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Series Editor

Harry L. Ioachim, M.D.
Clinical Professor of Pathology
College of Physicians and Surgeons
of Columbia University

Attending Pathologist
Lenox Hill Hospital
New York, New York

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Preface

Pathobiology Annual 1980 marks the tenth anniversary of this series and provides the occasion for a critical retrospective look. The ten volumes, contain a total of 137 articles that review a remarkably broad spectrum of biomedical topics.

The creative efforts of 231 authors, all well-known specialists in their respective fields of expertise, produced ten volumes of current medical knowledge that have been well received and highly praised by a great number of book reviewers. Diversity in Pathobiology Annuals has been expressed not only in the variety of topics that have been reviewed but also in the roster of contributors which represent some of the most prestigious universities of 18 different countries.

The articles in *Pathobiology Annual 1980,* in accordance with the style of this series, survey a broad spectrum of modern biomedical topics. Included are an impressive, in-depth, analysis of the pathogenesis of thalassemia, a beautifully illustrated study on the structure and function of the islets of Langerhans in diabetes, a paper investigating the mechanisms of metastasis and critical reviews on the melanins and melanogenesis, and on the nature of lipid pigments. Two papers are related to the mechanisms of hypertension and to the role of aldosterone in its pathogenesis while another article gives us practical clues for the interpretation of immunofluorescence staining in renal diseases. Different aspects of hepatitis are explored in an authoritative survey of posttransfusion hepatitis and in a study of the immunologic reactions of chronic active hepatitis. Finally, an excellent review on cryoglobulins and an interesting discussion on the factors that regulate the growth of cells *in vitro* complete the contents for 1980.

The list of contributors includes numerous, prestigious authors from universities in the United States and abroad. I hope that physicians and biologists in different areas of specialization will find the information and experience contained in this volume both beneficial and enjoyable.

The association with so many eminent scientists has been a continuous source of pride and pleasure for me over the past ten years. As for professional assistance, I enjoyed the dedicated help of Mrs. Berta Steiner Rosenberg, and of her colleagues at Raven Press, of an earlier technical team at Appleton-Century-Crofts and of my secretary, Mrs. Tove Bamberger.

We look forward to a second decade of Pathobiology Annuals in which our efforts will be directed toward continuously improving our present standards.

Harry L. Ioachim, M.D.

Contributors

Harvey J. Alter, M.D.
Department of Immunology, Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

Edward J. Benz, Jr., M.D.
Hematology Section, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Sharad D. Deodhar, M.D., Ph.D.
Department of Immunopathology, The Cleveland Clinic Foundation, Cleveland, Ohio 44106

Robert G. Dluhy, M.D.
Department of Medicine, Harvard Medical School, Peter Bent Brigham Hospital, Boston, Massachusetts 02115

Bernard G. Forget, M.D.
Hematology Section, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Arthur Grollman, M.D., Ph.D., F.A.C.P.
School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801

Paul V. Holland, M.D.
Department of Immunology, Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

Efthimios J. Kasambalides, M.D.
Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Karl W. Lanks, M.D., Ph.D.
Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Fiorenzo Paronetto, M.D.
Laboratory of Immunology, Bronx Veterans Administration Medical Center, Bronx, New York 10468

Robert H. Purcell, M.D.
Department of Immunology, Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

Patrick A. Riley, M.D.
Cell Pathology Unit, Department of Biochemical Pathology, University College Hospital Medical School, London WC 1E6 JJ, England

Evangelista Sagnelli, M.D.
Department of Pathology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10068

Rafael Valenzuela, M.D.
Department of Immunopathology, The Cleveland Clinic Foundation, Cleveland, Ohio 44106

Bruno W. Volk, M.D.
Department of Pathology, University of California Irvine Medical Center, California College of Medicine, Orange, California 92668

Leonard Weiss, M.D., Ph.D.
Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14263

Klaus F. Wellman, M.D.
Department of Pathology, Beekman Downstate Hospital, New York, New York 10038

Moshe Wolman, M.D.
Department of Pathology, Tel Aviv University, Sackler School of Medicine, Ramat Aviv, Israel, and Chaim Sheba Medical Center, Tel Hasomer, Israel

Horace H. Zinneman, M.D.
Department of Medicine and Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55417

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Pathogenesis of the Thalassemia Syndromes

Edward J. Benz Jr. and Bernard G. Forget

Hematology Section, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

The α- and β-thalassemia syndromes comprise a heterogeneous group of inherited hematologic disorders characterized by reduced or absent synthesis of the α- and β-globin polypeptide chains which form the apoprotein portion of normal adult hemoglobin, Hb A (Hb A: $\alpha_2\beta_2$) (1,2). In the α-thalassemias α-chain synthesis is impaired, whereas in β-thalassemia β-chain synthesis is diminished. The unaffected globin chains continue to be synthesized at nearly normal rates; thus, for example, β-chain synthesis proceeds at normal rates in α-thalassemia, and α-chain synthesis is normal in β-thalassemia. This review surveys the cellular and molecular pathophysiology of the thalassemias.

The hallmark of the thalassemia syndromes is an inherited defect causing reduced or absent synthesis of one or more of the normal polypeptide chains of hemoglobin. As a result, the erythrocytes have a low intracellular hemoglobin content (hypochromia) and are smaller than normal (microcytosis) because the decreased supply of globin subunits prevents adequate accumulation of functioning hemoglobin tetramers. In addition, continued normal synthesis of the unaffected globins leads to the accumulation of unstable aggregates of unpaired chains. In contrast to hemoglobin tetramers, which are highly soluble in the cytoplasm, globin chains are rather insoluble. These aggregates precipitate within developing erythroid progenitor cells in the bone marrow, alter intracellular metabolism, damage the cell membrane, and cause premature destruction of maturing erythroblasts (ineffective erythropoiesis). Cells surviving intramedullary maturation are released into the peripheral circulation with these globin precipitates (inclusion bodies). The inclusions are recognized by the spleen and removed, causing alteration of red cell size, shape, deformability, and metabolism. Ultimately, such red cells have a shortened survival in the peripheral circulation (hemolysis). All of these phenomena conspire to cause anemia. In general, the ineffective erythropoiesis and the hemolytic component are the predominant sources of disability in the most severe forms of thalassemia.

The hematologic syndromes observed in individual patients with thalassemia are quite heterogeneous with respect to severity, ranging from asymptomatic hypochromia in mildly affected heterozygotes to profound ineffective erythropoiesis, bone marrow expansion, and hemolytic anemia associated with growth

retardation and bony deformities in the more severely affected homozygotes. The severity of biosynthetic impairment and secondary factors which can alter the burden of unpaired globin chains influence the degree to which this phenotype is expressed in individual patients. The reduced accumulation of hemoglobin tetramers and the pathological accumulation of unpaired, insoluble globin chains which results from impaired biosynthesis are the unifying mechanisms from which these diverse clinical phenotypes arise.

At the molecular level, the thalassemia syndromes are characterized by a diversity of lesions with a common unifying theme. With few exceptions, patients with thalassemia produce structurally normal globin chains (i.e., globin chains having normal amino acid sequences) at reduced rates. Thus thalassemias are hemoglobinopathies distinct from disorders (e.g., sickle cell disease) characterized by production of globin chains having altered structure and physiologic function. Extensive family studies have established that the mutations responsible for thalassemia reside in, or very near to, the structural genes coding for the affected globin chain. Therefore these lesions specifically derange the processes which normally ensure adequate levels of globin gene expression during erythropoiesis. There is now abundant evidence that multiple lesions in the pathway of normal globin gene expression can give rise to defective globin synthesis and its attendant clinical complications.

In this review, we first survey the β-thalassemia syndromes and related abnormalities of non-α-globin synthesis, since these best illustrate most of the major molecular, genetic, and pathophysiologic principles useful for understanding hemoglobin synthesis. We then review the pathophysiology and molecular origins of the α-thalassemia syndromes, which were recently shown to be considerably more heterogeneous than previously appreciated. In each case we consider the pathophysiology of these disorders at the cellular and tissue level, and discuss recent progress made toward definition of their molecular origins.

In the following sections on the pathophysiology and pathogenesis of thalassemia, we cite only the most recent and/or relevant literature. A number of detailed reviews of the subject exist which can be referred to for more extensive bibliographic citations (1–11).

THE β-THALASSEMIA SYNDROMES

The biochemical abnormality common to all forms of β-thalassemia is decreased or absent synthesis of structurally normal β-globin; α-chain synthesis in these disorders proceeds normally. The major forms of β-thalassemia are classified in Table 1. As indicated in the first portion of Table 1, clinical terms such as thalassemia major, thalassemia intermedia, etc., describe only the severity of the anemia with respect to transfusion requirement. However, these terms retain their utility since considerable variability in clinical severity occurs within individual genetic and biochemical categories of β-thalassemia. For example, high hemoglobin (Hb) A_2-β^+-thalassemia is not a single disorder but a group

TABLE 1. *Classification of the major β-thalassemia syndromes*

I. **Clinical Classification**
- A. *"β-Thalassemia Major"*—Severe anemia and ineffective erythropoiesis, transfusion dependence. Also called "Cooley's anemia." Invariably homozygous.
- B. *β-Thalassemia Intermedia*—Significant anemia but no or only intermittent transfusion requirement. May be due to homozygosity for milder β-thalassemia genes, severe forms of heterozygous β-thalassemia, or interaction of two β-thalassemia genes.
- C. *"β-Thalassemia Minor"*—Invariably heterozygous (β-thalassemia trait). Asymptomatic hypochromic anemia.

II. **Genetic Classification**
- A. *High A_2 β^+-Thalassemia*—Partial reduction in β-globin production, elevated Hb A_2, no or modest increase in Hb F in heterozygous.
- B. *High Hb A_2 β^0-Thalassemia*—Like (A), but total absence of β-globin.
- C. *δβ-Thalassemia*—Total absence of Hb A, Hb A_2 production, moderate increase in Hb F ($\gamma/\alpha = 0.2$ to 0.4 in homozygote).
- D. *Hb Lepore*—α_2 $(\delta\beta)_2$; δβ-globin a "fused" chain due to crossover between δ and β genes. Reduced output of δβ-globin and absence of β-gene on affected chromosome causes thalassemia phenotype (see text).
- E. *Hereditary Persistence of Fetal Hemoglobin (HPFH)*
  1. *"Pancellular"*—Like δβ-thalassemia but with marked increase of Hb F ($\gamma/\alpha = 0.5$ to 1 in homozygotes) in all red cells. Homozygotes clinically well.
  2. *"Heterocellular"*—Heterogeneous cell-to-cell Hb F content with increased total Hb F due to increased "F" cells. Clinically benign

of mutations differing in the severity of globin synthetic impairment, the degree of increase of Hb F and Hb A_2 synthesis, etc.

For the purposes of characterizing the biosynthetic lesion and molecular mechanisms of disease in individual patients, it is more useful to utilize terminology based on genetic and biochemical criteria (Table 1, part II). In general, these syndromes are classified according to whether β-globin synthesis is entirely absent (β^0-thalassemia) or only partially reduced (β^+-thalassemia) as well as by associated abnormalities in the synthesis of other non-α-globin chains (the γ- and δ-chains of Hb F and Hb A_2, respectively) which may be present. Since the β-globin gene exists as a single copy within the haploid genome (discussed later), patients may be either heterozygous or homozygous for a given thalassemia mutation, doubly heterozygous for two forms of thalassemia, or doubly heterozygous for thalassemia and a structurally abnormal hemoglobin. Included in the second portion of Table 1 are some conditions (e.g., Hb Lepore syndrome) which are associated with structurally abnormal hemoglobins but resemble thalassemia at either the clinical or the molecular level. These are usually considered to be β-thalassemia syndromes. In addition, there are clinically benign conditions associated with disordered differential regulation of γ-, δ-, and β-globin synthesis. Called hereditary persistence of fetal hemoglobin (HPFH), these disorders are associated with high postnatal levels of Hb F, even in the heterozygous state. Various forms of HPFH are closely related to certain β-thalassemia syndromes at the molecular level and are usually classified with them.

Pathophysiology of the Anemia in Severe β-Thalassemia

Pathogenesis of the Clinical Phenotype

There are primary and secondary causes for the anemia observed in thalassemia. Reduced synthesis of β-globin chains leads to an overall intracellular deficit of Hb A synthesis, causing hypochromic microcytic anemia. In the homozygous state, another pathophysiologic process worsens the anemia and is responsible for the major clinical manifestations. The continued synthesis of α-globin in normal amounts results in the accumulation, within the red cell precursors, of excessive amounts of these normal chains during erythropoiesis. Not finding complementary globin chains with which to bind, the α-globin forms aggregates and precipitates within the cell. These precipitates lead to membrane damage and premature destruction, not only of circulating red cells which survive erythropoiesis and are released into the blood, but also of the majority of developing erythroblasts within the marrow itself.

Most of the erythroid progenitors are destroyed early during erythropoiesis by the accumulation of α-globin inclusions; very few achieve the status of circulating erythrocytes. The anemia fails to improve and erythropoietin stimulation persists. In an attempt to overcome this blockade in compensatory erythropoiesis, the bone marrow undergoes extraordinary hyperplasia, invading the bony cortex and not infrequently establishing sites of extramedullary hematopoiesis. This hyperplasia produces bony abnormalities (e.g., mongoloid facies and pathologic fractures), impaired growth, infiltration of the liver and spleen with erythropoietically active tissue, and intrathoracic, intra-abdominal, or intrapelvic masses of extramedullary erythropoiesis.

Like other disorders characterized by chronic anemia, defective iron utilization, and/or ineffective erythropoiesis, severe β-thalassemia is associated with chronically increased absorption of dietary iron. Some iron appears to be deposited in mitochondria of erythroblasts and may lead to decreased mitochondrial enzyme function, including those enzymes which are necessary for heme synthesis. Decreased heme synthesis may therefore contribute to a further decrease in Hb A synthesis and a further block in iron utilization.

The extreme expansion and turnover of the erythropoietic cell mass leads to a marked hypermetabolic state strongly resembling that seen in leukemias, lymphomas, and certain other conditions associated with extraordinarily high rates of cell turnover. Large folic acid requirements result from the cell turnover, so that folic acid deficiency is a frequent finding in untreated patients. The constant cell destruction promotes release of purines from cellular DNA, elevated serum uric acid levels, and occasionally clinical manifestations of gout; and increased bilirubin from heme catabolism causes calcium bilirubinate gallstones. In β-thalassemia these hypermetabolic conditions are aggravated by the massive expansion of bony spaces, liver, and spleen required to accommodate the erythroid cells. The additional blood supply required by these expanded organs

can increase the patient's blood volume by as much as 40% above normal, thus increasing total body oxygen delivery requirements and aggravating the physiologic effects of the reduced red cell mass.

The central role of globin inclusions in the pathophysiology of thalassemia has been convincingly demonstrated. Intracellular inclusions interfere with normal cell division and arrest cells at the G1 stage of the cell cycle; intracytoplasmic inclusions damage the cell membrane and derange the internal sodium–potassium environment. Moreover, the very heterogeneous distribution of Hb F, which is known to be present in β-thalassemia, appears to correlate well with the postulated role for inclusion bodies. Those cells which have the most fetal hemoglobin are those which have the least relative excess of α-chains, since more γ-chains are available to combine with α-chains to form Hb F. It has been demonstrated in β-thalassemia that Hb A has a more rapid turnover (shorter half-life) than Hb F. There is also a good positive correlation between the severity of the disease in β-thalassemia and the size of the free α-chain pool.

These findings emphasize the relationship of α-chain inclusions to the pathogenetic process and the beneficial role of γ-chain synthesis in lessening the imbalance of globin chain accumulation, decreasing the formation of α-inclusions and increasing the red cell survival. Similarly, removal of α-chain inclusions almost certainly contributes to the hemolytic process in the spleen. Prior to splenectomy, the inclusion bodies are practically never seen in peripheral red blood cells but are abundant in erythroblasts. Following splenectomy, inclusions appear in large numbers in erythrocytes. This observation correlates well with the role of the spleen and reticuloendothelial system in removing inclusion bodies from red cells, thereby damaging and/or destroying these cells.

Comparative analyses of globin chain imbalance in α-and β-thalassemia is instructive for appreciating the preeminence of inclusion body formation in determining clinical severity. The α-globin inclusions in β-thalassemia are extraordinarily insoluble, accumulating at a very early stage of erythropoiesis when only a small amount of hemoglobin has been produced within the erythroblast. In contrast, the excess β-chains accumulating in Hb H disease, the most severe form of α-thalassemia seen in adult life, are rather more soluble. These β-chains form moderately unstable β_4 tetramers called Hb H, which slowly precipitate within the circulating red cell as it ages. Hb H disease is thus a considerably milder condition than β-thalassemia even though the ratios of α- to β-globin synthesis may be comparable in the two disorders. There is also much less ineffective erythropoiesis in Hb H disease.

Since the inclusion body formation is the primary determinant of severity in thalassemia, the clinical status of the patient depends more on the net degree of inclusion body formation than on the actual amounts of hemoglobin produced or the β/α ratio alone. Patients homozygous for β-thalassemia who also inherit the heterozygous state for α-thalassemia have been identified; these patients have a much milder anemia and much less ineffective erythropoiesis even though hypochromia and microcytosis are severe because of the "double" impairment

in Hb A formation. Conversely, a family has been described in whom all the features of moderately severe β-thalassemia exist *without microcytosis or hypochromia* (12). In these heterozygotes imbalance of α/β synthesis is encountered, and excess α-globin and α-globin inclusions accumulate. It is likely that these patients suffer from a β-thalassemia-like disorder because of an absolute increase in α-chain production rather than a decrease in β-chain production. Thus the absolute burden of inclusions is greater even though the β/α ratio is that of β-thalassemia minor (β/α = 0.5). Degradation (proteolysis) of free globin chains also occurs in normal and thalassemic erythroblasts, reticulocytes, and erythrocytes (13,14). The efficiency of this proteolysis may play a role in determining the relatively benign clinical course of heterozygous thalassemia in most patients and the somewhat more severe disease seen in a few heterozygotes who have more inclusions and β-thalassemia intermedia.

At the present time, transfusion with red blood cells represents the only acceptable form of therapy for severe β-thalassemia. Transfusion not only increases the oxygen-carrying capacity of the blood but also serves to suppress the erythroid hyperplasia, reducing bone marrow expansion and the hypermetabolic state which complicate the anemia in untreated patients. Hypertransfusion (maintenance of transfusion schedules assigned to maintain hemoglobin levels at 9 to 11 g% or higher) has become the generally accepted mode of transfusion therapy. The limiting factor in this therapeutic approach is iron overload, leading to cardiac hemosiderosis and death during the second or third decade of life in the majority of cases. Recent therapeutic efforts have focused on the development of improved transfusion regimes (e.g., "supertransfusion") whereby hemoglobin levels are maintained at or very close to the normal range. This achieves complete suppression of the bone marrow, reducing blood volume and, according to preliminary data, resulting in a blood requirement equal to, or only slightly greater than, previous hypertransfusion regimens. In addition, use of chronic subcutaneous infusions of desferrioxamine has been shown to greatly enhance the efficiency of iron excretion in transfused patients. Chronic iron chelation combined with rational hyper- or supertransfusion therapy has been said to result in negative iron balance in many of these patients. Further trials are required to determine whether these newer methods of therapy will alter long-term morbidity and survival.

Molecular Pathogenesis of the Thalassemia Syndromes

Mutations responsible for β-thalassemia syndromes lie within or very near the structural gene coding for β-globin. Numerous families in whom β-thalassemia and gene for a structurally abnormal β-globin variant coexist permit many opportunities to observe informative matings in which offspring due to crossover and recombination events could occur. No recombination between β-thalassemia mutations and β structural variants has yet been observed in such families, even though recombination between β-thalassemia and the closely linked δ-

globin gene has been observed. The β-thalassemia mutation has also been shown to be only "*cis* dominant" (i.e., affecting only the β-globin gene on the same chromosome on which the mutation is inherited). For example, patients inheriting β-thalassemia on one chromosome and the gene for sickle hemoglobin (β^S) on the homologous chromosome "in *trans*" (sickle–β-thalassemia) produce β^A-globin in reduced or absent amounts but the β^S globin at normal rates. Thalassemia mutations have no deleterious effect on the activity of globin genes located "in *trans*" on the homologous chromosome.

For all of the above reasons, the thalassemias are considered regulatory mutations because they generally result in altered synthesis of a structurally normal protein. Moreover, the molecular lesions affect the processes governing expression of these specific genes. Therefore the thalassemias have been intensively investigated with the expectation that they might provide insights into normal regulatory mechanisms. Considerable recent progress suggests that this hope may soon be realized. In order to review these advances, it is necessary to survey briefly recent technological advances permitting rigorous analysis of globin genes and their messenger RNA(s) and to review the normal molecular biology of globin gene expression.

Techniques Used to Study Globin Gene Structure and Function

The ability to analyze the structure and function of individual genes, including globin genes, has centered around several general methodologies: (a) isolation of mRNA and translation of the mRNA in mRNA-dependent cell-free protein-synthesizing systems: (b) synthesis of specific complementary DNA molecules (cDNAs) from mRNA templates and the use of cDNAs in molecular hybridization reactions; (c) restriction endonuclease mapping of structural genes; and (d) molecular cloning of DNA sequences into bacterial hosts. Partially purified mRNAs can be translated in a variety of mRNA dependent cell-free protein-synthesizing systems. The proteins whose synthesis is promoted in these *in vitro* systems constitute an indicator of the types of mRNA present in the preparation. However, strict quantitation of the amounts of each mRNA species cannot always be inferred from the relative amounts of individual proteins synthesized. Variation in the incubation conditions tend to favor the translation of different mRNAs, frequently in an unpredictable fashion. Thus molecular hybridization techniques based on direct chemical measurement of the amount of mRNA, independent of its function in cell-free systems, have been developed.

The poly(A) "tail" of mRNAs allows them to be utilized as a template for synthesis of a DNA strand having the complementary base sequence (cDNA) in the presence of the enzyme RNA-dependent–DNA polymerase from avian myeloblastosis virus (reverse transcriptase). In the presence of an oligo-d(T) primer, the poly(A) tail of the mRNA and the oligo-d(T) primer form a short double-stranded region which serves as the initiation point for the reverse transcriptase enzyme. If radioactive deoxynucleotide triphosphate precursors are

included in the reaction, radioactive cDNA copies of the mRNA are obtained. The RNA template can be completely removed from the radioactive cDNA strand by alkaline hydrolysis, thus providing a highly specific, radioactive, single-stranded DNA probe capable of forming cDNA–RNA, or cDNA–DNA, double-stranded hybrid molecules with, but only with, DNA or RNA molecules having the complementary base sequence.

The techniques of molecular hybridization provide quantitative assays which exploit the specificity of double-stranded hybrid molecule formation inherent in the base sequence of the radioactive cDNA probe. During incubation in an appropriate ionic environment, globin cDNA anneals by formation of hydrogen bonds to DNA or RNA molecules whose base sequences are complementary. By adjusting the salt concentration and the temperature of the incubation medium, one can make these reactions highly specific. For example, conditions can be devised so that human β-globin cDNA reacts only with the β-globin gene or with β-globin mRNA sequences, whereas human γ-globin cDNA reacts only with γ sequences. The binding of radioactive cDNA into duplexes with the DNA or RNA sample is quantitated during a subsequent incubation with a nuclease which selectively digests nonannealed single-stranded molecules. The amount of cDNA protected from the nuclease digestion is a measure of the quantity of complementary gene or mRNA sequence present in a sample being analyzed. Quantitation is permitted because the specific activity of the cDNA can be determined during its synthesis with reverse transcriptase. Thus the absolute amount of cDNA included in the reaction can be controlled by adjusting the number of counts per minute added. By simultaneously synthesizing two cDNAs in parallel reactions (e.g., α-cDNA and β-cDNA), one obtains two probes of identical specific activities which can be used to quantitate the relative amounts of complementary sequences (e.g., α- and β-mRNA) in the same sample.

The restriction endonucleases have become powerful tools for investigating gene structure and the molecular mutations which cause thalassemia. These bacterial enzymes recognize only certain small (oligonucleotide) sequences in DNA and cut only at, or very near, those sites. For example, Eco RI, which is isolated from *Escherichia coli,* recognizes the sequence 5′-GAATTC-3′. The substrate for this enzyme is double-stranded DNA, so that the actual structure it cleaves is

$$\begin{array}{c} \downarrow \\ \text{5′-GAATTC-3′} \\ \text{3′-CTTAAG-5′} \\ \uparrow \end{array}$$

The arrows indicate the bond in each DNA strand which is actually hydrolyzed by this enzyme. This enzyme cuts DNA molecules only at those positions which contain this precise sequence. As of this writing, over 50 restriction endonucleases have been characterized; each has a unique recognition sequence. Thus each

enzyme cuts a given DNA molecule at different positions. These fragments can be separated by gel electrophoresis according to molecular weight. For example, digestions of human DNA with the enzyme Eco RI and Bam HI yield different patterns of fragments, and "double digestion" of a single sample with both enzymes yields still another pattern. It can readily be appreciated that digestion of a DNA molecule with a number of these enzymes can be used to "map" the sites within the DNA sequence.

Incubation of cellular DNA with a particular restriction endonuclease yields a large but reproducible number of fragments which may range in size from a few hundred to several thousand base pairs. For example, human DNA contains several billion base pairs, within which there are approximately 500,000 sites recognized by the enzyme Eco RI. These fragments may be fractionated roughly according to size by electrophoresis in an agarose gel; however, no distinct resolution of any single band can be achieved because of the many thousands of fragments generated. To learn about the relationship between the globin genes and the sites for the particular enzyme used to digest the DNA, it is necessary to identify those few DNA fragments which contain globin gene sequences among the many which do not. This is accomplished by transfer of the DNA fragments to a nitrocellulose filter; transfer is accomplished by "blotting" the agarose gel with the filter. DNA in the gel elutes onto, and binds tightly to, nitrocellulose and can then be annealed *"in situ"* to radioactive cDNA. The positions on the filter where cDNA is bound can then be visualized by radioautography as distinct bands corresponding to a certain molecular weight or chain length. This method identifies the position and size of fragments containing globin gene sequences. Figure 1 summarizes the use of the gel blotting technique to detect DNA fragments containing specific gene sequences.

The terms "molecular cloning" and "recombinant DNA" refer to a methodologic approach by which two DNA sequences from dissimilar species can be joined together (recombined) *in vitro* to form a single, biologically active recombinant DNA molecule capable of infecting a bacterial host cell. One of the DNA components utilized is the vector, invariably a bacterial plasmid or bacteriophage DNA, which serves as the biologically active (infective) DNA cloning vehicle for the other component, the inserted DNA. The latter consists of a DNA preparation containing fragments on which reside all or part of the DNA sequences one wishes to study. For example, total human cellular DNA serves as a suitable inserted DNA preparation for molecular cloning of the human globin genes; alternatively, one could create recombinant DNA molecules from globin cDNAs rendered double-stranded by additional enzymatic treatment of the cDNA strands, etc. The "inserted DNA" fragments are rendered suitable for *in vitro* recombination by treatments which generate single-stranded "sticky ends" at the end of each double-stranded fragment. One such method for generating sticky ends—digestion with certain restriction endonucleases—was mentioned earlier. The vector is treated so that it acquires sticky ends having the complementary base sequence. The two preparations, vector and "inserted

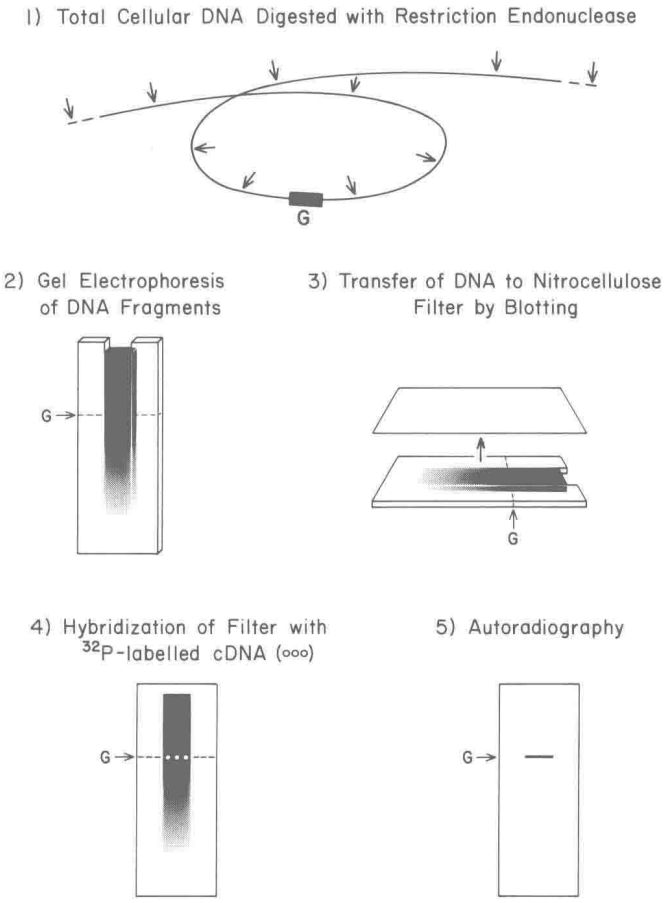

FIG. 1. Method for gene mapping by the gel blotting procedure. (From ref. 10.)

DNA," are incubated together under conditions that permit annealing of these complementary sticky ends. These techniques yield vector DNA-inserted DNA recombinant molecules; each recombinant DNA molecule contains a single fragment from the initial "inserted DNA" preparation.

Molecular cloning of the recombinant DNA molecules is accomplished by infecting an excess number of cells of a suitable bacterial host strain with the recombinant DNA molecules. In this manner, the likelihood that a single cell will become infected with more than one recombinant DNA molecule is minimal. Thus each recombinant DNA molecule is "cloned" in its own bacterial host cell. The recombinant DNA molecules are then propagated within the host cells, in semisolid media, so that each infected host cell yields a single colony or bacteriophage plaque, which can be recognized and isolated from the others. In order to identify those colonies or plaques which contain the inserted DNA