INSTRUCTOR GUIDE

IND-6B. Analysis of a Mutant Hemoglobin Gene

I. Chemicals and Materials Provided

	Amount
Hae III	25µl (250 units)
EcoR1	35µl (350 units)
Restriction Nuclease Buffer	500µl
Normal Globin DNA	120µl
Mutant Globin DNA	120µl
Sample Buffer	300µl

II. Materials that are Needed but Not Provided

PROCELL Electrophoresis Unit and MB-170 Power Supply or equivalent
Accessory Kit or equivalent
Water Bath
Agarose, Electrophoresis Buffer and Gel Stain from Electrophoresis Package 3/4. Instructions for preparing the buffer and stain are provided with Electrophoresis Package 3/4.

III. Preparation of Solutions

Hae III - The restriction enzyme Hae III is provided in a glycerol solution. Immediately before the laboratory session, add 100µl of the Restriction Nuclease Buffer to the tube containing the Hae III. Mix well the contents of the tube by rotating the tube on its side to ensure that the enzyme comes in contact with the buffer. Place the tube in an upright position in the refrigerator or in an ice bath.

EcoR1 - The restriction enzyme EcoR1 is provided in a glycerol solution. Immediately before the laboratory session, add 200µl of the Restriction Nuclease Buffer to the tube containing the EcoR1. Mix well the contents of the tube by rotating the tube on its side to ensure that the enzyme comes in contact with the buffer. Place the tube in an upright position in the refrigerator or in an ice bath.

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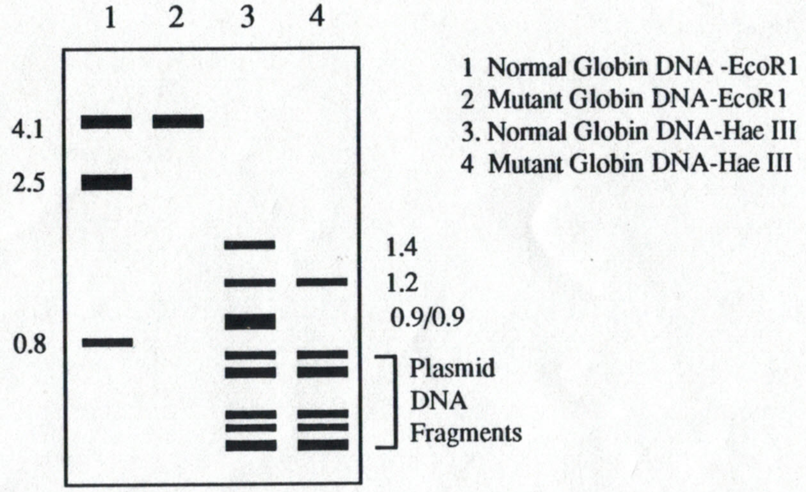
IV. Experimental Results - Diagram of a stained gel.



V. Answers to Study Questions

1. See the above gel.

2.

Restriction Enzyme	Band #	Band length (base-pairs)	Position in β Globin DNA (see Figure 4)
EcoR1 (tube 1 and 5)	1	4.1	3..3 KB to 4.7 KB from site 0
	2	2.5	0.0 KB to 2.5 KB from site 0
	3	0.8	2.5 KB to 3.3 KB from site 0
Hae III (tube 3 and 7)	1	1.4	0.0 KB to 1.4 KB from site 0
	2	1.2	3.5 KB to 4.7 KB from site 0
	3/4	0.9	1.6 KB to 2.5 KB or 2.6 KB to 3.5 KB from site 0
	3/4	0.9	

3. The Mutant DNA lacks the 2.5 KB and 0.8 KB bands in the EcoR1 digest that are found in the normal globin DNA. The mutant DNA lacks the 1.4 KB and both 0.9 KB bands in the Hae III digest that are found in the normal globin DNA. Thus, the mutation is a deletion which includes the entire β globin gene and the nontranscribed DNA flanking the globin gene on the 5' side. That is, the mutant lacks the DNA that is found between 0 KB to 3.3 KB from the site 0 on the map in Figure 4.

4. β-thalassemia (See Background Information Section I, The Structural Basis of Mutation)

IND-6B. Analysis of a Mutant Hemoglobin Gene

Background Information

I. The Structural Basis of Mutation

A mutation is a change in the nucleotide sequence of DNA which leads to an inherited change in an organism. A mutation within or near a gene may change the phenotype of an organism by altering the level of the protein product specified by the gene or by changing the properties of the protein product. The most common types of mutations are point mutations, deletions, and insertions.

Point mutations result from a change in a single base-pair in DNA. If the change occurs within a gene, it can result in a single base change in the mRNA, and thus to the replacement of one amino acid by another in the sequence of the polypeptide. In humans, a point mutation is responsible for the disease sickle cell anemia, where the sequence GAG is changed to GTG in the gene that codes for the beta polypeptide chains of hemoglobin. This change results in the replacement of a single glutamic acid residue on the beta chains by valine and the resulting hemoglobin crystallizes in the red blood cells, leading to a distortion of the red cells into a sickle shape. Point mutations have now been implicated in a number of human diseases including some forms of cancer.

Deletion and insertion mutations result from the removal or the addition of DNA base-pairs into the genome. Some forms of thalassemias provide examples of deletion mutations in humans. In these patients, all or part of the genes for the beta chain of hemoglobin are deleted which prevents synthesis of normal hemoglobin. In some β-thalassemias, the deletion extends beyond the β globin gene. In these patients, the DNA that flanks the β globin gene is also absent. Insertion of DNA segments near or within gene loci is also responsible for numerous mutations in eukaryotes and is likely to be a causative factor in some human cancers. More than 50 mutations that cause genetic diseases in humans have now been characterized at the DNA level.

II. Detecting Mutants by Restriction Nuclease Mapping

Restriction endonucleases are valuable tools for characterizing mutations at the DNA level. Figure 1 illustrates a strategy that could be used to identify point mutations, deletion mutations, and insertion mutations in a simple genome such as that from a virus. The restriction enzyme used in the analysis is called Hind III which recognizes the DNA sequence AAGCTT. In the Figure, the following DNAs were digested with Hind III: Wild-type DNA (that is, the original nonmutant), DNA with a point mutation in the Hind III recognition site (this mutation blocks cutting by Hind III), DNA with a 2 KB (2 kilobase-pairs or 2,000 base-pairs) insertion element, and DNA with a 1 KB deletion. After digestion, the DNA fragments are electrophoresed on an agarose gel and the size of the fragments determined. Inspection of the diagram reveals that the type of mutation can readily be deduced from the sizes of the DNA fragments. You will use this strategy in today's laboratory to characterize a mutation in a specific gene.

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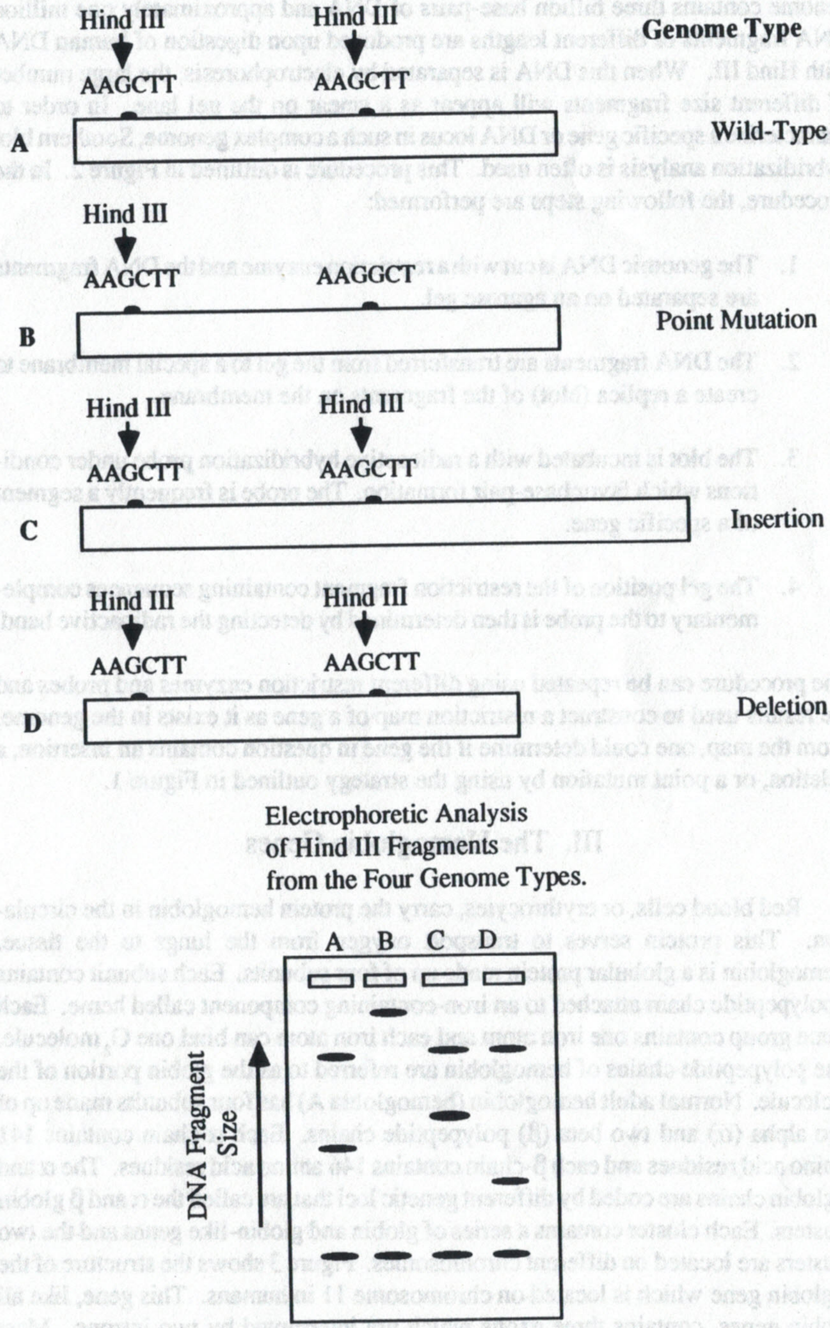
Figure 1 Strategy for Characterizing Mutations.



In organisms with genomes larger than viruses, the number of DNA fragments produced by restriction nuclease digestion is generally too great to detect one specific DNA band in an agarose gel by gel staining. For example, the human genome contains three billion base-pairs of DNA and approximately one million DNA fragments of different lengths are produced upon digestion of human DNA with Hind III. When this DNA is separated by electrophoresis, the large number of different size fragments will appear as a smear on the gel lane. In order to characterize a specific gene or DNA locus in such a complex genome, Southern blot hybridization analysis is often used. This procedure is outlined in Figure 2. In the procedure, the following steps are performed:

1. The genomic DNA is cut with a restriction enzyme and the DNA fragments are separated on an agarose gel.

2. The DNA fragments are transferred from the gel to a special membrane to create a replica (blot) of the fragments on the membrane.

3. The blot is incubated with a radioactive hybridization probe under conditions which favor base-pair formation. The probe is frequently a segment of a specific gene.

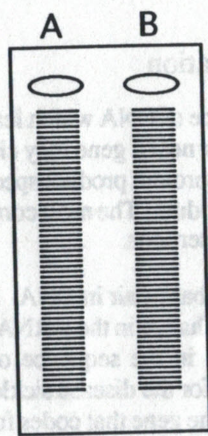
4. The gel position of the restriction fragment containing sequences complementary to the probe is then determined by detecting the radioactive band.

The procedure can be repeated using different restriction enzymes and probes and the results used to construct a restriction map of a gene as it exists in the genome. From the map, one could determine if the gene in question contains an insertion, a deletion, or a point mutation by using the strategy outlined in Figure 1.

III. The Hemoglobin Genes

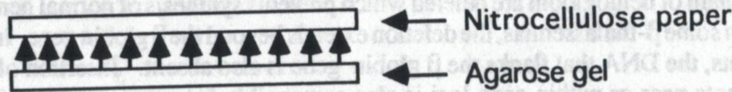
Red blood cells, or erythrocytes, carry the protein hemoglobin in the circulation. This protein serves to transport oxygen from the lungs to the tissue. Hemoglobin is a globular protein made up of four subunits. Each subunit contains a polypeptide chain attached to an iron-containing component called heme. Each heme group contains one iron atom and each iron atom can bind one O_2 molecule. The polypeptide chains of hemoglobin are referred to as the globin portion of the molecule. Normal adult hemoglobin (hemoglobin A) has four subunits made up of two alpha (α) and two beta (β) polypeptide chains. Each α-chain contains 141 amino acid residues and each β-chain contains 146 amino acid residues. The α and β globin chains are coded by different genetic loci that are called the α and β globin clusters. Each cluster contains a series of globin and globin-like genes and the two clusters are located on different chromosomes. Figure 3 shows the structure of the β globin gene which is located on chromosome 11 in humans. This gene, like all globin genes, contains three exons which are interrupted by two introns. Most

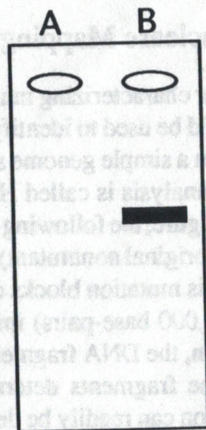
Figure 2. Procedure for Blot Hybridization Analysis



Cellular DNA from yeast (lane A) and human (lane B) are digested with a restriction enzyme. The DNA fragments are then separated on an agarose gel. Note the smears on each gel lane. The smears represent large numbers of different length DNA fragments.

The separated DNA in the gel is then denatured and transferred to a membrane made of nitrocellulose or nylon. The replica of the DNA fragments on this membrane is called a blot.

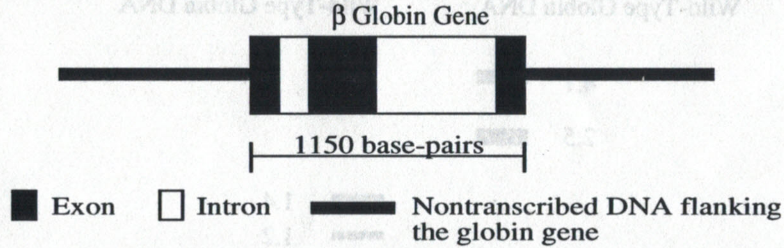




The blot is incubated with a radioactive alpha-globin DNA probe. The probe hybridizes (binds) to the restriction fragment in human DNA that contains the alpha-globin gene. The blot is then washed and exposed to X-ray film in order to visualize the restriction fragment containing the globin sequence.

eukaryotic genes contain introns which are segments of DNA within genes that do not code for proteins. During transcription, the gene gives rise to an RNA species which is a precursor to the mRNA. The intron sequences are then removed from the precursor and the remaining segments (coded by the exons) are spliced together to form the mature mRNA. The mRNA then enters the cytoplasm and is translated into a polypeptide chain.

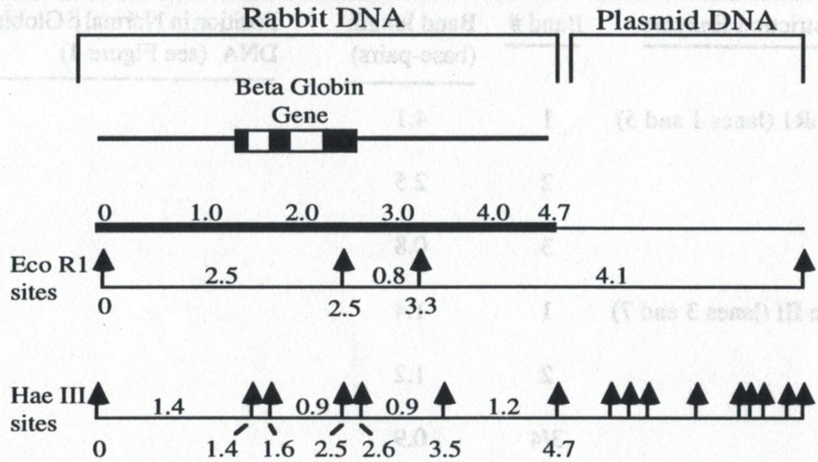
Figure 3. Organization of the β Globin Gene.



IV. Description of this Laboratory Exercise

In this exercise, you will study the organization of the normal β globin gene and a mutant β globin gene from the rabbit. The globin genes have been inserted into bacterial plasmids (pUC18) and you will study the globin gene-plasmid complex. Figure 4 shows a map of the region of the normal β globin cluster that will be analyzed. You will compare the organization of this normal gene region to a mutant gene region using the restriction enzymes EcoR1 and Hae III. The positions of the restriction sites for these enzymes are shown in Figure 4.

Figure 4. A Map of Rabbit β Globin DNA



The numbers in this figure are given in kilobase pairs (KB).
One KB = 1,000 base pairs.

Objective: In this exercise you will digest the normal β globin gene region and a mutant β globin gene region with EcoR1 and Hae III and then analyze the DNA fragments from each by electrophoresis. The results of the analysis should enable you to identify the type of mutation and the position of the mutation in the β globin cluster.

Materials Provided

Normal β Globin DNA
Mutant β Globin DNA
EcoR1 and Hae III: The restriction enzymes to be used in this laboratory should be made up immediately before use as described in the Instructor Guide. The enzymes should then be placed in a beaker containing ice chips. The solutions contain the enzymes suspended in a nuclease digestion buffer. The solutions of enzyme and DNA must not be contaminated, so use a fresh micropipet whenever you remove the enzyme and DNA from the stocks.
Sample Buffer - The buffer contains electrophoresis buffer, glycerol, and bromophenol blue.

Materials Not Provided

The solutions and materials required for electrophoresis, sample handling and gel staining (See Instructor Guide)
Water bath for tube incubation maintained at 37°C.
Microtubes (0.5ml)

Procedure

The experiment was designed for 8 students working individually or 16 students working in teams of two.

A. Preparing the DNA samples

1. Number four 0.5ml tubes 1 to 4 with a water-proof marking pen.
2. Place 5μl of the normal β globin DNA into tube 1.
3. Place 5μl of the mutant β globin DNA into tube 2.
4. Place 10μl of normal β Globin DNA into tube 3.
5. Place 10μl of mutant β Globin DNA into tube 4.
6. Place 10μl of EcoR1 into tubes 1 and 2.
7. Place 5μl of Hae III into tubes 3 and 4.
8. Cap the tubes and tap them with the tip of your index finger to mix the solutions. Incubate the tubes for 50 minutes at 37°C.

9. While the tubes are incubating, prepare 1.2% agarose gels as described in Part A - page 16.

10. At the end of the 50 minute incubation period, add 5µl of sample buffer to each of the four tubes.

B. Electrophoresis

1. Load 15µl of the following samples from the above section into the sample wells

Sample Well	Sample	
1	Tube 1	
2	Tube 2	
3	Tube 3	Group 1
4	Tube 4	
5	Tube 1	
6	Tube 2	
7	Tube 3	Group 2
8	Tube 4	

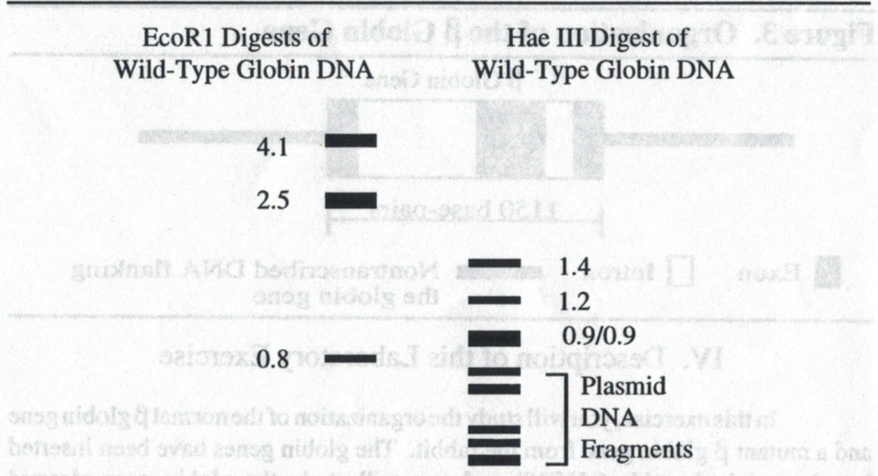
2. Seal the wells with agarose and electrophorese until the bromophenol blue in the samples has migrated to within 1mm of the positive electrode end of the gel. At 170 volts, this should take about one hour. If the rapid (in gel) staining procedure described in Section IV is used, electrophorese until the bromophenol blue has migrated about half way to the positive electrode. At that time, view the gels and then resume electrophoresis until the bromophenol blue has migrated to 1mm of the positive electrode end.

3. Remove the gels from the unit and stain them overnight at 4°C with methylene blue as described in Section IV.

4. Measure the distance of the DNA bands in mm from the sample wells and draw a picture of the DNA bands in each gel lane.

Data Analysis and Study Questions

1. The Figure below shows a diagram of DNA bands that should have been produced by digestion of the normal β globin DNA with EcoR1 and Hae III. The sizes of the bands, in kilobase-pairs, are also shown. Identify these bands on lanes 1,3, 5, and 7 of your gel.



2. A map of the restriction sites for EcoR1 and Hae III in the normal β globin DNA is shown in Figure 4. With this information, identify the position along the rabbit globin DNA of the largest fragments on your gels (lanes 1, 3, 5, 7) and record your results in the table below.

Restriction Enzyme	Band #	Band length (base-pairs)	Position in Normal β Globin DNA (see Figure 4)
EcoR1 (lanes 1 and 5)	1	4.1	
	2	2.5	
	3	0.8	
Hae III (lanes 3 and 7)	1	1.4	
	2	1.2	
	3/4	0.9	
	3/4	0.9	

3. Compare the sizes and numbers of fragments in the EcoR1 and Hae III digests of the mutant DNA to the normal DNA. With this information describe the type of mutation and the position of the mutation in the β globin DNA.

4. Give the name of the disease that is caused by this type of mutation in the β globin gene in human DNA.