CURRENT HEMATOLOGY

VOLUME 1

Edited by

Virgil F. Fairbanks, M.D.

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Preface

Two decades ago, when I first became acutely aware of the publication explosion in medicine and the biological sciences, it was said that there were more than 10,000 journals in these related fields, which contained at least a million articles published annually. The situation has not changed and, indeed, even more articles are now being published. I suspect that at least one factor that helped persuade many of us to narrow our interests to a subspecialty such as hematology was the notion that at least we could stay abreast of a more restricted field. If we read *Blood* and the *British Journal of Haematology* plus some general internal medicine journals, we believed we could cope successfully. However, times change and now it is easy to list more than 20 journals specifically devoted to hematology and its branches plus several seminars, clinics, and other volumes in hematology. Further, add the fact that many critical hematology articles do not appear in purely hematology journals, but may be found in the *Journal of Clinical Investigation* or *FEBS Letters*, among many others. Can one devise a strategy to cope with the complexity and scattered sources of the ever-burgeoning hematology literature?

We have tried in this volume to provide an answer. I have asked 20 distinguished colleagues, each particularly knowledgeable in one area of hematology, to review the literature of the last few years and to indicate where notable progress has been made. Each was to add his or her own unique perspective in a critical interpretive review of the most recently published articles. I deliberately avoided being too arbitrary in defining "most recently," in the belief that, particularly for Volume 1 of this series, it would be better to leave that to the judgment of each contributor. *Current Hematology*, Volume 1, the result of this endeavor, is a distillate of more than 3,000 articles, of which about 80% were published in the years 1977 to 1981, and particularly in the years 1978 to 1979. A casual review of the bibliographies of the 17 chapters in this volume indicates some sources few readers would likely encounter otherwise: *Tropical and Geographical Medicine; Journal of Experimental Zoology; ASCD Journal of Dentistry for Children; Acta Haematologica Polonica; Journal of Molecular Catalysis; Journal of Agriculture and Food Chemistry; Poultry Science; Central African Journal of Medicine; Aviation, Space and Environmental Medicine; Journal of Protozoology; Verhandlungen der deutschen Gesellschaft für innere Medizin.*

We initially attempted to cover all areas of hematology in Volume 1 of this series, but came to realize that this could not be accomplished within the constraints of time and space. Therefore, the current literature of chronic lymphocytic leukemia, congenital dyserythropoietic anemias,

and storage disorders was not reviewed in this volume. These omissions will be rectified in Volume 2.

Besides reviewing the major areas of hematology, such as lymphomas, leukemias, coagulation, and so forth, we have included 3 "special-topic" chapters which present in depth subjects that have exhibited extraordinary growth during the past few years. In Chapter 1, Drs. Phillips and Kazazian lucidly review the methods and applications of restriction endonuclease studies of the hemoglobin chain DNA genomes and the new vistas that such studies have opened. The extraordinary advances that these techniques brought earned the 1978 Nobel Prize for fellow workers at Johns Hopkins, Drs. Smith and Nathans. In Chapter 6, Dr. Greenberg reviews the methods and applications of in vitro granulocytopoietic studies, a field in which he has become a prime mover. In Chapter 8, Dr. Trujillo, one of the best-known American cytogeneticists, presents a concise and easy-to-assimilate review of the findings and implications of cytogenetic studies in hematology.

Hematology is unique among medical disciplines in that it is both a clinical and a laboratory science. In recognition of this, we have included chapters that reflect advances in morphology and hematopathology and a chapter on laboratory instrumentation.

Both the publishers and I will be very receptive to comments and suggestions from readers as we strive to make *Current Hematology* an effective strategy to help you keep current in this field.

I wish to acknowledge the help of many persons without whom this volume could not have been completed. My wife and family have shown patience and fortitude during my many weekends and evenings of editing and proofreading. I wish to thank the patience and hard work of my secretary, Miss Dawn Stangler, the help and tolerance of Mayo Clinic librarians, and the thoughtful suggestions of many colleagues who have reviewed with me the submitted manuscripts, in particular Drs. Breanndan Moore, Lawrence Solberg, Robert L. Phyliky, Gerald Holcomb, and Robert M. Petitt. Ms. Libby Feinblatt, copyeditor, is due an accolade for the way she has polished phraseology and syntax of manuscripts when needed. I especially want to thank the many contributors to *Current Hematology*, Volume 1, who have given of their precious time and talents to join in this endeavor.

Dr. William F. Taylor provided invaluable help with statistical interpretation.

Virgil F. Fairbanks

Contents

1

Globin Gene Analysis by Restriction Endonuclease Mapping

JOHN A. PHILLIPS III
and
HAIG H. KAZAZIAN, Jr

The purpose of this chapter is to introduce the reader to recent advances in deoxyribonucleic acid (DNA) technology that have allowed globin gene analysis by restriction endonuclease mapping. These advances have already led to clinical applications, and further progress in diagnosis of hematologic as well as other inherited disorders seems imminent. The molecular basis of the thalassemias is now discussed in terms of DNA nucleotides deleted, added, or substituted, and the origins of these mutations are being elucidated.

Restriction endonuclease polymorphisms are now important to the hematologist. These polymorphisms are frequently occurring variations (> 1% incidence) in DNA sequences of "normal" humans. When these variations are in close physical proximity to mutations producing disease, they can be used as markers for identifying these mutations. Such close linkage of markers with mutant genes has enabled prenatal diagnosis of sickle cell anemia and certain thalassemias by analysis of DNA from fetal cells of amniotic fluid.

We begin this review with a glossary of terms (Table 1), followed by a discussion of the methods for restriction endonuclease analysis currently in use, normal globin gene organization, known globin gene mutations, and useful restriction endonuclease polymorphisms. Diagnosis of thalassemic states by these methods and prenatal diagnosis of various hemoglobinopathies are two clinical applications that will be discussed in detail.

METHODS

Restriction Endonuclease Mapping

The technique of restriction endonuclease mapping requires a combination of

TABLE 1. GLOSSARY OF TERMS

Allele—A gene occupying a specific locus. The β globin locus may be occupied by any of a large number of alleles (e.g., β^A, β^S, β^C, β^D, etc.).

Chromosomal homologue—One of a chromosome pair. Since humans are diploid (chromosomes are present in pairs), each non-sex chromosome has a homologue.

Deletion—The loss of genetic material, whether of one DNA nucleotide pair or several thousand.

5′ and 3′ ends of DNA fragments—By convention, the 5′ end refers to the left end of a DNA fragment and the 3′ end refers to the right end. The γ-δ-β gene complex has the order 5′-$^G\gamma$-$^A\gamma$-δ-β-3′. Biochemically, 5′ and 3′ refer to the points of attachment of phosphate to ribose on the two ends of the coding strand.

Genome—All the DNA sequences found in a complete set of chromosomes in an individual.

Kilobase (kb)—One thousand base (nucleotide) pairs of DNA.

Linkage—The close physical association of one locus with another on a particular chromosome. For example, γ, δ, and β loci are closely linked on chromosome 11.

Linkage disequilibrium—The association of 2 or more linked genes at nonrandom frequencies. Genes for 2 closely linked traits (e.g., ABO blood groups and the nail-patella syndrome), occur with a random association demonstrating linkage equilibrium. If blood group O were seen in greater frequency with nail-patella syndrome than in the general population, these two genes would be in linkage disequilibrium.

Locus—A specific site in the genome (e.g., the β globin locus on chromosome 11).

Molecular hybridization—Hydrogen bonding between one strand of a DNA molecule and its complementary strand. Under stringent conditions this hybridization can be very specific, so that one strand of the β globin gene will hybridize with its complementary β globin strand, but not with either strand of α globin DNA.

Nucleotide—Chemical unit of DNA composed of a base (adenine, guanine, cytosine, or thymine), deoxyribose, and phosphate. RNA nucleotides differ in that ribose replaces deoxyribose and uracil replaces thymine.

Polymorphism—Two or more common sequences or genes that occur at a particular locus in the genome of individuals in a population, the rarest of which has a frequency of 1% or greater.

Restriction endonuclease—An enzyme found in bacteria that cleaves DNA at or near specific recognition sites. These sites are usually designated by 4 or 6 base pairs of double-stranded DNA. For example, the enzyme *Eco*RI (isolated from *Escherichia coli*) cleaves DNA wherever the sequence 5′-GAATTC-3′ is found (see Table 2).

Restriction endonuclease polymorphism—Gain or loss of a restriction endonuclease site due to an alteration of the DNA sequence at a specific position in the genome. To qualify as a polymorphism, the altered restriction site must occur at a frequency of 1% or greater in the genome of different individuals in a population group. For example, the *Hpa*I site adjacent to the β globin gene is polymorphic. It is absent in most β^S-bearing chromosomes in American blacks but present in nearly all β^A-bearing chromosomes in this population group.

Restriction map—A map of a segment of DNA with the position and order of various restriction endonuclease sites noted. Map distances are given in kb.

Southern transfer—Procedure described by E.M. Southern for transfer from electrophoresis gels to filter paper of DNA fragments generated by restriction endonuclease cleavage of genomic DNA.

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine.

several steps. These steps will be discussed in some detail to acquaint the reader with their complexity, potential applications, and limitations.

Genomic DNA preparation

The first step in restriction mapping is the preparation of DNA from the nuclei of patients' leukocytes or cultured fibroblasts. Unless the patient is leukopenic, sufficient DNA (usually 25 to 60 μg/ml of whole blood) can be obtained from 15 to 20 ml of fresh blood that has been anticoagulated with the potassium salt of ethylenediaminetetraacetic acid (K_3EDTA).

The packed cells are first homogenized at 0° C in a buffer containing 1% Triton X-100 to release the nuclei. After centrifugation, the pelleted nuclei are lysed, freeing the DNA. Pronase is added, and the mixture is incubated at 37° C for 4 hours to digest nuclear proteins and membranes. Following phenol and chloroform extractions to remove peptides, the aqueous phase is made 0.2 M in KCl, and 2 volumes of ethanol are added. DNA relatively free of ribonucleic acid (RNA) is removed from the interphase with a glass rod and, after washing with ethanol, is stored in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) (1).

Restriction endonuclease digestion

After preparation, the genomic DNA is subjected to restriction endonuclease digestion. Each of the many restriction endonucleases has a specific oligonucleotide recognition sequence or site (2, 3). For example, *Eco*RI recognizes the DNA sequence 5'-GAATTC-3' (G, guanine; A, adenine; T, thymine; C, cytosine) and cleaves DNA as shown by the arrows in Table 2 whenever such sequences appear. Two other endonucleases and their recognition sequences are *Bam*HI—5'-GGATCC-3', and *Hpa*I—5'-GTTAAC-3'. (Sites of cleavage for *Bam*HI and *Hpa*I are also shown in Table 2.) Restriction endonuclease digestion, therefore, produces specific DNA fragments of reproducible size. The size of these fragments is determined by the number and locations of recognition sites in the DNA sequences.

Agarose gel electrophoresis

Following restriction endonuclease digestion, agarose gel electrophoresis is used to separate the DNA fragments by size (Fig. 1). Samples of DNA (5 to 10 μg) and molecular weight markers are loaded on 3-mm-thick vertical 0.8% agarose gels. Following electrophoresis, the gels are stained in an aqueous solution of ethidium bromide. Genomic and marker DNA patterns are visualized under an ultraviolet light and photographed.

Southern transfer

The DNA is then transferred from the agarose gels to a nitrocellulose filter. For transfer, the DNA must first be denatured (i.e., rendered single stranded) by treatment in 0.5 N NaOH and 0.6 M NaCl for 1 hour at 25° C. The gel is then neutralized in 1 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) and 0.6 M NaCl for 40 minutes, and the DNA fragments are "Southern transferred" for 24 hours or longer at 25° C to a nitrocellulose filter using 20 × SSC (4) (Fig. 1). The process involves the drawing up of the buffer by capillary action from a lower reservoir through the gel and the nitrocellulose filter into blotting paper. The flow of buffer transfers DNA fragments from the gel onto the filter. Next, the filter is gently washed in 3 × SSC, air dried, and baked overnight at 70° C to fix the transferred DNA fragments to the filter.

Preparation of a recombinant DNA probe

Probes for various globin genes can be obtained by the following methods. For α globin, the recombinant plasmid-

TABLE 2. RESTRICTION ENZYMES AND THEIR RECOGNITION SITES

Restriction Enzyme	Sequence at Sites of Recognition
*Acc*I	5′-GTCTAC-3′[a] or 5′-GTAGAC-3′ 3′-CAGATG-5′ 3′-CATCTG-5′
*BAM*HI	5′-GGATCC-3′ 3′-CCTAGG-5′
*Bgl*II	5′-AGATCT-3′ 3′-TCTAGA-5′
*Eco*RI	5′-GAATTC-3′ 3′-CTTAAG-5′
*Hind*II	5′-GTPyPuAC-3′ 3′-CAPuPyTG-5′
*Hind*III	5′-AAGCTT-3′ 3′-TTCGAA-5′
*Hinf*I	5′-GANTC-3′ 3′-CTNAG-5′
*Hpa*I	5′-GTTAAC-3′ 3′-CAATTG-5′
*Mbo*II	5′-GAAGANNNNNNNN-3′ 3′-CTTCTNNNNNNNN-5′
*Pst*I	5′-CTGCAG-3′ 3′-GACGTC-5′
*Sac*I	5′-GAGCTC-3′ 3′-CTCGAG-5′
*Taq*I	5′-TCGA-3′ 3′-AGCT-5′
*Xba*I	5′-TCTAGA-3′ 3′-AGATCT-5′

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; Py, pyrimidine (C or T); Pu, purine (A or G); N = A, G, T, or C.

[a]Arrows represent sites of cleavage.

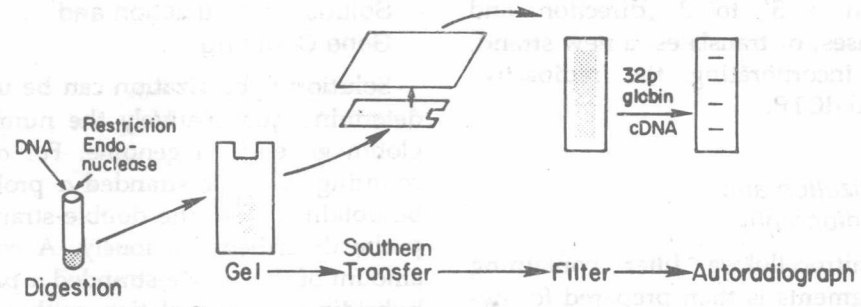

FIG. 1. Steps in restriction endonuclease analysis. These include restriction endonuclease digestion of genomic DNA, electrophoretic separation of the DNA fragments in agarose gels, Southern transfer of the DNA to filters, and identification of portions of structural genes in these fragments by hybridization of gene-specific probes and autoradiography. The probe shown in the figure, ^{32}P globin cDNA, is a DNA complementary to a specific globin, mRNA.

containing α globin gene sequence (JW 101) is cleaved with restriction endonucleases *Hinf*I and *Mbo*II to yield a double-stranded complementary DNA copy of human α globin messenger (mRNA) within a 960–base pair (bp) fragment (5, 6) (Fig. 2). To produce probes for specific portions of the α gene, this fragment can be isolated from an agarose gel and redigested with *Hpa*II to yield 2 smaller fragments, 1 of which contains the 5′ portion (left half) and the other the 3′ portion (right half) of the α sequences. Similarly, β globin sequences can be obtained from the recombinant plasmid JW 102 in a 1,200-bp fragment after *Hind*III and *Mbo*II digestion, and γ globin sequences can be obtained in a 1,100-bp fragment after *Taq*I digestion of the plasmid JW 151 (5). These various fragments containing α, β, or γ globin sequences can be labeled in vitro with $\alpha[^{32}P]$-deoxyadenosine triphosphate (dATP) and $\alpha[^{32}P]$-deoxycytidine triphosphate (dCTP) to a specific activity of approximately 10^8 counts per minute (cpm)/μg by the "nick translation" function of *Escherichia coli* DNA polymerase I (7). In this reaction the DNA polymerase I first binds at a nick or break in 1 of the 2 strands of the DNA. Then the enzyme simultaneously hydrolyzes, or breaks down, the nicked

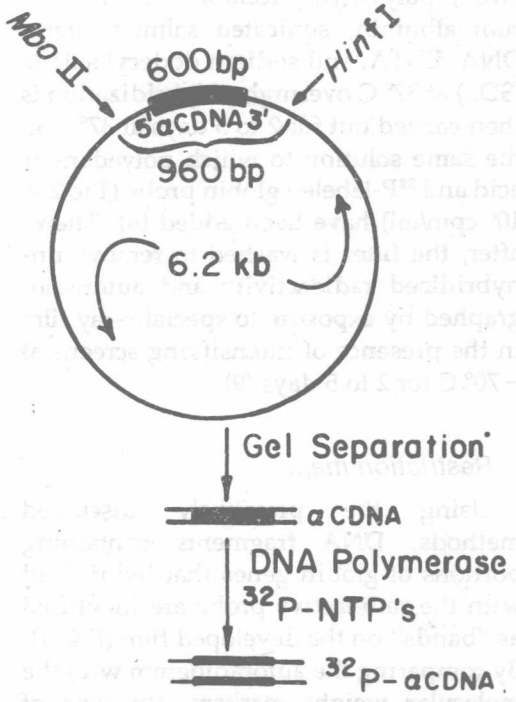

FIG. 2. An example of gene-specific probe preparation. DNA from the plasmid JW 101, which contains human α globin gene sequences, is digested with the restriction endonucleases *Mbo*II and *Hinf*I. A fragment of about 960 pairs (bp) containing the α globin gene sequence is recovered from preparative gels. This fragment is used as an α probe after radiolabeling with the deoxynucleoside triphosphates (NTPs) $\alpha[^{32}P]$-dATP and $\alpha[^{32}P]$-dCTP by the "nick translation" function of *Escherichia coli* DNA polymerase.

strand in a 5′ to 3′ direction and polymerases, or translates, a new strand, thereby incorporating the radioactive dATP and dCTP.

Hybridization and autoradiography

The nitrocellulose filter containing DNA fragments is then prepared for hybridization. To reduce nonspecific binding of the radioactive probe to the filter during hybridization, the filter is placed in a polyethylene bag containing buffered deionized formamide, NaCl, sodium citrate, Hepes, sodium pyrophosphate, Ficoll, polyvinylpyrrolidone, bovine serum albumin, sonicated salmon sperm DNA, EDTA, and sodium dodecyl sulfate (SDS) at 37° C overnight. Hybridization is then carried out for 2 to 3 days at 37° C in the same solution to which polyadenylic acid and ^{32}P-labeled globin probe (1 to 2 × 10^6 cpm/ml) have been added (8). Thereafter, the filter is washed to remove unhybridized radioactivity and autoradiographed by exposure to special x-ray film in the presence of intensifying screens at −70° C for 2 to 5 days (9).

Restriction maps

Using the previously discussed methods, DNA fragments containing portions of globin genes that hybridized with the radioactive probe are identified as "bands" on the developed film (Fig. 1). By comparing the autoradiogram with the molecular weight markers, the size of hybridizing DNA fragments can be estimated. Using a number of different restriction endonucleases, a map of the restriction endonuclease sites in the DNA regions containing α or β, $^{G}\gamma$, $^{A}\gamma$, δ, and ϵ genes can be derived. As will be shown, such maps are helpful in characterizing deletions in these genes.

Solution Hybridization and Gene Counting

Solution hybridization can be used to determine quantitatively the number of globin genes in a genome. For α gene counting, a single-stranded α probe can be obtained from the double-stranded α probe described previously. A constant amount of this single-stranded α probe is hybridized to completion with a sonicated genomic DNA from control subjects and from patients with α-thalassemic states (Fig. 3). The ratio of genomic DNA to probe is chosen so that normal DNA, containing 4 α genes per diploid cell, will hybridize 50% of the probe at the completion of the reaction. Under these conditions, the percent of probe expected to be hybridized by genomes with 0, 1, 2, and 3 α globin genes is 0%, 20%, 33%, and 43%, respectively, by calculation. After hybridization, the percentage of probe hybridized is calculated from the ratio of radioactivity in double-stranded DNA (hybridized probe) to total radioactivity (unhybridized and hybridized probe) recovered (6). As will be shown in the section *Human Globin Gene Deletions* this method can be useful in identifying α globin gene deletions in α-thalassemia. Solution hybridization analysis of β globin genes is technically more difficult than solution hybridization analysis of α gene number because the homology between $^{G}\gamma$, $^{A}\gamma$, δ, and β genes can lead to significant cross-hybridization.

HUMAN GLOBIN GENE ORGANIZATION

Recombinant DNA techniques, including the cloning of specific human DNA fragments and restriction endonuclease mapping, have been used to determine the structural organization of vari-

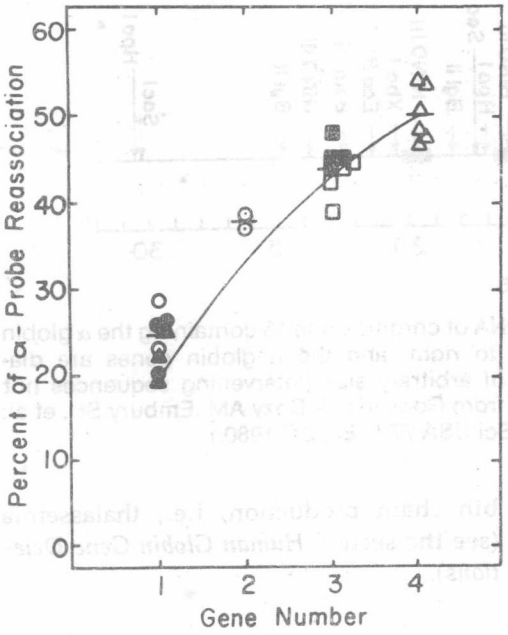

FIG. 3. Percentage of single-stranded α probe annealed by DNA from black individuals with α-thalassemia and healthy control subjects. Horizontal lines represent the observed means, and the solid line represents the percent hybridization theoretically expected with 1 to 4 α genes per diploid genome. Triangle, normal control subjects with 4 α genes; solid and open squares, 2 unrelated black silent carriers (α-thal 2) with 3 α genes (see lane 2, Fig. 6); circle with dot, a black α-thalassemic trait with 2 α genes (α-thal 1); solid circle and triangle, 2 unrelated blacks; and open circle, an Asian with hemoglobin H (Hb H) disease, all having a single α gene (see lanes 3 to 7, Fig. 6). (Reprinted with permission from Phillips JA III, Scott AF, Smith KD, et al: A molecular basis for hemoglobin H disease in American blacks. Blood 54:1439–1445, 1979. Courtesy of Grune & Stratton.)

ous globin genes. Two closely linked α globin genes are normally present on chromosome 16 (10, 11). These α genes are 3.7 kb from center to center (10, 12) (Fig. 4). Loci that contain ζ globin structural genes (embryonic α-like genes) lie 5′ to the α loci (13).

The non-α globin gene complex is found on the short arm of chromosome 11 (14–16). Although the correct chromosome assignment was first made using mouse-human hybrid cell panels and solution hybridization, confirmation of this result and more exact localization of this important gene complex has been achieved by restriction endonuclease analysis (17). In the first such experiment, mouse fibroblasts selected for the presence of a single human translocation chromosome bearing most of chromosome 11 and the distal portion of the long arm of the X chromosome were shown to contain the human β and δ genes. After selection against the human X chromosome, cells were isolated that lacked the human translocation chromosome. These cells also lacked the human β and δ globin genes (8). Further localization to a region of the short arm of chromosome 11 has been performed using cell lines having different deletions of chromosome 11 (14–16).

The arrangement of genes in the $^{G}\gamma$-$^{A}\gamma$-δ-β gene region, as derived by restriction endonuclease analysis, is shown in Figure 5 (18, 19). The centers of $^{G}\gamma$ and $^{A}\gamma$ genes are separated by about 5 kb, while the δ and β genes are about 7 kb apart (18–21). About 14 kb separate the $^{A}\gamma$ and δ genes, and regulatory sequences for both γ and β genes have been hypothesized to reside in this region (18). Genes responsible for ϵ chains, the embryonic β-like chains, have recently been identified 5′ to the $^{G}\gamma$ locus (19).

Data of several investigators have indicated that the translated portions of globin genes are present in 3 pieces and that these 3 pieces are separated in 2 places by intervening sequences (21–24). For β globin, these intervening sequences are located between the nucleotides coding for amino acids 30 and 31 and 104 and 105 of the β chain (24). The first β intervening sequence is about 150 nucleotides long, and the second is about 900 nucleotides

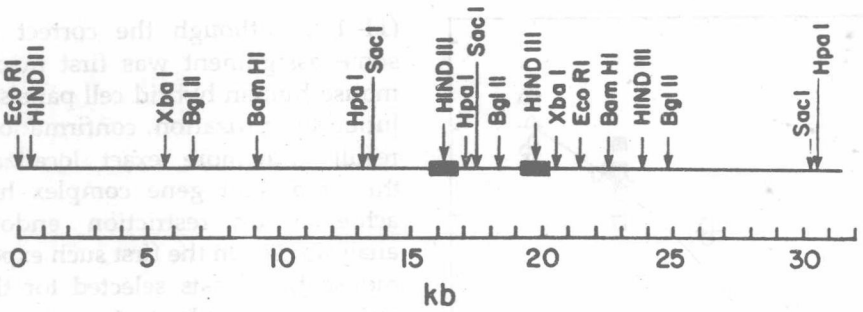

FIG. 4. Map of restriction endonuclease sites in DNA of chromosome 16 containing the α globin genes. The 5′ and 3′ orientation is from left to right, and the α globin genes are diagrammatically represented by solid rectangles of arbitrary size (intervening sequences not shown). Kb, kilobase. (Modified with permission from Goosens M, Dozy AM, Embury SH, et al: Triplicated α-globin loci in man. Proc Natl Acad Sci USA 77:518–521, 1980.)

long. Similar intervening sequences are found in the γ and δ genes (20, 21), but in the human α genes they are both shorter than 150 nucleotides in length (12). These intervening sequences are transcribed into mRNA precursor, but are then precisely spliced out of the precursor in the synthesis of mature globin RNA (25). These processing steps are clinically important because they offer opportunities for errors that could result in reduced globin chain production, i.e., thalassemia (see the section *Human Globin Gene Deletions*).

HUMAN GLOBIN GENE DELETIONS

α Globin Gene Deletions

After *Bam*HI digestion of genomic DNA, the two linked α globin genes are

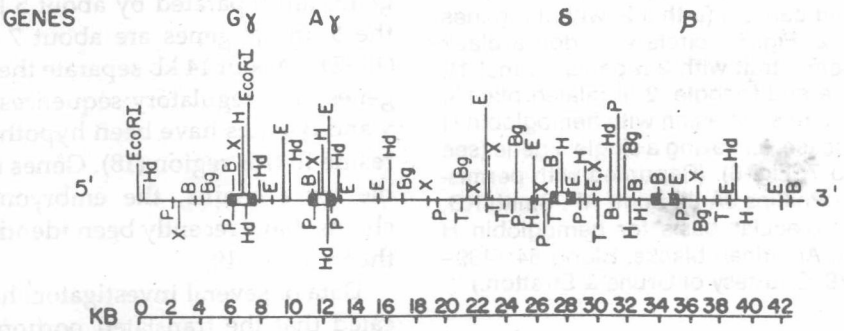

FIG. 5. Map of restriction endonuclease sites in DNA region of chromosome 11 containing the $^{G}\gamma$, $^{A}\gamma$, δ, and β globin genes. The 5′ and 3′ orientation is from left to right, and the various globin genes are diagrammatically represented by rectangles (dark portions indicate coding sequences, light portion indicates one of the intervening sequences). The scale represents size in kilobases (kb). Restriction endonuclease sites shown include: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hind*III (Hd), *Hpa*I (H), *Pst*I (P), *Taq*I (T), and *Xba*I (X). (The genes and the regions of DNA between them are drawn to scale in this figure.) (Data taken from Fritsch EF, Lawn RM, Maniatis T: Characterization of deletions which affect expression of fetal globin genes in man. Nature 279:598–603, 1979, and Ven der Ploeg LHT, Konings A, Oort M, et al: γ-β-thalassemia studies showing that deletion of the γ- and δ-genes influences β-globin gene expression in man. Nature 283:637–642, 1980.)