mutations that prevent its expression. A locus control region (LCR), a powerful enhancer located upstream (5′) of these six genes, controls the rate of transcription of the entire β-globin gene cluster. Deletions (solid darker bars, lower) within the β locus cause β-thalassemia (deficiency or absence [β^0] of β-globin). Meiotic recombination between δ and β causes hemoglobin Lepore, and results in DNA deletion and δ-β coding sequence fusions reducing the levels of HbB (see Figures 6–7 and 35–10). An inversion $(A\gamma\delta\beta)^0$ in this region (largest bar) disrupts gene function and also results in thalassemia (type III). Each type of thalassemia tends to be found in a certain group of people, for example, the $(A\gamma\delta\beta)^0$ deletion inversion occurs in persons from India. Many more deletions in this region have been mapped, and each causes some type of thalassemia.

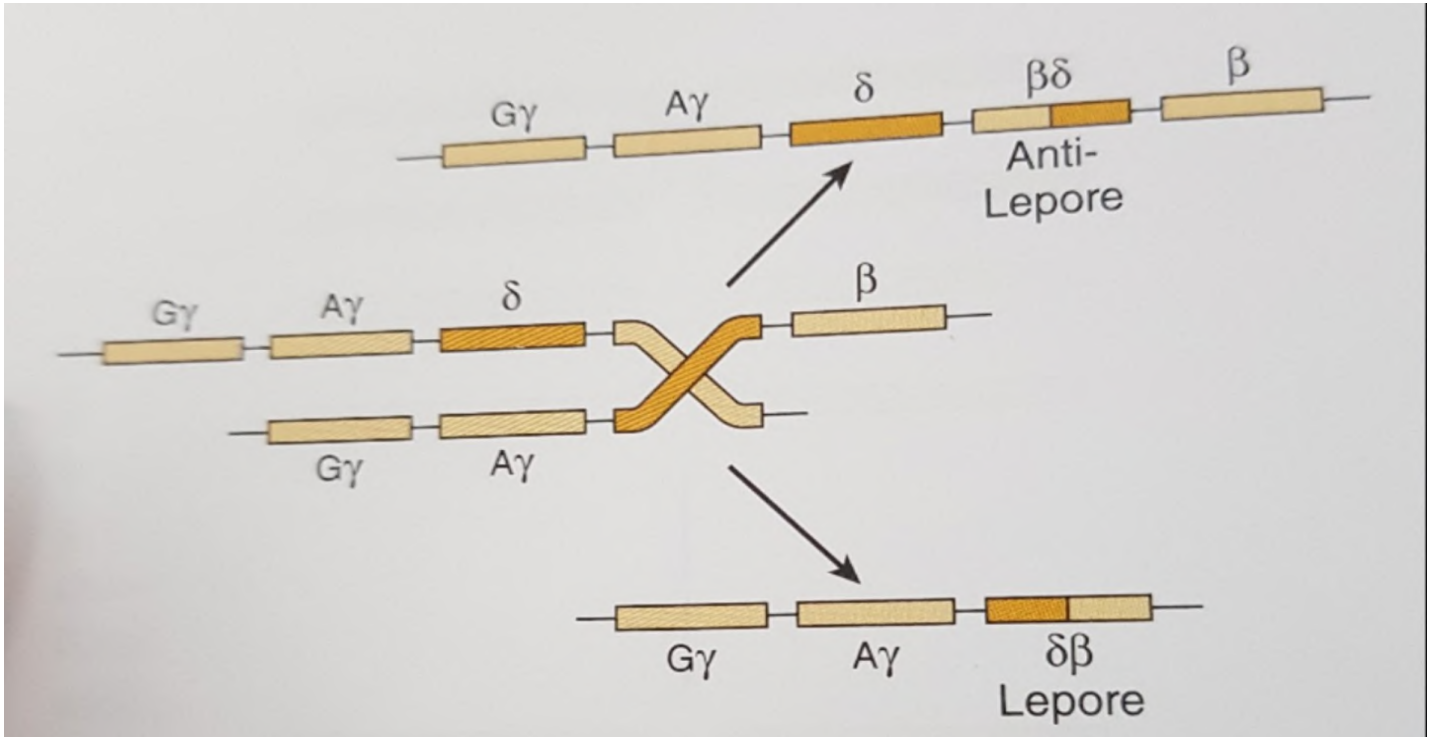


FIGURE 35–10 **The process of unequal crossover in the region of the mammalian genome that harbors the structural genes encoding hemoglobins and the generation of the unequal recombinant products hemoglobin delta-beta Lepore and beta-delta anti-Lepore.** The examples given show the locations of the crossover regions within amino acid coding regions of the indicated genes (ie, β and δ globin genes). (Redrawn and reproduced, with permission, from Clegg JB, Weatherall DJ: β^0 Thalassemia: time for a reappraisal? Lancet 1974;2:133. Copyright © 1974. Reprinted with permission from Elsevier.)

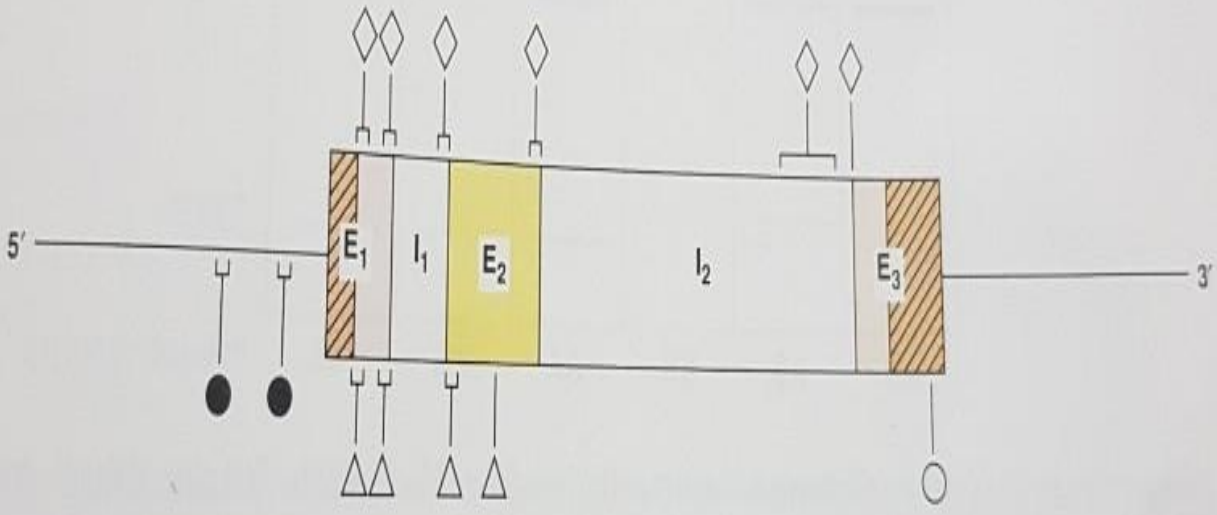


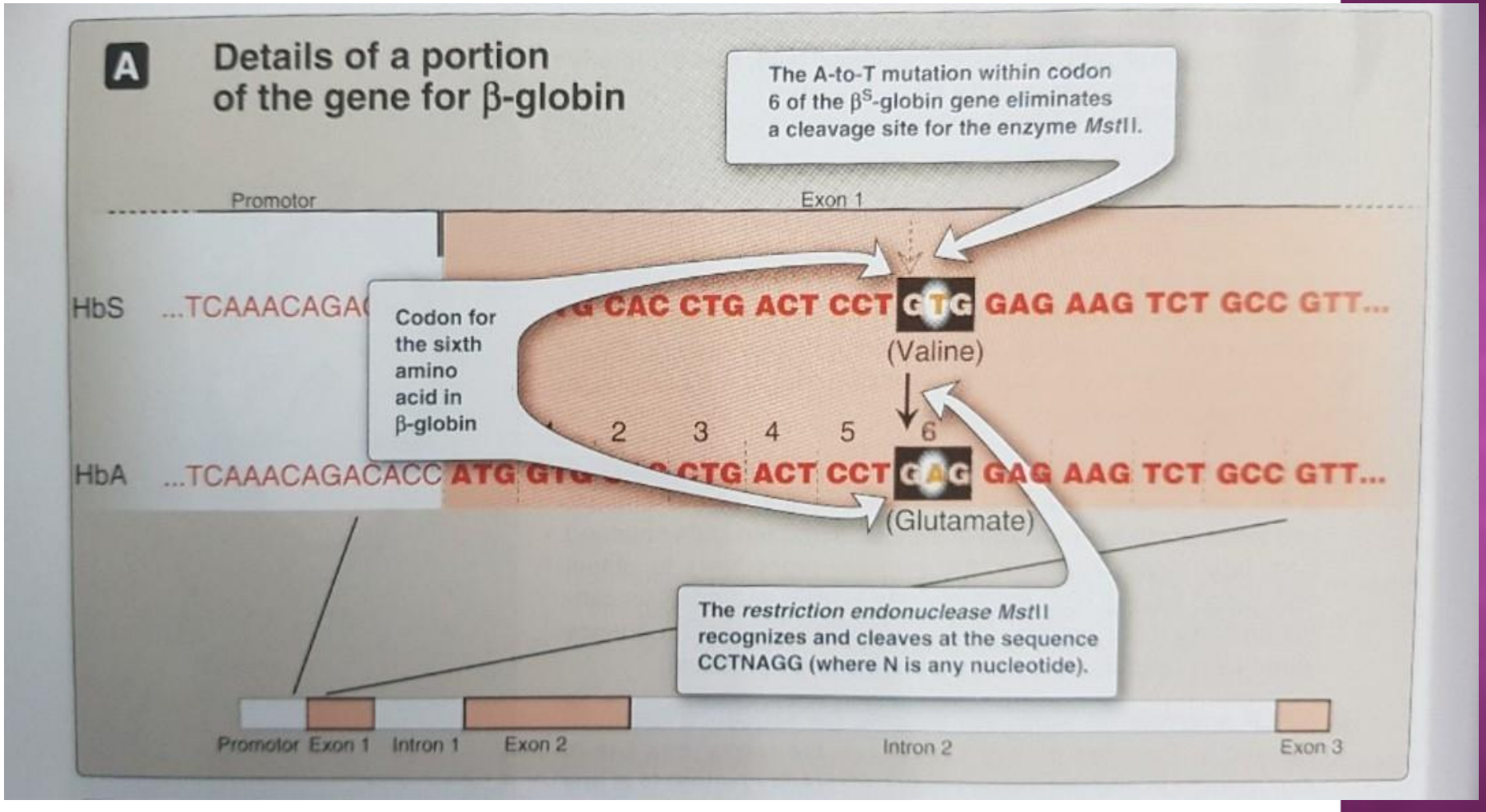
FIGURE 39–9 Mutations in the α-globin gene causing β-thalassemia. The β-globin gene is shown in the 5′ to 3′ orientation. The cross-hatched areas indicate the 5′ and 3′ untranslated regions (UTRs). Reading from the 5′ to 3′ direction, the shaded areas are exons 1 to 3 (E_1, E_2, E_3) and the white areas between exons are introns 1 (I_1) and 2 (I_2). Mutations that affect transcription control (•) are located in the 5′ flanking-region DNA. Examples of nonsense mutations (△), mutations in RNA processing (◇), and RNA cleavage mutations (○) have been identified and are indicated. In some regions, many distinct mutations have been found. These are indicated by the size and location of the brackets.

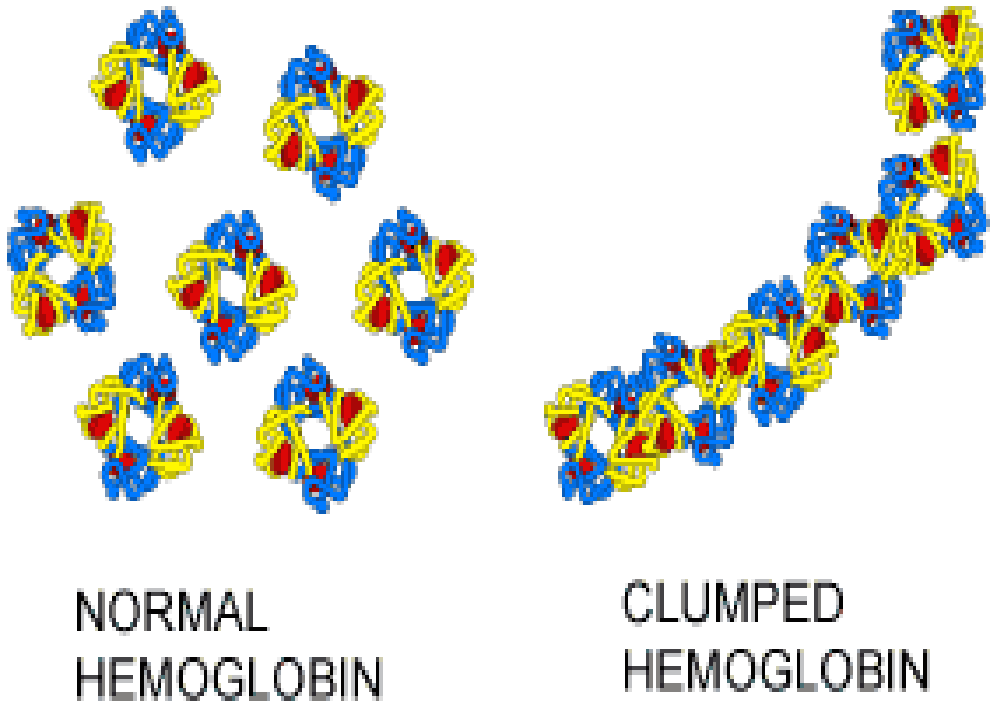
TABLE 39–5 Structural Alterations of the β-Globin Gene

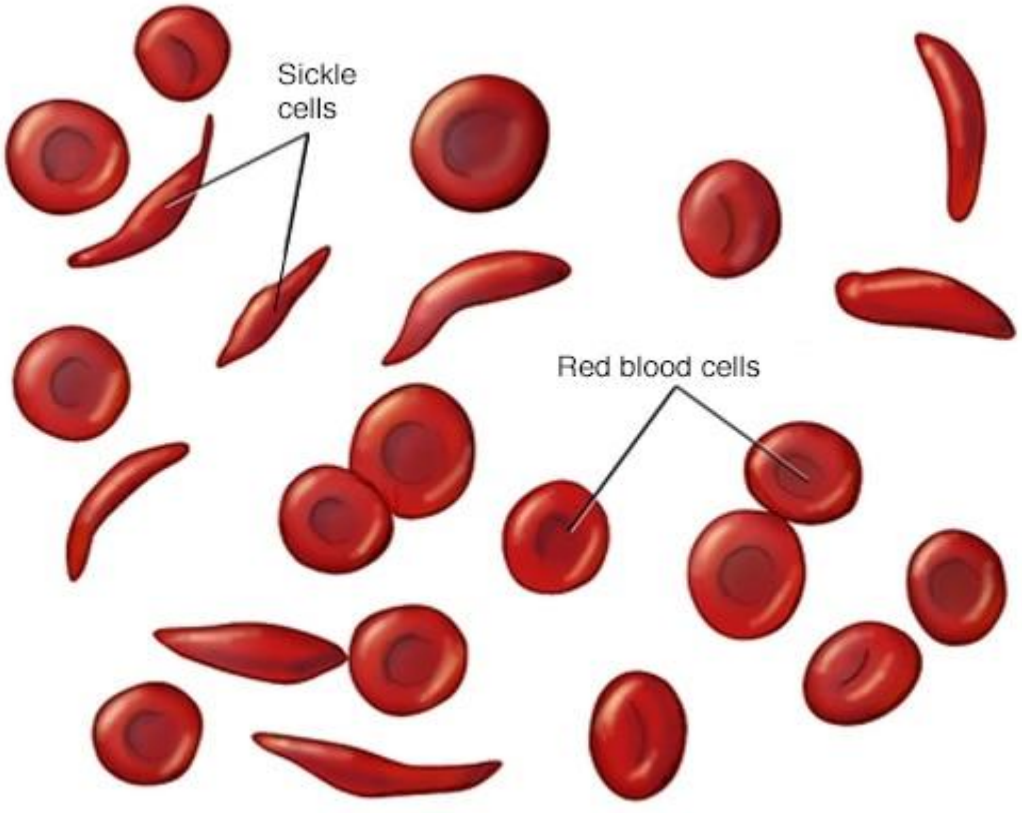
Alteration	Function Affected	Disease
Point mutations	Protein folding	Sickle cell disease
	Transcriptional control	β-Thalassemia
	Frameshift and nonsense mutations	β-Thalassemia
	RNA processing	β-Thalassemia
Deletion	mRNA production	β^0-Thalassemia
		Hemoglobin Lepore
Rearrangement	mRNA production	β-Thalassemia type III

Point Mutations

- The classic example is sickle cell disease, which is caused by a mutation of a single base i.e. **A-to-T DNA substitution**
- This in turn results in an **A-to-U** change in mRNA corresponding to the **sixth codon of the beta-globin gene.**
- The altered codon specifies a different amino acid i.e **GLU to VAL**
- This causes a **structural abnormality** of the beta globin molecule leading to hemoglobin aggregation and red cell **"sickling".**







- Other point mutations in and around Beta globin gene result in decreased or, in some instances, no production of beta globin causing beta thalassemia

- The thalassemias are characterized by defects in the **synthesis of haemoglobin subunits**, and so beta thalassemia results when there is insufficient production of beta-globin.

Deletions, Insertions and Rearrangements of DNA

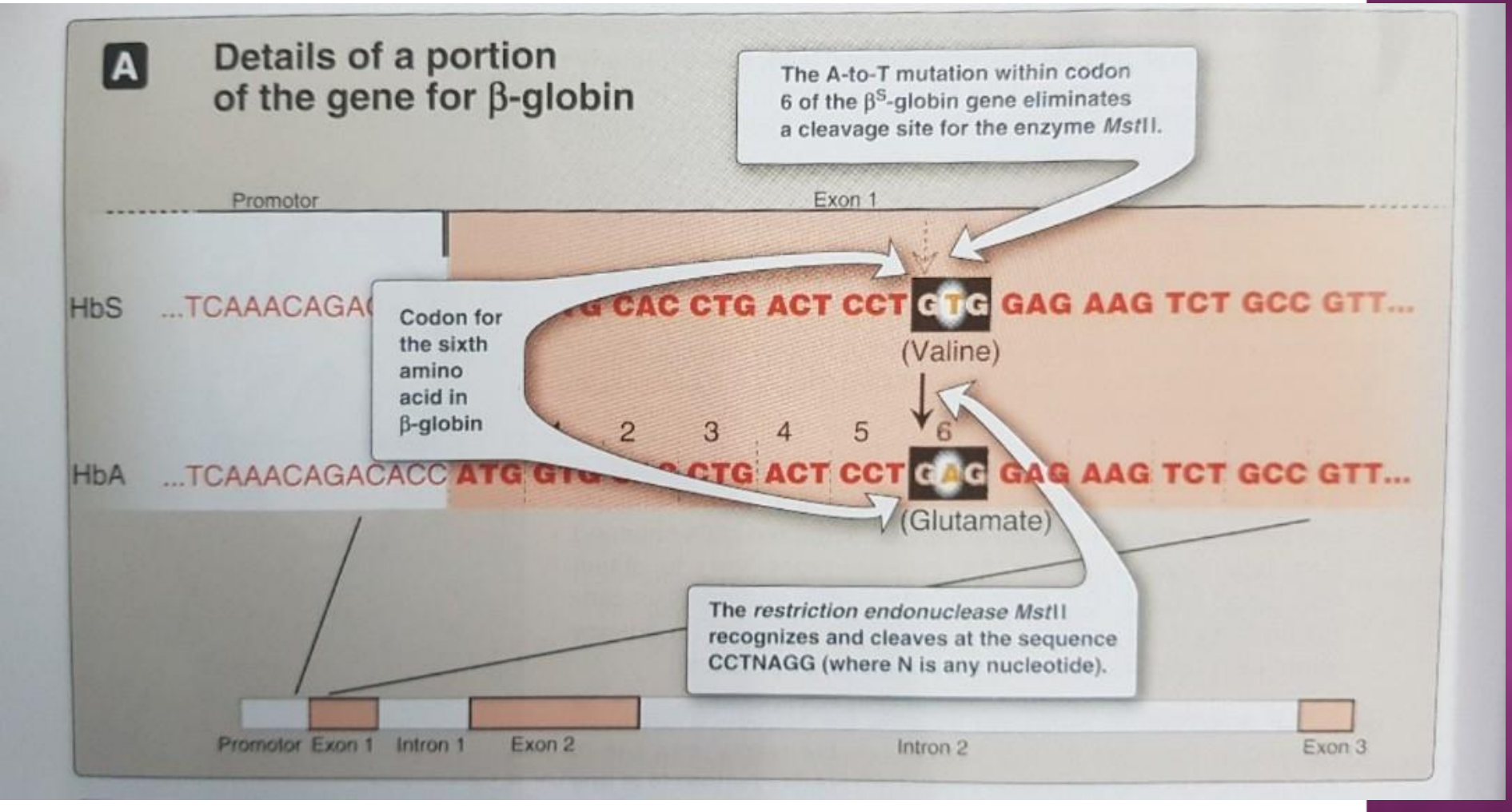
- Studies of bacteria,viruses,yeasts,fruit flies, and now humans show that pieces of DNA can move, or transpose from one place to another within a genome via a process of DNA transposition.

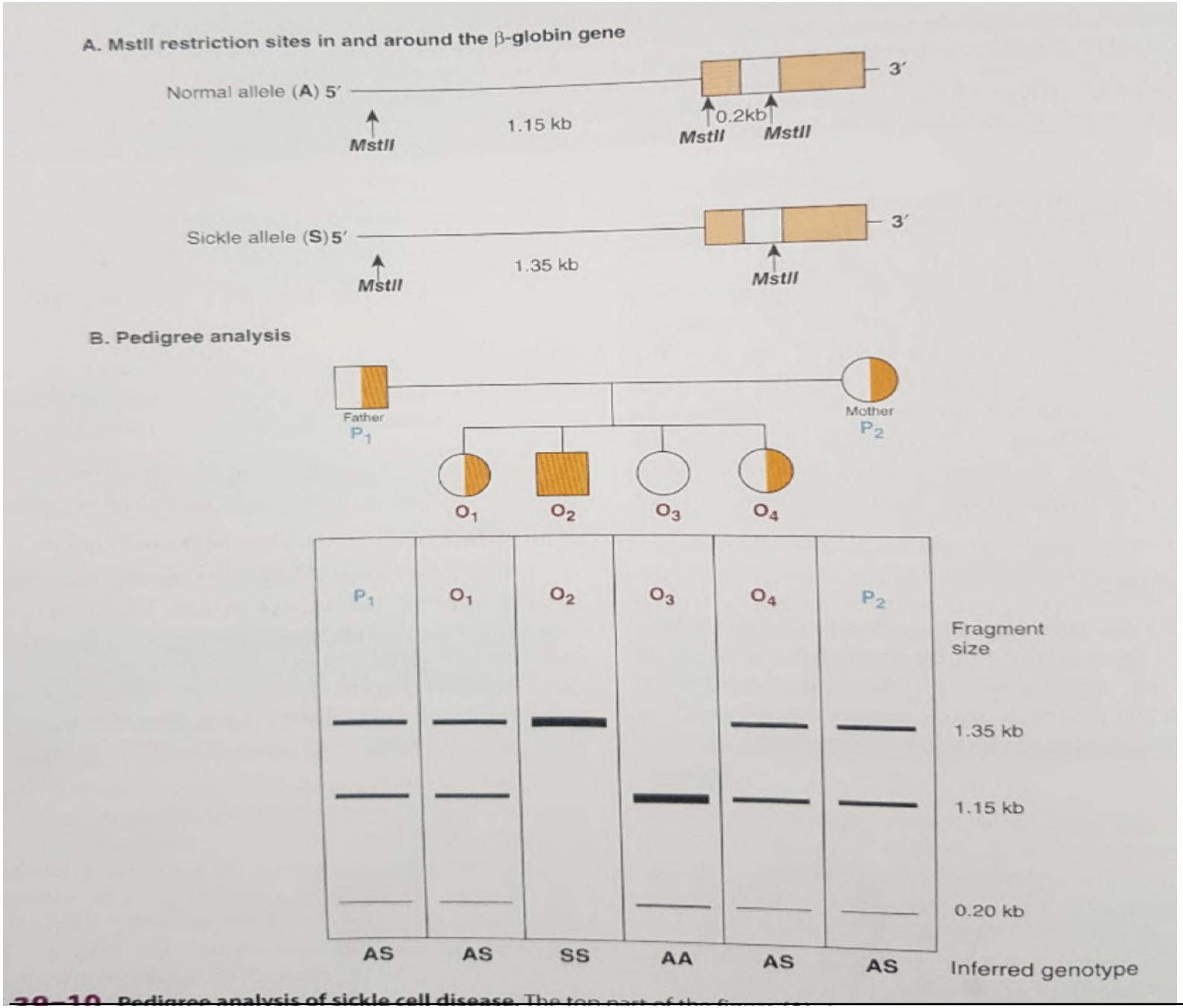
- The deletion of critical piece of DNA, the rearrangements of DNA within a gene, or the insertion or amplification of a piece of DNA within a coding or regulatory region can all cause changes in gene expression resulting in disease

- Molecular analysis of thalassemias produces numerous example of these processes-particularly deletions-as cause of disease
- Deletions in the alpha-globin cluster, located on chromosome 16, cause alpha-thalassemia.
- A similar analysis could be made for a number of other diseases.
- If the mutation destroys or creates a restriction enzyme site, the technique of RFLP can be used to pinpoint the lesion
- Deletions or insertions of DNA larger than 50 bp can often be detected by southern blotting while PCR can detect much smaller change in DNA structure

Pedigree Analysis

- Sickle cell disease again provides an excellent example of how RDT can be applied to the study of human disease
- The substitution of T for A in template strand of DNA in beta globin gene changes the sequence in the region and destroys a recognition site for restriction enzyme MstII.
- Pedigree analysis has been applied to a number of genetic diseases and is most useful in those caused by deletions and insertions or rare instances in which restriction endonuclease cleavage site is affected
- Such analyses are now facilitated by the PCR reaction, which can amplify and hence provide sufficient DNA for analysis from just a few nucleated cells.





20–10 Pedigree analysis of sickle cell disease. The top part of the figure [illegible]

Prenatal Diagnosis

- If the genetic lesion is understood and a specific probe is available, prenatal diagnosis is possible
- DNA from cells collected from small volume of amniotic fluid can be analyzed by Southern blot transfer, and much smaller volume if PCR –based assays are used
- Fetus with the restriction pattern AA is normal, if with the SS pattern will develop the disease.

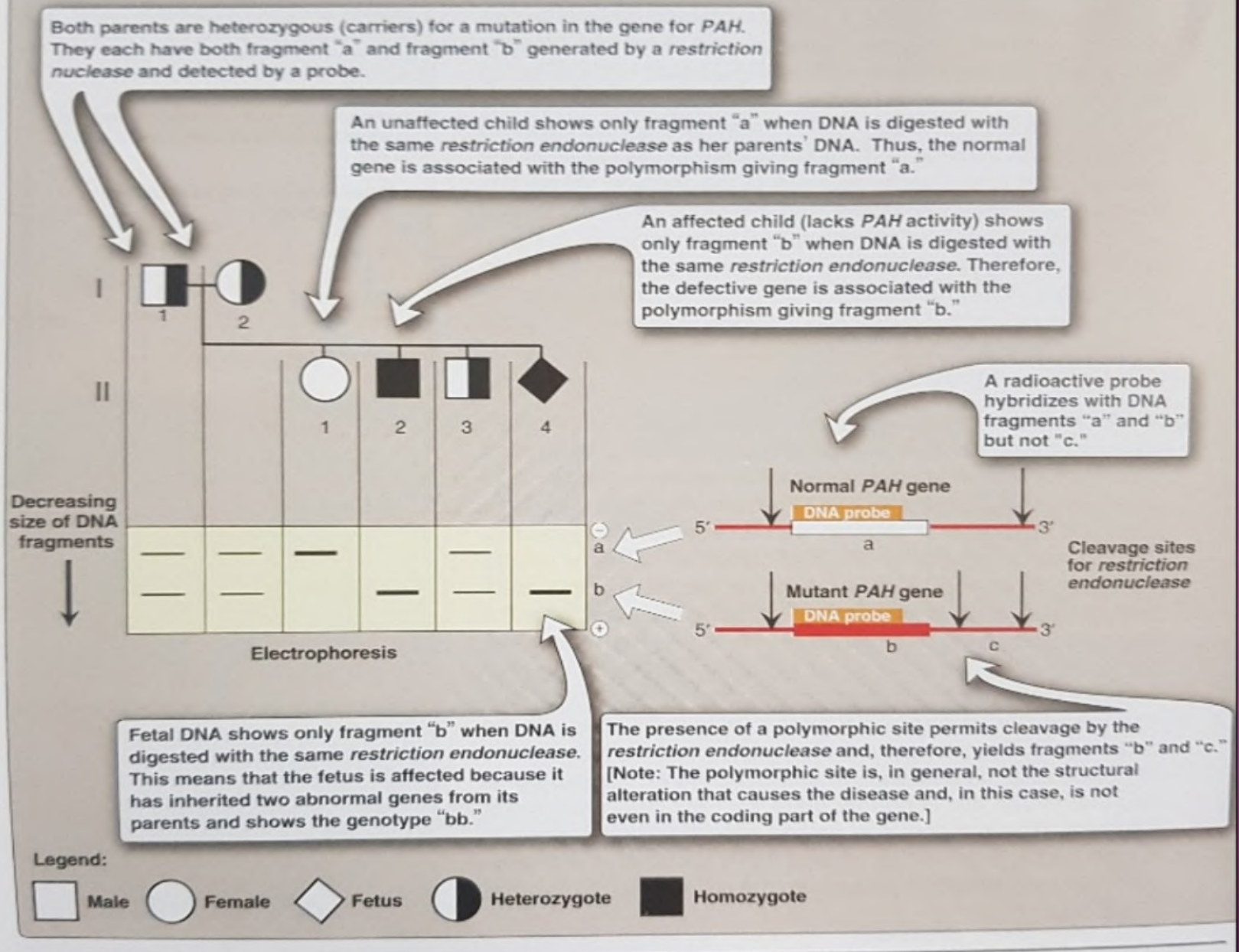
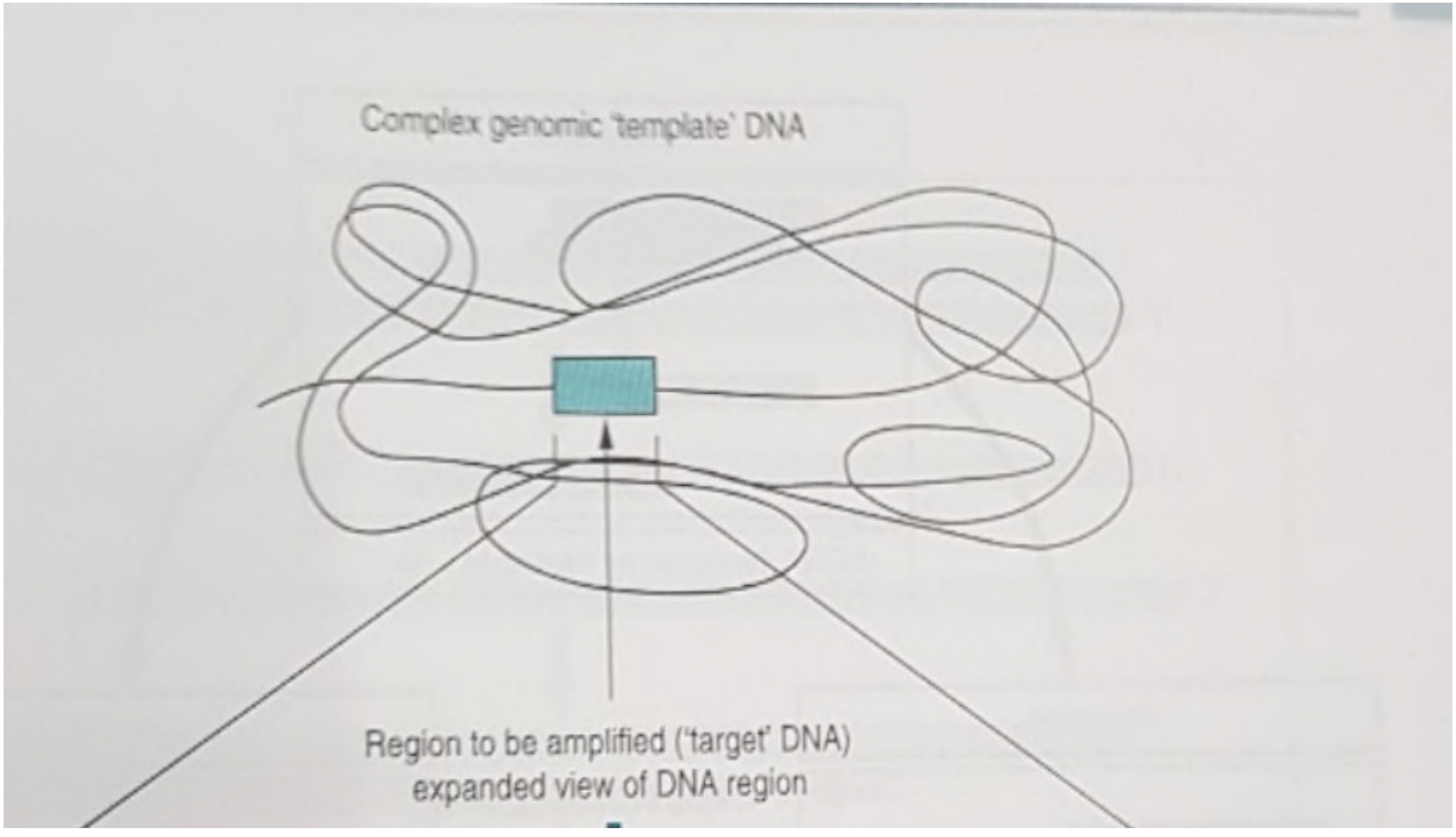


Figure 34.19
Analysis of restriction fragment length polymorphism in a family with a child affected by phenylketonuria (PKU), an autosomal-recessive disease. The molecular defect in the gene for *phenylalanine hydroxylase* (*PAH*) in the family is not known. The family wanted to know if the current pregnancy would be affected by PKU.

POLYMERASE CHAIN REACTION

- PCR is an in vitro method for amplifying a selected DNA sequence
- PCR permits the synthesis of millions of copies of a specific nucleotide sequence in few hours
- It can amplify the sequence, even when the targeted sequence makes up less than one part in a million of total initial sample
- The method can be used to amplify DNA sequences from any source, including viral,bacterial,plant or animal



Procedure

1. **Constructing primer**

- It is not necessary to know the entire nucleotide sequence of the target DNA in the PCR method.
- However, it is necessary to know the nucleotide sequence of short fragment on each side of target DNA
- The nucleotide sequence of the flanking regions are used to construct two, single-stranded oligonucleotides,which are complementary to the respective flanking sequences
- The 3'OH end of each oligonucleotide points towards the target sequence

2. **Denaturing DNA**

- The target DNA to be amplified is heated to 95 degree Celsius to separate dsDNA in to single strands

3. **Annealing primers**

- The separated strands are cooled to 50 degree Celsius and the two primers anneal to a complementary sequence on the DNA.

4. **Extending primers**

- DNA pol and DNTP are added to the mixture to initiate the synthesis of two new strands which are complementary to the original DNA strands.

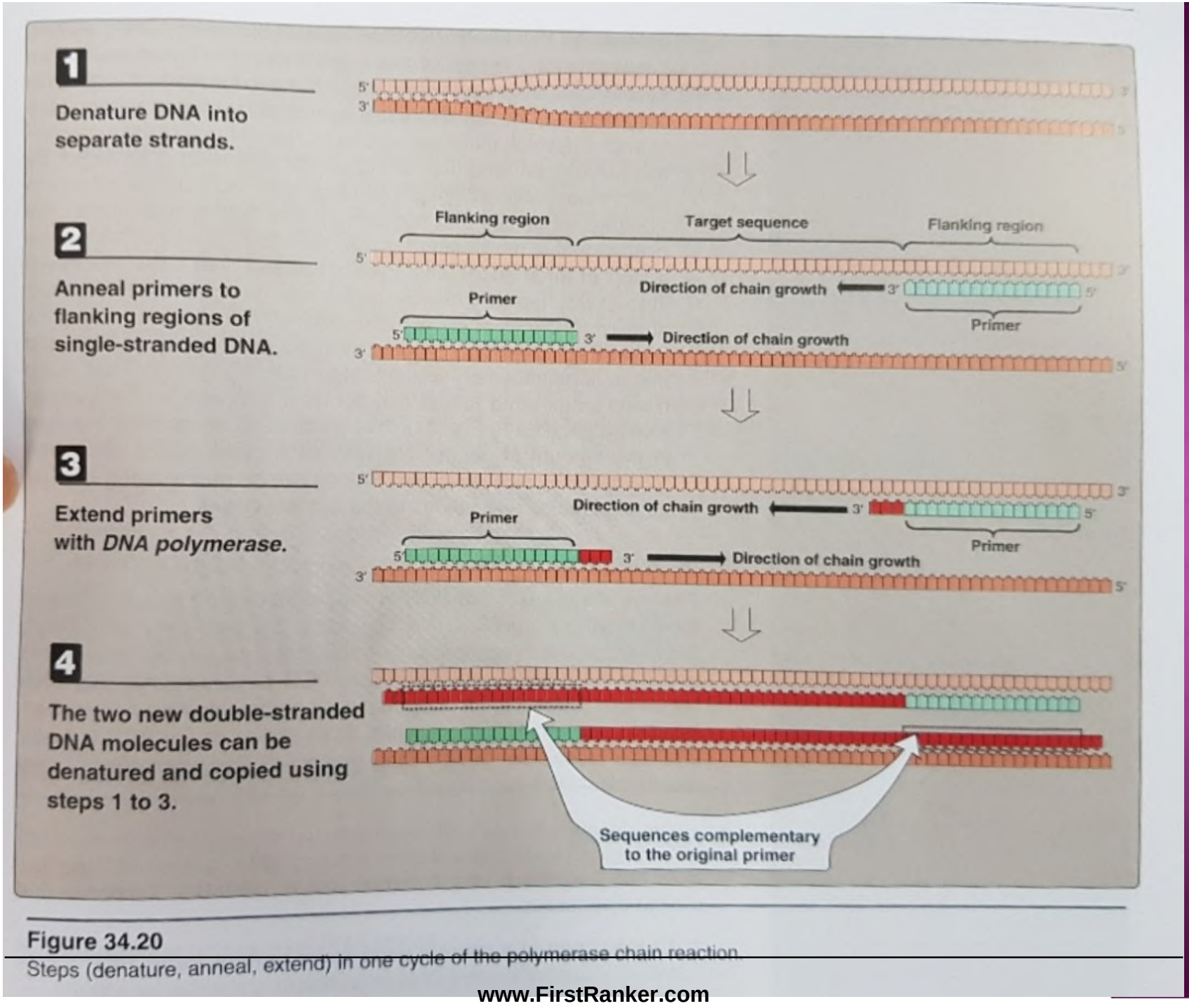


Figure 34.20
Steps (denature, anneal, extend) in one cycle of the polymerase chain reaction.

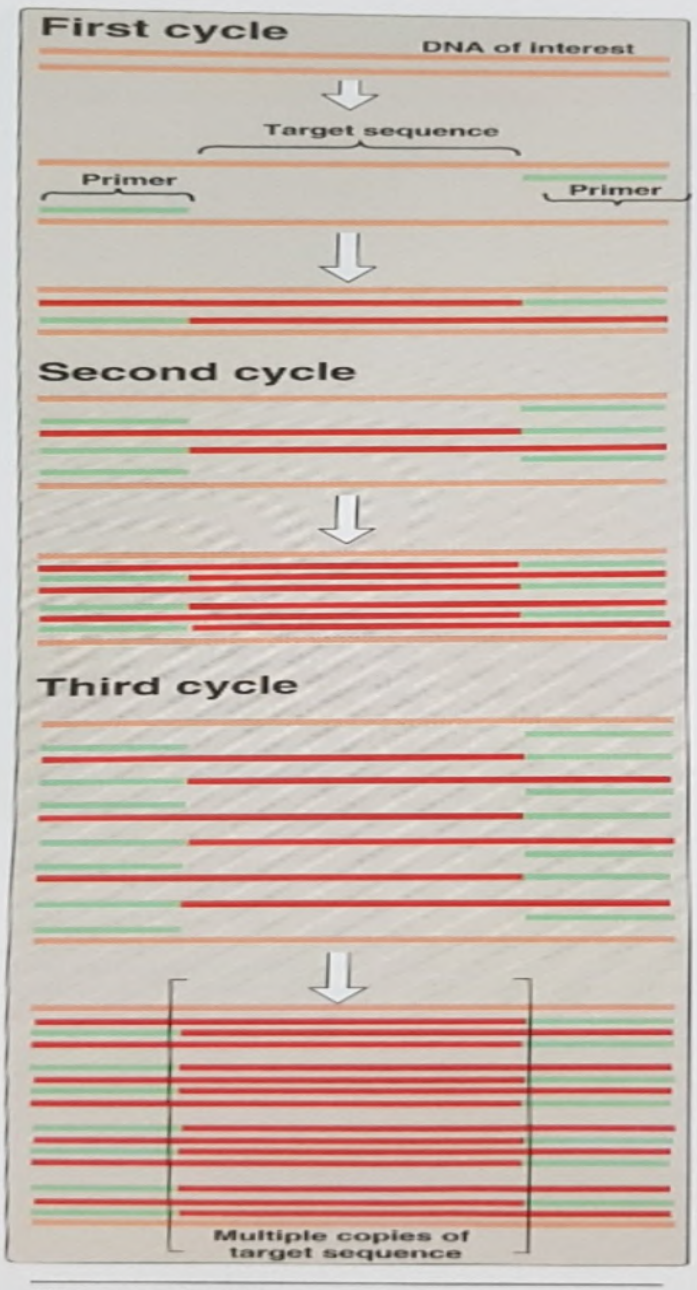


Figure 34.21
Multiple cycles of the polymerase chain reaction.

Applications

- 1. Comparison of a normal gene to its mutant forms
- Forensic analysis of DNA samples
- Detection of low-abundance nucleic acid sequences
- Prenatal diagnosis and carrier detection of cystic fibrosis

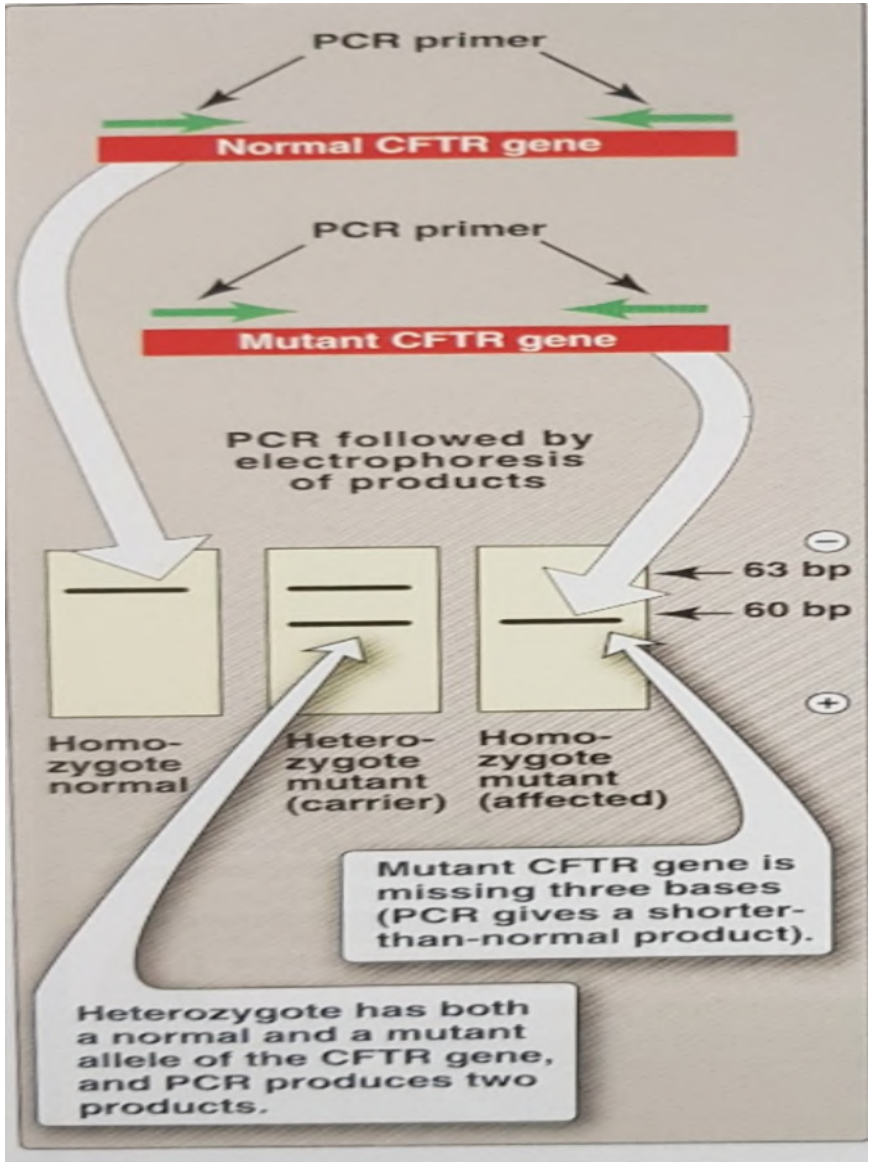


Figure 34.22
Genetic testing for cystic fibrosis (CF) using the polymerase chain reaction (PCR). [Note: CF is also diagnosed using allele-specific oligonucleotide analysis (see p. 488).] CFTR = cystic fibrosis transmembrane conductance regulator; bp = base pairs.