THE MOLECULAR BIOLOGY OF THE THALASSEMIA SYNDROMES*

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INTRODUCTION

The thalassemias are a group of inherited anemias characterized by a decrease or absence of α or β globin chain production of normal hemoglobin (hemoglobin A, HbA, $\alpha_2\beta_2$).[1-3] The thalassemias differ from the hemoglobinopathies, in that structural changes in the globin chains do not appear to occur; however, a change in the amount of either α or β globin present does exist. These disorders may, therefore, represent models of disordered regulation of the biosynthesis of specific mammalian proteins.

Detailed information available from genetic studies of the human hemoglobins provides a powerful basis for determining the regulation of specific globin chains at the molecular level. These studies demonstrate that the γ, δ, and β globin genes are linked on a single chromosome, in a specific arrangement (Figure 1). Two types of γ globin genes have been identified, while there appears to be only one structural β and δ globin gene per haploid chromosome set. The α globin genes appear to be multiple in most populations and are on a different chromosome than the γ-δ-β complex; the linkage of the α globin genes with each other has not been established. The genes for α and β thalassemia are closely linked to the structural α and β globin genes, respectively.[3]

In fetal life, γ globin gene expression predominates, and hemoglobin F (HbF, $\alpha_2\gamma_2$) is the major hemoglobin synthesized. Late in fetal life, γ globin biosynthesis decreases markedly, and δ and β globin production increases. Production of δ globin chains is always limited, even under the stress of severe anemia and seldom represents more than 5% of the total hemoglobin. The switch from γ to β globin synthesis is thus the major change accompanying human erythroid development. The regulation of this switch is poorly understood at the molecular level and has great potential significance, since reversion to γ globin synthesis to a maximal extent would alleviate the severe anemia present in patients with disorders of human hemoglobin synthesis such as the β thalassemias and sickle cell anemia.

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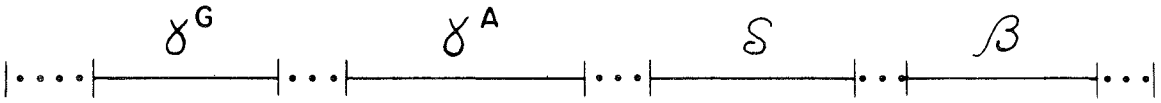

FIGURE 1. Arrangement of human β-like genes on a single chromosome. γ^G represents the gene for γ globin with glycine at position 136; γ^A represents the gene for γ globin with alanine at position 136. The dotted areas represent postulated areas of DNA containing regulatory gene material.

During the 3 years since the thalassemias were last reviewed in this series,[4] progress has been made in more precisely defining specific defects at the RNA and DNA level in patients with the thalassemia syndromes. The use of specific globin complementary DNAs (cDNAs)[5-7] has permitted a further analysis of the heterogeneity of the defects in the β thalassemias at the RNA[8-15] and gene level.[13,16-21] The use of these cDNAs has resulted in the detection of deletions of globin genes in cellular DNA of patients with certain of the thalassemia syndromes and related disorders.[16-19,21,22] This review will summarize briefly the molecular biology of the thalassemias at the globin chain level, and will concentrate on an analysis of more recent work utilizing globin cDNA to characterize globin mRNA content and globin genes in the thalassemia syndromes and related disorders.

TYPES OF THALASSEMIA

There are several types of β thalassemia, the two most common being β^+ and β^0 thalassemia (Table 1). In the heterozygous state of β^+ thalassemia, abnormalities in red cell morphology are present, and there is an increase in the amount of hemoglobin A_2 (Hb A_2, $\alpha_2\delta_2$) and a small and variable increase in HbF. The homozygous state of β^+ thalassemia is accompanied by severe anemia, since there is inadequate compensation by HbF or HbA_2 synthesis for the marked diminution in the amount of β^A and, therefore, HbA. In the homozygous state of β^+ thalassemia, there is always some detectable β globin and HbA, indicating that the structural gene sequences coding for β globin are intact in this disorder. By contrast, there is neither detectable β globin nor HbA in patients homozygous for β^0 thalassemia. Heterozygotes for β^0 have morphologic changes and elevations of HbA_2 similar to those seen in heterozygotes for β^+ thalassemia. The severity of the anemia in β^+ and β^0 thalassemia homozygotes is roughly comparable, suggesting that β globin synthesis in β^+ thalassemia is inadequate.

Two other disorders, δβ thalassemia and hereditary persistence of fetal hemoglobin (HPFH), are related to the β thalassemia syndromes. In homozygous δβ thalassemia, there is no HbA or HbA_2 production in homozygotes. However, in contrast to the β^+ and β^0 thalassemias, there is only a mild anemia present. This is due to a marked increase in HbF as compared to β^+ and β^0 thalassemia. In δβ thalassemia heterozygotes, there is a normal or decreased HbA_2 and a higher level of hemoglobin F than in β^+ or β^0 thalasemia. In homozygotes for HPFH, there is no anemia despite the absence of hemoglobins A and A_2. HPFH represents the extreme situation in which complete

TABLE 1

States of β Thalassemia and Related Disorders

Type	Anemia	HbA	HbA_2	HbF(%)
β^+ Heterozygous	Absent	Normal	Increased	1—3
β^+ Homozygous	Severe	Decreased	Increased	10—90
β^0 Heterozygous	Absent	Normal	Increased	1—3
β^0 Homozygous	Severe	Decreased	Increased	10—98
δβ Heterozygous	Absent	Decreased	Decreased	5—10
δβ Homozygous	Mild	Absent	Absent	100
HPFH Heterozygous	Absent	Decreased	Decreased	10—40
HPFH Homozygous	Absent	Absent	Absent	100

compensation by HbF production for the absence of hemoglobins A and A_2 exists. HPFH heterozygotes are of several types and have little or no red cell abnormalities and marked increases in their levels of HbF. The distribution of HbF in the cells of patients with HPFH is uniform, suggesting that in this condition all cells are capable of continuous, relatively constant production of HbF. By contrast, in β^+, β^0, and $\delta\beta$ thalassemia, the content of HbF varies markedly from cell to cell and suggests that there is either repression or inadequate biosynthesis of HbF in these erythroid populations.

Anemia in the β thalassemias and HPFH is well correlated with the relative excess of α globin chains.[3] Alpha globin does not have a stable molecular configuration and has been shown to aggregate and precipitate in red cells in bone marrow and blood when present and unassociated with γ or β chains and results in the premature destruction of the cells containing them.[23,24] In β^+ and β^0 thalassemia, α globin excess is extreme, as α globin continues to be produced in normal amounts, while inadequate numbers of β and γ globin chains are synthesized. In $\delta\beta$ thalassemia and HPFH, the increased γ globin synthesis leads to a marked decrease in α globin chain excess.

The availability of different types of mutants in the β thalassemia syndromes and HPFH, which are vastly different in their ability to synthesize HbF, makes a comparison of the molecular defects in patients with these syndromes of special interest in attempting to relate the variations in HbF biosynthesis to specific changes at the gene level.

The α thalassemias appear to be caused by defects in one of four α globin loci (Table 2).

TABLE 2

Types of α Thalassemia

Type	Anemia	α Globin genes[a]
Silent carrier state	Absent	3
α Thalassemia trait	Absent	2
HbH Disease	Mild	1
α thalassemia/hydrops fetalis	Severe	0

[a] It is presumed that in most human populations four α globin genes are present, two in each haploid chromosome set.

The mildest form, the silent-carrier state of α thalassemia, is associated with no hematologic abnormalities. A second state, the α thalassemia trait, is associated with red cell abnormalities, but not anemia. By contrast, the apparent loss of three of the four α loci leads to hemoglobin H disease, a disorder with moderate anemia. HbH is an unstable tetramer of β globin chains (β_4) which accumulate due to the lack of adequate numbers of α globin chains. The precipitated HbH causes premature destruction of these cells (primarily by the spleen) and some anemia. In the most severe form of α thalassemia, hydrops fetalis, no α globin is present and only hemoglobin Bart's (γ_4) and HbH are found. This disorder is incompatible with postnatal survival.

GLOBIN BIOSYNTHESIS IN INTACT CELLS

The β Thalassemias

Globin synthesis is measured by incubating either peripheral blood or bone marrow samples with a radioactive amino acid (usually ^{3}H-leucine) and quantitating the amount of radioactivity in α, β, and γ globin chains by column chromatography.[25,26] The peripheral blood of normal human subjects contains 0.5 to 2% reticulocytes. These cells contain polyribosomes and globin mRNA and permit the measurement of globin biosynthesis in blood samples, despite the presence of large amounts of mature red cells which are incapable of globin synthesis. In the thalassemia syndromes, the percent of reticulocytes may be quite low, especially if patients are undergoing intensive transfusion therapy; however, the radioactive incorporation of amino acids into globin is sensitive enough to measure globin biosynthesis in the presence of less than 1% reticulocytes. Bone marrow samples of normal and thalassemia subjects have adequate numbers of nucleated erythroid precursors, which are capable of 10 to 50 times the amount of globin synthesis as reticulocytes. Over 95% of hemoglobin biosynthesis occurs in bone marrow erythroid cells, as compared to reticulocytes. Studies over the past decade indicate that, in most cases, globin biosynthesis by reticulocytes accurately reflects overall erythroid cell activity. The chromatographic method originally described by Clegg et al.,[25] in which globin chains are separated on a column

of carboxymethyl cellulose (CMC) in the presence of 8 *M* urea and mercaptoethanol and are eluted by changing the sodium phosphate concentration, is the standard method of quantitation of individual globin chains. This technique has been proven to be remarkably specific for the separation of individual globin chains which differ by as little as one charge group and selective in separating globins from nonglobin proteins in bone marrow and peripheral blood samples.

From many studies of patients without thalassemia, the ratio of α globin synthesis to that of β globin (α to β ratio) is close to 1.0. In reticulocytes of patients with heterozygous β^0 and β^+ thalassemia of non-black origin, the α to β ratio is close to 2.0, consistent with the decreased or absent function of one β globin allele.[2,26,27] One recent study[28] suggests that the α to β ratios in β^+ and β^0 thalassemia can be distinguished. The α to β ratios in β^0 thalassemia average 2.3, while those in β^+ thalassemia average 2.0. In most other series, the variations in the α to β ratios do not permit a distinction of heterozygotes with these disorders in the heterozygous state. In some heterozygous black patients, α to β ratios of close to 1.0 have been reported.[29,30]

The α to β ratios in homozygotes for β^+ thalassemia vary markedly between 5 and 25.[2,26] It is still unclear whether this variation is due to different levels of β globin production by different β^+ thalassemia genes or is due (at least in some cases) to double heterozygosity for β^+ and β^0 thalassemia. Similar α to β ratios have been found in siblings with homozygous β^+ thalassemia.[31] Only α and γ globin chains are synthesized in the cells of patients with homozygous β^0 and $\delta\beta$ thalassemia and HPFH. Measurements of globin chain biosynthesis in reticulocytes permit the distinction between individuals with homozygous sickle cell disease (possessing two β^s genes) from those with sickle cell β^0 thalassemia (possessing one β^s gene). The α to β^s ratio in homozygous sickle cell disease is 1.0, while in sickle cell β^0 thalassemia, the ratios are closer to 2.0.[26,27]

The relative amounts of α and β globin synthesis in bone marrow erythroblasts largely reflect that of reticulocytes. There is an increase in the α to β globin ratio in total bone marrow erythroblasts of β^+ thalassemia homozygotes. In addition, in one study in which early erythroid precursors were separated from thalassemia bone marrow by selective antibody lysis of late erythroid cells, the results indicated that β globin biosynthesis is decreased at all stages of erythroid cell maturation.[32] In β^0 thalassemia homozygotes, no β globin synthesis is seen in nucleated red cells in bone marrow. There has been some controversy as to the possible contamination of β globin peaks on CMC chromatograms by nonglobin proteins in human bone marrow. However, when total human bone marrow from β^0 thalassemia patients is used to prepare globin and the acid-acetone insoluble material is separated by CMC chromatography, no radioactivity is seen in the β globin region of the chromatogram, thus indicating little, if any, contamination of this region by nonglobin proteins.[33] Although it is possible that measurements of globin biosynthesis in bone marrow specimens may reflect some contamination of these specimens with reticulocytes, the 50- to 100-fold greater biosynthesis of globin per erythroblast compared to that in a reticulocyte makes this unlikely in most studies.

One anomalous finding in β thalassemia is the approximately equal α and β globin synthesis in the bone marrow of patients heterozygous for β^+ or β^0 thalassemia.[34] This finding has been reproduced in other laboratories[33] and is also demonstrable in patients with sickle cell-β^0 thalassemia.[27,35] While the cause of the balanced α and β globin synthesis in intact reticulocytes in heterozygous β thalassemia is still obscure, several studies have indicated that there is a reduced amount of β globin mRNA as compared to α globin mRNA in these erythroid precursor cells. It is possible that post-translational events, such as the proteolysis of α globin chains may occur in thalassemia[36] or feedback inhibition of α globin translation by the excess α globin present in cells, could account for the balanced synthesis.

A vast excess of α globin, as compared to β globin synthesized in homozygous β thalassemia, is only seen when hemolysates of red cells or bone marrow specimens are used.[26] If hemoglobin is isolated from whole hemolysates prior to globin chain chromatography, there is a marked decrease in the relative excess of α globin.[37] This finding initially suggested that free α globin chains were present in other than the tetrameric form.[38]

The production of γ globin chains in the β thalassemia syndromes is quite variable. There is little increase in γ globin chain synthesis in heterozygous β thalassemia. By contrast, there is an increase in γ globin synthesis in homozygous β thalassemia, and the ratio of α to non-α globin synthesis can approach two. However, only in HPFH is the total non-α to α globin ratio close to unity. In homozygous β thalassemia, the cells in peripheral blood synthesize a relatively increased amount of γ globin chains as compared to those in bone marrow erythroblasts.[39] This is most probably due to selection of those cells containing the largest amounts of γ globin and presumably the smallest amount of α globin excess.[40] Heterogeneity of γ globin production in different cells in β thalassemia remains largely unexplained.

The α Thalassemias

In the α thalassemias, a decrease in α globin as compared to β globin production in both reticulocytes and bone marrow cells is characteristic.[41] The α to β ratio in the silent carrier state varies between 0.8 and 0.9; in α thalassemia trait, the α to β ratios are 0.7 to 0.8; in hemoglobin H disease, 0.3 to 0.6;[41] and in hydrops fetalis, no α globin is synthesized.[42] The decrease in α globin synthesis in reticulocytes closely parallels that present in bone marrow precursors in these conditions. Since hemoglobin H, the tetramer of β globin chains (B_4), is a relatively stable structural hemoglobin as compared to free α globin, it is presumed that there is a lesser toxic effect of β globin in bone marrow cells as compared to reticulocytes.

Prenatal Diagnosis

The accuracy and reproducibility of measurements of α, β, and γ globin biosynthesis has provided the biochemical basis for the prenatal diagnosis of homozygous β thalassemia.[43,44] Improved methods for fetal blood sampling have recently become available and have been used to obtain specimens of fetal blood during the 18th to 20th week of gestation.[45] The relative amounts of γ and β globin synthesis at different times during gestation have been quantitated, and nomograms have been obtained in which a comparison can be made between the γ to β ratios of synthesis at various gestational ages between normal fetuses and those under investigation.[46] Using this methodology, accurate diagnosis of homozygous β thalassemia has been made in nine cases to date, and over 75 analyses of pregnancies at risk have been performed.[43,44] Increased experience with methods for obtaining fetal blood samples and for performing biochemical analyses can be expected to lead to widespread use of this procedure in the future.

Although CMC chromatography continues to be the single most accurate method of quantitating globin chains, the procedure is time-consuming and only a limited number of samples can be analyzed at one time. More recently, another method has been described in which α, β, and γ globin chains are separated by cellulose acetate electrophoresis and quantitated by radioautography.[47] In these studies, ^{35}S methionine is used to radioactively label newly synthesized globin chains in reticulocyte-rich samples. The results are comparable to those obtained by CMC chromatography. However, this method is limited in that at least 2% reticulocytes are required to obtain enough radioactivity to quantitate globin chains in the small amounts of blood that are utilized. It will be necessary to concentrate the reticulocytes before this method can be routinely used to establish the diagnosis of β thalassemia trait, since most of these patients have less than 2% reticulocytes. However, it may be of use in the screening of patients with homozygous β thalassemia for the presence of β^+ or β^0 thalassemia and to compare α, β, and γ globin synthesis in different patients with β thalassemia. As little as 50 μl of blood can be used in this procedure and as many as 24 samples can be analyzed in a single experiment.

TRANSLATION OF GLOBIN mRNA

Studies to date indicate that the β globin synthesized in β^+ thalassemia appears to be structurally identical with that of normal β globin.[3] In addition, the time required to translate a β globin chain in β^+ thalassemia is similar to that of normal reticulocytes.[48,49] In these latter studies, short-term labeling of globin chains is used to determine the time required from initiation of globin peptide synthesis to the time of completion and release of globin chains. A defect in the rate of initiation of β globin synthesis would not be detected by this methodology. With this limitation in mind, these initial data

suggested that β globin mRNA in β^+ thalassemia was qualitatively normal within the nucleotide sequence required to code for globin.

Early studies using crude cell-free systems, in which ribosomes and supernatant fractions were isolated from normal and β^+ thalassemia cells, indicated that the ribosomal fraction of β^+ thalassemia cells had a decreased ability to support normal globin biosynthesis.[50] By contrast, when a synthetic mRNA, polyuridylic acid, was added to the thalassemia ribosomes, they appeared to have a normal ability to stimulate the synthesis of polyphenylalanine. These results indicated that the underlying defect in β^+ thalassemia cells was associated with either a decrease in amount or an abnormal β globin mRNA.

The isolation of functional globin mRNA and the demonstration that this mRNA could direct the biosynthesis of globin in heterologous cell-free systems has led to the characterization of globin mRNA function in the thalassemia syndromes. Biologically active globin mRNA was initially isolated by sucrose density-gradient centrifugation as a 9 to 10S RNA.[51] Subsequently, the use of oligodeoxythymidylate (oligodT) chromatography to bind polyadenylate (poly A) containing RNA has led to further purification of human globin mRNA from reticulocytes.[52] Faithful translation of human globin mRNA in cell-free systems was first demonstrated using rabbit reticulocyte fractions[53] and mouse ascites tumor cells.[54] In these studies, it was shown that the defect in β globin synthesis in intact cells could be reproduced by isolated globin mRNA. Subsequently, it has been shown that ribosomal salt-wash factors isolated from rabbit reticulocytes enhance the ability of mouse ascites lysates to translate human globin mRNA;[55] cell-free systems, derived from wheat germ[56,57] and frog oocytes,[58] also permit the accurate translation of human globin mRNA. In all experiments reported to date,[33,53-56] the isolated mRNA from homozygous β^+ thalassemia cells has led to α to β ratios of biosynthesis which are increased and comparable to those obtained in intact cells. Thus, there appears to be little evidence in homozygous β thalassemia for translational control of α vs. β globin synthesis; in addition, the accurate relative translation of human α and β globin mRNAs appears to require no specific factors present in erythroid cells other than intact globin mRNA. These studies strongly suggest that the underlying defect in the β thalassemias is primarily due to abnormalities in the production of β globin mRNA in the nuclei of cells. They do not, however, distinguish between the presence of abnormal β globin mRNAs in the cytoplasm of cells in β thalassemia and a decrease in the amount of β globin mRNA present in these cells.

Isolated bone marrow mRNA from β thalassemia cells also results in the decreased translation of β as compared to α globin comparable to that present in intact bone marrow cells.[33,58] There is reduced translatable β globin mRNA in heterozygotes for β^+ thalassemia as well.[33,59] The mRNA from cells of patients with β^0, $\delta\beta$, and HPFH lead to absent β globin synthesis in cell-free translational systems.[13,22,33,56] An observation of great interest, although still unconfirmed by other laboratories, is that using crude cell-free systems derived from reticulocytes of β^0 thalassemia patients from the Ferrara region of Italy, there is stimulation of β globin synthesis by the addition of normal supernatant fractions to the ribosome fractions of these patients.[60,61] In addition, stimulation of β globin synthesis by transfusion has also been reported in these patients.[61] However, no β globin translation has been demonstrated using the isolated mRNA from these patients to date.[13,56] In α thalassemia, there is decreased α globin synthesis by isolated mRNA demonstrable using cell-free systems.[14,62,63] The decrease in α synthesis relative to β synthesis is much greater using isolated RNA than in intact cells of HbH patients.[62,63]

QUANTITATION OF GLOBIN mRNA CONTENT IN THALASSEMIA CELLS

To distinguish between abnormal and decreased β globin mRNA in β^+ thalassemia, it is necessary to measure the actual amount of β globin mRNA sequences present in these cells as compared to normal cells. This requires the biosynthesis of a specific molecular probe for detection of specific globin mRNA nucleotide sequences. To accomplish this, the enzyme reverse transcriptase, an RNA-directed DNA polymerase from avian myeloblastosis cells is used to synthesize a complementary DNA (cDNA)

using globin mRNA as a template.[5-7] The presence of a long poly A sequence at the 3′ end of human globin mRNA provides a structural feature which allows the controlled biosynthesis of cDNA starting at the 3′ end of the mRNA. OligodT is added to the reaction mixture to serve as a primer for synthesis by binding to the polyA-rich region of globin mRNA. In the presence of the four deoxyribonucleotide triphosphates and reverse transcriptase, a complete or nearly complete copy of globin cDNA is obtained. This in vitro reaction involves the use of relatively purified components. Highly radioactive-labeled deoxyribonucleotide triphosphates can be used in the reaction, and the specific activity of the globin cDNA product is 1.4 to 5 × 10^7 cpm/μg using ^{3}H-deoxyribonucleotide triphosphates. The cDNA can be used to detect the presence of 10 to 100 pg of globin-specific mRNA or globin DNA complementary sequences.

The specificity of globin cDNAs has now been largely demonstrated. In initial studies it could be shown that human globin cDNA did not hybridize with ribosomal RNA, transfer RNA, or viral or bacterial RNA species.[5] More recently, it has been shown that α globin cDNA does not hybridize with β, δ, or γ globin mRNAs or gene DNA, and vice versa.[13,57] Also, β cDNA does not hybridize with α or γ mRNA or DNA sequences, and vice versa.[13,64] The lack of significant cross-hybridization between γ and β nucleotide sequences is significant, since the polypeptide structure of these two globin chains differ by only 38 amino acids.[1] The stringency of the hybridization conditions may be responsible for the low apparent degree of homology detected by these methods.

In the usual reaction mixtures for measuring hybrid formation, a fixed amount of cDNA is added to increasing amounts of RNA containing specific globin mRNA sequences in 0.3 *M* NaCl. Since the melting temperature of cDNA-mRNA hybrids under these conditions is approximately 90°C, a stringent temperature that permits only the hybridization of strongly complementary molecules is used, most commonly 68°C, or 43°C in 50% formamide. The precise degree of homology of nucleotide sequences required to form a stable hybrid under these conditions is unknown.

The usual method of assay for specific globin mRNA content is to add increasing amounts of RNA to a tracer amount of cDNA and allow hybridization to occur. Following hybridization, the reaction mixture is treated with enzymes which specifically degrade single-stranded cDNA, but will not attack double-stranded nucleic acids (cDNA which is hybridized to specific globin mRNA).[5-15] The commonly used enzymes of this type are S_1 nuclease and micrococcal nuclease. In the authors' laboratory, after hybridization is complete, one half of the hybridization mixture is subjected to nuclease digestion and the other half is analyzed without nuclease and the percent of cDNA resistant to nuclease digestion determined.[8,11,13,14] The hybridization reaction appears to be dependent upon two factors: the concentration (C_o) of RNA used and the time (t) of hybridization.

The relative amounts of two globin RNAs for example, α and β mRNA, in a sample of RNA can be determined by using purified α and β cDNAs. The relative amounts of RNA input at which the α and β cDNAs are completely protected from degradation by nuclease are found.[8,11,12] A convenient measure of this reaction is a comparison of $C_ot_{1/2}$ (the C_ot value at which 50% of the cDNA is hybridized) for each cDNA.[65]

Using purified globin mRNA, it has been found that a $C_ot_{1/2}$ of about 10^{-3} mol sec/l is required.[8]

Relatively few studies have been done to carefully examine the time course of hybridization of globin RNA preparations to cDNA. Theoretically, if 2500 cpm of globin cDNA with a specific activity of 1.4 × 10^7 cpm/μg are used, 175 pg of globin mRNA should be detectable in a sample of RNA. This calculation assumes that globin cDNA and mRNA interact in an equimolar ratio. Ramirez et al. have shown that globin cDNA will protect ^{125}I-labeled globin mRNA from nuclease digestion to greater than 95% at ratios of one-to-one of the two reactants.[66] If the hybridization reaction is carried out in a 10 μℓ volume, then the 175 pg of mRNA is at a concentration of 17.5 ng/mℓ and at 5 hr; a C_ot of approximately 10^{-3} is reached, and at 10 hr (C_ot of 2 × 10^{-3} mol sec/l) all of the cDNA is hybridized. Longer times of incubation result in no further hybridization. If less than 175 pg of mRNA are present in the RNA sample, not all of the cDNA will be hybridized

even after prolonged times of hybridization, since the hybridization is limited by the amount of available globin mRNA.

In practice, two different methods have been employed to measure the relative amounts of globin mRNA present in different samples of RNA. In both methods it is assumed that the relative amount of an RNA sample required to protect a tracer amount of globin-specific cDNA from degradation is an accurate measure of the amount of the globin-specific mRNA sequences present in that sample. In one technique, increasing amounts of RNA are added to a tracer amount of globin cDNA and the reaction allowed to proceed for approximately 4 hr.[8] In this method, the assumption is not made that the reaction between globin mRNA and the cDNA is complete. It is rather presumed that, at lower amounts of RNA, there will be insufficient RNA present to hybridize all of the cDNA. Increasing amounts of globin cDNA will become hybridized as the RNA is increased, and the cDNA will become completely hybridized when greater than 175 pg of globin mRNA in 10 μl is added per 2500 cpm of cDNA. The use of relatively short hybridization times in this method prevents any artifact which may be due to RNA degradation.

Another method can be used in which varying amounts of RNA are incubated with a fixed tracer amount of cDNA for extremely long times to insure that all of the cDNA that can be hybridized is, in fact, hybridized.[9,15] Periods of up to 14 days have been used in hybridization experiments.[15] No evidence of linearity in the time course of hybridization over prolonged periods of time has been obtained. The authors have compared the relative amounts of α and β globin mRNAs in samples of RNA from mouse and human cells using different times of hybridization.[67] No differences are detectable in the relative amounts of α and β globin mRNAs by varying the time of hybridization between 4 and 24 hr. The percent of hybridization of a given amount of RNA is linear with time for between 2 and 4 hr.

There has also been some controversy as to the appropriate method of plotting hybridization results. The authors have preferred to plot their data on a semilog scale usually used in measurements of C_0t.[65] It has been suggested that the use of a log scale will obscure one- to ten-fold differences in measurements of the relative amounts of RNAs present in hybridization reactions.[4] However, since the actual values are plotted on both log and linear scales, the $C_0t½$ values are easily measured, and the log scale permits a greater range of RNA values to be presented on a single curve (see Figures 2 and 3). The relative amounts of α and β mRNA in normal reticulocyte mRNA is close to 1.0 (Figure 2).[8,11,13]

A decrease in the content of β globin mRNA sequences in β^+ thalassemia was first demonstrated using α and β globin cDNAs prepared from purified rabbit α and β globin mRNAs.[8,9] The peculiar distribution of isoleucine residues in α and β globin chains of rabbits permits the use of a competitive inhibitor of isoleucyl tRNA, O-methylthreonine (OMT) to separate the polyribosomes containing α globin mRNA from those containing β globin mRNA on the basis of their size.[68] β globin mRNA accumulates on heavy polyribosomes because the isoleucine residues are nearer the carboxyl terminal end of the β globin chain. α globin mRNA is associated with lighter ribosomes in the presence of OMT because isoleucine residues are grouped toward the amino-terminal end of the α globin chain.[69] Hybridization of the α and β cDNAs prepared from each of the separated RNAs to α and β mRNA preparations indicate that each of cDNAs is enriched at least fivefold with respect to the content of RNA of the opposite chain.[8,70] The extent of protection of each of the rabbit cDNAs by an excess of human mRNA is approximately 50 to 60%, indicating the relative extent of homology between rabbit and human globin nucleotide sequences. Using ^{32}P-labeled α cDNA and ^{3}H-labeled β cDNA in a single reaction mixture with normal or thalassemia RNA, it was shown that (1) there are relatively similar amounts of α and β mRNAs present in normal human reticulocyte RNA and (2) there is a significant decrease in the relative amount of β mRNA as compared to α mRNA in five of seven patients with β^+ thalassemia studied.[8] In two other patients, there was no significant difference in the relative hybridization to α and β cDNA from normal, and these results were consistent with the presence of an abnormal untranslated β mRNA in these patients.

More recently, several other methods have been used to prepare purified human α and β globin cDNAs.[11,16,17,57,71] In one procedure, the

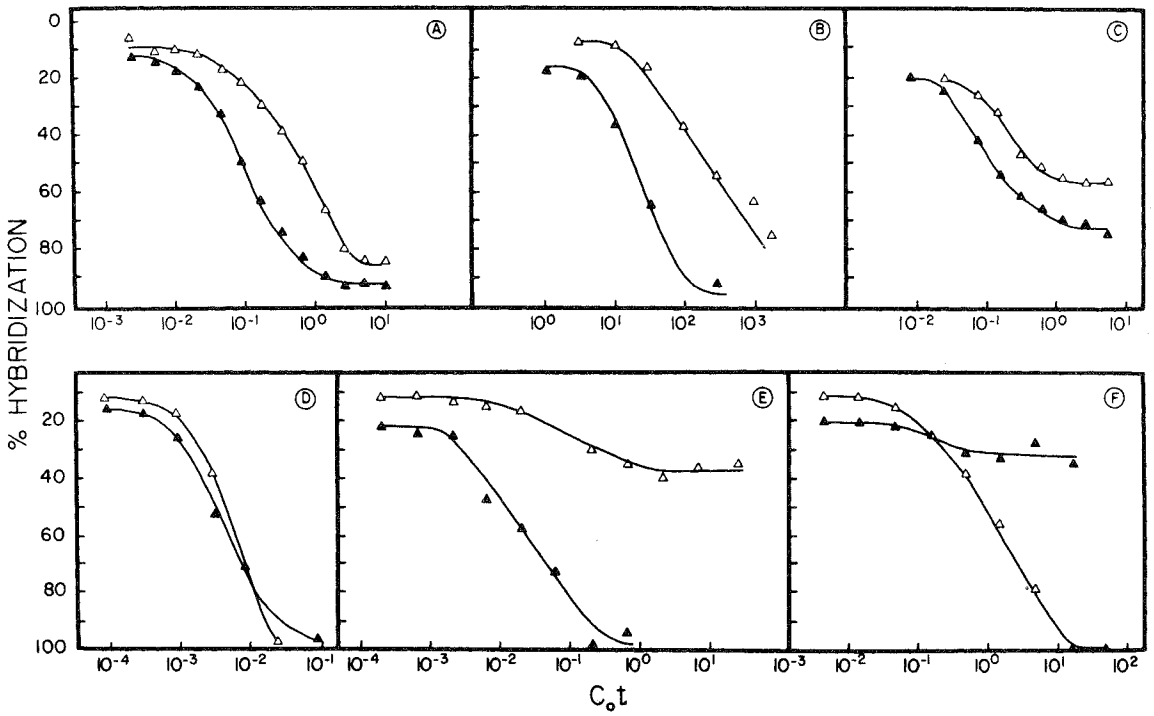

FIGURE 2. Hybridization of human reticulocyte 10S RNA preparations to purified α and β cDNAs. Assays are done as described in Reference 8. Increasing amounts of RNA are hybridized to 2000 to 2400 ^{3}H cpm of α or β cDNA (specific acitivity: 1.4×10^7 cpm/μg) for 4 to 24 hr. After hybridization, half of the sample is treated with micrococcal nuclease and the other half analyzed directly. The percent of hybridization is the nuclease resistant cpm/untreated sample cpm × 100; (A) homozygous β^+ thalassemia mRNA; (B) β^0 thalassemia (Catania pt.); (C) homozygous β^0 thalassemia (Ferrara pt.); (D) normal reticulocyte; (E) homozygous $d\beta$ thalassemia; (F) hydrops fetalis mRNA, ▲,αcDNA; Δ, βcDNA.

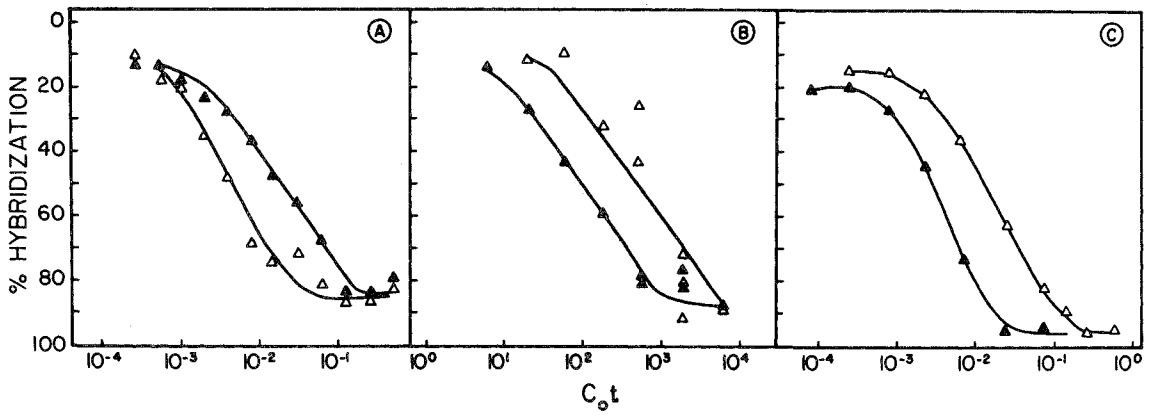

FIGURE 3. Hybridization of human reticulocyte 10S RNA to α and β cDNAs. Conditions of assay are described in Figure 2; (A) HbH mRNA, (B) homozygous β^+ thalassemia RNA; (C) homozygous Lepore mRNA. ▲, αcDNA; Δ, βcDNA.

mRNA from a fetus with hydrops fetalis has been used to isolate α and β cDNAs as follows. Hydrops fetalis RNA contains only β and γ mRNA and no α mRNA. When hydrops fetalis RNA is added to a mixture of α and β cDNAs (prepared from normal reticulocyte RNA), the β cDNA hybridizes to the hydrops fetalis RNA, while the α cDNA remains single-stranded.[11,17] Hydroxylapatite chromatography at 68°C elutes single-stranded nucleic acids at 0.12 *M* phosphate and double-stranded nucleic acids at 0.4 M sodium phosphate and, thus, is used to

separate the α cDNA (single-stranded) from the β cDNA (double-stranded) hybridized to β mRNA. With the α and β cDNAs obtained by this method, the decrease in amount of β globin mRNA in the RNA of β^+ thalassemia patients has been confirmed (Figures 2A and 3B).[11] In these studies, the relative decrease in β mRNA content in β^+ thalassemia is proportional to the decrease in the translation of β mRNA in cell-free systems.

RNAs enriched for α and β sequences have been prepared by other methods. RNA from patients with hemoglobin H disease has been used as a source of β globin mRNA to synthesize β cDNA.[16] In addition, RNA from a patient with $\delta\beta/\beta^0$ thalassemia has been used to synthesize α cDNA.[17] The potential pitfall of using such unfractionated cellular RNAs is that they may contain abnormal untranslated α or β mRNA sequences which may be transcribed into cDNA. Physical separation of α and β mRNAs has been accomplished by using formamide gels.[57,71,72] Initially, α and β globin mRNAs were separated by gels on a relatively small scale, and the RNA eluted from the gels used to synthesize globin-specific cDNAs.[71] More recently, the authors have used a polyacrylamide gel preparative column to separate α and β globin mRNAs.[57] Using this procedure, 50 to 150 μg of poly A-containing RNA can be fractionated into α and β globin mRNA fractions. The fractions eluted from the column are assayed for their globin mRNA content by hybridization to ^{3}H-polyuridylic acid. Appropriate fractions are pooled, passed through an oligodT column, and assayed for their specific α and β globin mRNA content by translation in a wheat germ cell-free system with ^{35}S methionine; the cell-free product is analyzed by cellulose acetate electrophoresis and autoradiography.[57] The recovery of translatable α and β globin mRNAs with this procedure is close to 50%. With the purified α and β cDNAs obtained from mRNA using this method, the decrease in β mRNA content in β^+ thalassemia RNA has been confirmed. The purity of the α and β cDNAs have been analyzed by hybridization to purified α and β mRNAs. Greater than 20-fold differences in $C_0t_{1/2}$ are obtained indicating that by these criteria the cDNAs are greater than 95% pure. In a vast excess of hydrops fetalis RNA (containing no α mRNA), less than 20% of the α cDNA above background is hybridized, while over 90% of the β cDNA is hybridized.[57] When δβ thalassemia RNA (which contains no δ or β mRNA) is hybridized to β cDNA, less than 10% of the β cDNA is hybridized, indicating that the β cDNA is greater than 90% pure. By contrast, only 70% of the cDNA is hybridized, indicating that it is probably contaminated with 20% β cDNA, a conclusion consistent with data from hybridization to hydrops fetalis RNA. Experiments using δβ thalassemia and hydrops fetalis RNA are shown in Figure 2.

The RNA from patients homozygous for δβ thalassemia and HPFH have no RNA sequences complementary to β cDNA.[10,13,22] In β^0 thalassemia, the situation appears to be more complicated, and the results from different laboratories are inconsistent. In some Italian β^0 thalassemia homozygotes, no β mRNA is demonstrable.[10,20] In other β^0 patients of Sicilian and Chinese extraction, structural β globin mRNA sequences appear to be present, as indicated by the complete or nearly complete hybridization of the RNA to β cDNA (Figure 2B).[12,13] In some cases, the β mRNA content by hybridization is similar to that of α mRNA,[12] while in others, the β mRNA content is 2- to ten-fold less than that of α mRNA.[13,73] It is probable that δ mRNA sequences will interact with β cDNA, since the δ and β globin chains differ by only nine amino acids. However, the relatively high amount of β-like mRNA is unlikely to be δ mRNA, since δ globin synthesis represents less than 2% of the total globin synthesis and is particularly low in reticulocytes.[74,75] However, it is possible that δ mRNA sequences exist in cells in a higher concentration than the relative amount of δ globin synthesis or content in cells. In a recent study of one β^0 patient, it has been shown by RNA nucleotide sequence analysis that intact β mRNA sequences are present in these cells.[73] The finding of some intact normal amounts of β mRNA sequences in cells of patients with β^0 thalassemia who have no detectable β globin synthesis suggests the presence of an abnormal untranslated β globin mRNA in these patients. This mRNA may either not bind to ribosomes normally or may be defective in its ability to initiate translation. Since globin mRNA is known to contain at least 50 untranslated nucleotides at its 5′ end, as well as a methylated cap structure, and at least 100 nucleotides

which are untranslated at its 3′ end adjacent to the poly A region, it is possible that these regions may be defective in β^0 thalassemia mRNA. The authors have recently found a significant variation in the relative amount of the β mRNA in different patients with β_o thalassemia from Catania and in the same patient studied at different times.[75] This may be due to an instability in the β mRNA present in these patients. Since the time and conditions of hybridization vary significantly in different laboratories, the different results may be due to relative instability of certain of these β_o mRNAs, as well as the length and purity of the cDNA probes used.

Another unresolved issue is whether intact β mRNA sequences are present in the cells of β^0 thalassemia patients from the Ferrara region of Italy.[13,15,76] Results from the authors' laboratory indicate that there are no intact β mRNA sequences in β^0 Ferrara mRNA, since only 45 to 55% of β cDNA can be protected in a vast excess of β^0 Ferrara mRNA in five different patients (Figure 2C).[13] Mixing of different β^0 Ferrara mRNAs did not increase the hybridization plateau of the β cDNA, indicating that a similar defect is probably present in the RNA of all of these patients. These results suggest that either grossly abnormal β-like mRNA is present in these patients or that β mRNA is completely absent and the hybridization observed due to homology between β cDNA and δ mRNA. The relatively high amounts of β-like mRNA as compared to δ globin in these cells makes this latter possibility unlikely. In a recent study using homozygous Lepore globin mRNA, we have found almost complete homology (greater than 90% hybridization) between Lepore mRNA sequences and those of β cDNA, indicating that at least the δ sequences present in Lepore mRNA are homologous to β cDNA.[75] Whether all δ mRNA sequences are hybridized by β cDNA remains to be determined. In another study of β^0 Ferrara thalassemia patients, the presence of 4 to 10% as many intact β mRNA sequences as α mRNA sequences has been reported on the basis of the complete protection of β cDNA by an excess of RNA.[15] Another study of these same patients does not permit a conclusion as to whether intact β mRNA sequences are present in β^0 Ferrara.[76] In this latter study, there was significant hybridization of β cDNA with the Ferrara RNA, but only 60% of the β cDNA was protected at high RNA inputs. It is important to note that extrapolation of hybridization curves to determine RNA content can be made only if it is known that intact mRNAs are present in the sample under analysis. Since it is possible that in β^0 Ferrara thalassemia cells there are incomplete β mRNA sequences, it is not possible to extrapolate the curve from 60 to 100% without demonstrating the ability of the β^0 Ferrara mRNA to completely hybridize to β cDNA under some conditions. Again, it is possible that some of these conflicting results are due to differences in the conditions of hybridization, instability of β mRNA in β^0 Ferrara, and the quality of the cDNA probes.

It has also been possible to isolate γ globin specific cDNA and quantitate the relative amount of γ globin mRNA in normal and thalassemia cells.[64,77] Gamma globin cDNA has been prepared as follows: cord blood mRNA known to contain γ, β, and α mRNAs has been used to synthesize a mixture of cDNAs. This cDNA is then hybridized to an excess of mRNA obtained from normal reticulocytes which contains α and β mRNA in significant amounts. The γ cDNA remains unhybridized, while the α and β cDNA hybridize to their complements in normal mRNA. The single-stranded γ cDNA is separated from the double-stranded α and β cDNA-mRNA hybrids by hydroxylapatite chromatography. The γ cDNA will not hybridize to normal reticulocyte mRNA except at extremely high C_0t values. By contrast, the γ cDNA hybridizes at low C_0t values to mRNA samples containing large amounts of γ mRNA, such as that from patients with β thalassemia and hydrops fetalis as well as from cord blood.[64] The lack of contamination of the γ cDNA with nonglobin cDNA has been demonstrated by its failure to hybridize with an excess of human fibroblast RNA.[64] The lack of hybridization between γ mRNA and β cDNA under stringent conditions has been shown using RNA from patients with $\delta\beta$ thalassemia.[64] This RNA does not hybridize to β cDNA, although it does hybridize to γ cDNA at low C_0t values.

GLOBIN GENE CONTENT AND GENE DELETION IN THE THALASSEMIA SYNDROMES

The specificity of hybridization of globin cDNAs has been most dramatically demon-

strated by hybridization studies using human cellular DNA. The ability to detect specific α globin gene deletions in the α thalassemias[11,16,17,19] and β-like gene deletions in $\delta\beta$ thalassemia and HPFH[13,18,21,22] have confirmed the specificity of these probes. A single globin gene in human cellular DNA represents approximately 1 part in 10 million. If one or two β globin genes are present in human cellular DNA, this indicates that globin cDNA is capable of specifically detecting a single globin gene in a mixture of between 1 and 10 million non-globin gene sequences.

Several different methods have been used to measure the number of globin genes and detect deletions of globin genes in cellular DNA. All methods to date use cellular DNA that has been sheared either by sonication or by alkaline treatment to a size comparable to that of the cDNA. The cellular DNA and cDNA are mixed in an appropriate salt solution, denatured by heating, and allowed to rehybridize. The specific hybridization of cDNA to its complement in cellular DNA is usually measured by hydroxylapatite chromatography, which will detect both partial and complete hybrids of the cellular DNA and cDNA. Alternatively, a single-stranded nuclease such as S_1 can be used to detect only the amount of double-stranded regions between cellular DNA and cDNA. The methods used to date differ in the relative amounts of cDNA and cellular DNA added.

Vast Excess Cellular DNA Hybridization

In these experiments, a vast excess of cellular DNA is added to cDNA to insure that all of the cDNA sequences which can be hybridized are hybridized. Since unfractionated cellular DNA is used, it is necessary to add a greater than 10^7-fold amount of cellular DNA compared to cDNA to obtain DNA excess for the specific gene sequence being measured. For example, if 500 counts of cDNA are used in a hybridization reaction and represent 35 pg of cDNA, then one equivalent of a single globin gene in cellular DNA would be 35×10^7 or 350 μg of DNA. If a 10- to 100-fold excess of cellular globin gene is to be achieved, then 3.5 to 35 mg of cellular DNA would have to be added. Although using ^{32}P-labeled cDNA, it is possible to diminish the amount of cellular DNA required by a factor of ten because of its higher specific activity; these experiments still require large amounts of cellular DNA in the hybridization reactions. To quantitate the reaction of cellular DNA with cDNA using this methodology, two parameters can be used: the $C_ot_{1/2}$ of the reaction or the hybridization obtained at saturation in a vast excess of DNA. The interpretation of $C_ot_{1/2}$ in reactions between a double-stranded cellular DNA and a single-stranded cDNA probe is complex. The $C_ot_{1/2}$ can be used to determine the reiteration frequency of the gene being measured, as compared to that of the bulk of unique cellular DNA sequences. However, differences in the $C_ot_{1/2}$ of two- or threefold are difficult to quantitate and depend on the relative concentrations of the reactants.

The extent of hybridization of the cDNA at very high C_ot values (saturation hybridization plateau) is more informative and has been used to determine the presence of deletions of α globin genes in hydrops fetalis α thalassemia[16,17] and deletion of β-like globin genes in homozygous HPFH.[22] In studies with hydrops fetalis DNA, the α cDNA probe was hybridized to 25 and 40% in two studies,[16,17] as measured by hydroxylapatite chromatography. Under the same conditions using normal DNA, the α cDNA probe was hybridized to over 60% indicating deletion of part or all of the α globin gene sequences. The β cDNA probe was hybridized to approximately 60% with both normal and hydrops fetalis DNA. It has been unusual to obtain more than 75% hybridization of α and β cDNA using cellular DNA. This may be due to preferential reassociation of the two cellular DNA strands as compared to that between cDNA and its complement in cellular DNA. It is difficult to detect deletion of globin genes in heterozygotes using a vast excess of cellular DNA, since the presence of half of the normal complement of globin genes would permit relatively complete hybridization of the cDNA at saturation (high C_ot values) and can only be expected to change the $C_ot_{1/2}$ by a factor of 2. Use of this method has been reported to detect deletions in HbH disease.[19]

Relative Excess of cDNA

In these experiments, the relative amounts of cDNA and DNA used in hybridization reactions are calculated, such that a competition is established between cDNA and the two strands of cellular DNA.[78-80] Hybridization of the cDNA will be limited by the amount of its com-

plement in cellular DNA. Consequently, it is expected that a predictable decrease in the percent of cDNA hybridized should occur as the amount of cDNA is increased relative to the amount of cellular DNA added. When equal amounts of cDNA and cellular globin genes are mixed, approximately 50% of the cDNA should be hybridized, since the cDNA is competing with an equivalent amount of cellular globin gene for the complementary cellular globin DNA strand. When ten times as much cDNA as cellular globin genes are present, then one part of cellular DNA and ten parts of cDNA are competing for one part of cellular DNA strand and, therefore, one eleventh of the cDNA should be hybridized. Similarly, when three parts of cDNA are added to one part of calculated globin gene in DNA, then one fourth of the cDNA should be hybridized.

In practice, the amount of cDNA used is calculated from the specific activity of the cDNA; the amount of cellular DNA added is measured directly. The assumption is made that a single globin gene represents 1.1×10^{-7} of the total cellular DNA and, therefore, the amount of cellular DNA globin gene copies added can be calculated. If there is more than a single globin gene in cellular DNA then the percent of cDNA hybridized under the calculated conditions should reflect the increase in the number of globin genes in the cellular DNA. For example, if 50% of the cDNA is hybridized at a calculated input of three times as much cDNA as the calculated amount of a single globin gene in the cellular DNA, then there are three copies of globin genes in the cellular DNA. The relative numbers of α, β, and γ globin genes in cellular DNA have been measured using this method.[11,64,80]

In initial experiments, total cDNA was hybridized to varying inputs of human cellular DNA. In all these experiments, saturation hybridization values are measured and confirmed by determining two values at high C_0t (> 1000) at which there is no further increase in the hybridization of the cDNA.[11,13,78] Fifty percent hybridization was achieved at approximately three times the amount of cDNA added per amount of calculated globin genes in cellular DNA, indicating that there were approximately three globin genes in cellular DNA. It is assumed in these studies that the relative rates of hybridization of the two cellular DNA complementary strands of cellular DNA and that of cDNA with its complementary strand in cellular DNA are similar. The results indicated that as the relative amount of cDNA was increased, the calculated gene copy number in cellular DNA increased as well.[80] This might have several explanations: relative rates of hybridization of the two cellular DNA strands as compared to that between cellular DNA and cDNA could differ significantly, the size of the cellular DNA and cDNA might not be comparable, and the calculated inputs of cellular DNA and cDNA might not be accurate. In addition, the calculation of globin gene copy is made assuming that 100% of the cDNA can be hybridized to cellular DNA. If, as determined experimentally, only 75% of the cDNA is hybridized at vast excesses of DNA, then the calculated globin gene copy numbers with different inputs of cellular DNA are more comparable and are between three and ten copies. In an analysis in which no assumptions are made as to the relative rates of hybridization of the two cellular DNA strands, as compared to that between cellular DNA and cDNA, it was calculated that there are less than 20 globin genes per cellular human genome.[80]

In subsequent studies in which purified α and β cDNAs were used with varying inputs of cellular DNA, it was determined that normal human spleens contain between one and five α globin genes and between two and five β globin genes per haploid genome.[11] The variation in gene numbers with different inputs of cellular DNA was explored because the accuracy at any particular cDNA to cellular DNA input could not be determined. In studying the numbers of globin genes in thalassemia DNA, a range of inputs of cDNA and cellular DNA was used and the relative extents of hybridization compared.[11,13,78] It was found that β^+ thalassemia DNA hybridizes to total cDNA[80] and purified β cDNA[11,13] to an extent similar to normal DNA, indicating that there is no detectable deletion of β globin genes in β^+ thalassemia DNA. In more recent experiments with other cellular DNAs obtained from blood buffy coats, spleen, and lymphocyte cell lines, it has also been determined that β^+ and β^0 thalassemia DNA hybridize with β cDNA to an extent similar to normal cellular DNA, again suggesting no detectable deletion of β globin genes in β^+

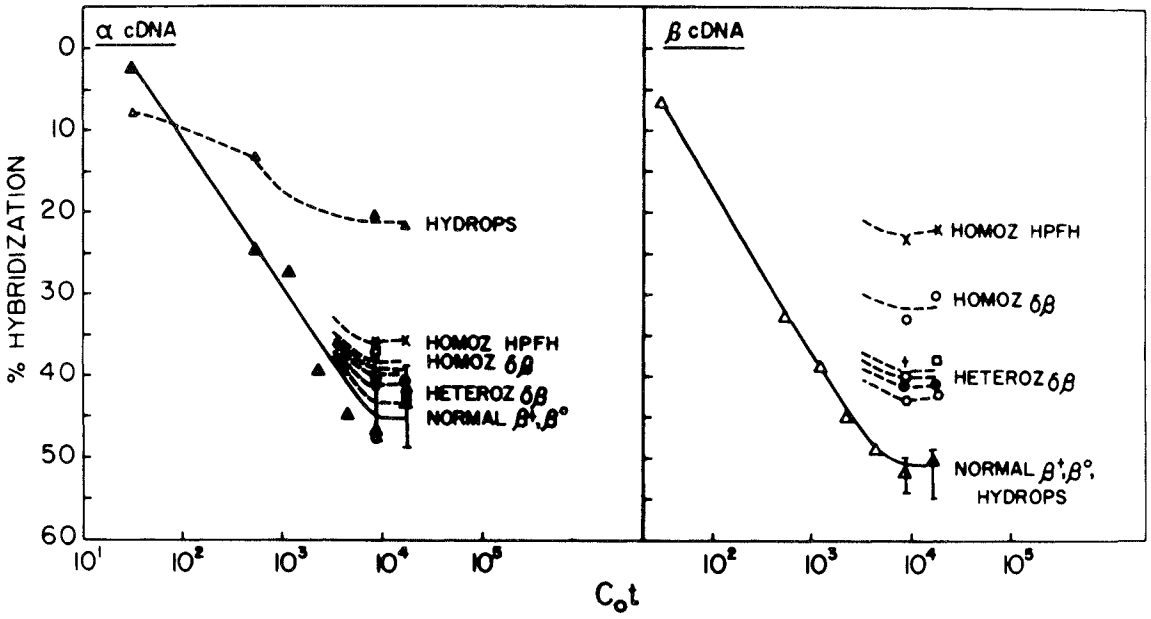

FIGURE 4. Hybridization of human cellular DNA samples to purified α and β cDNAs. For each point, 150 μg of cellular DNA was mixed with 700 cpm of α or β cDNA (1.4×10^7 cpm/μg) at a DNA concentration of 3 mg/ml. The time of hybridization was varied to obtain the desired C_0t values. Hybridization was measured as described[80] using hydroxylapatite to separate hybridized and unhybridized cDNA. β^+ refers to homozygous β^+ thalassemia DNA; β^0 to homozygous β^0 thalassemia DNA; homozygous (homoz) HPFH DNA is from a fibroblast cell line;[13] homoz δβ DNA is from white blood cell DNA and has been repeated with lymphocyte cell line DNA; heterozygous (hetero) δβ thalassemia DNA is from white blood cell DNA; Hydrops fetalis (hydrops) DNA is from liver; and normal DNA is from white blood cell, spleen, and lymphocyte cell liver DNAs.

thalassemia (Figure 4).[81] Normal numbers of β-like genes are also present in β^0 thalassemia DNA using these techniques.[13] Similar results have been reported by others in β^0 thalassemias.[20,82]

By contrast, when homozygous δβ thalassemia DNA is hybridized to β cDNA, only 31 to 33% of the β cDNA is hybridized, while 50 to 55% of the β cDNA is hybridized with similar inputs of normal cellular DNA[13] (Figure 4). In addition, DNA from a patient homozygous for hereditary persistence of fetal hemoglobin HPFH hybridized only 22 to 23% of β cDNA (Figure 4). The relative hybridization of all δβ, HPFH, and normal DNAs to α cDNA were comparable.[13] In these experiments, the amount of α and β cDNA added was approximately three times the amount of calculated single globin gene cellular DNA added. The decreased hybridization of β cDNA to HPFH and δβ thalassemia DNA is consistent with deletion of some or all of the β-like genes in these conditions. The results obtained by this methodology are in agreement with similar data reported from other laboratories on deletion of β-like genes in these disorders.[18,21,22]

The extent of deletion of β-like globin genes in δβ thalassemia appears to be less than that in HPFH, only 22 to 23% of the β cDNA with HPFH DNA and 31 to 33% with δβ thalassemia DNA. In addition, when equal amounts of homozygous δβ thalassemia and HPFH DNA are mixed, only 37% of the β cDNA is hybridized, while 67% of the β cDNA is hybridized when a similar amount of normal DNA is used.[13] These results indicate that the deletions in δβ thalassemia and HPFH are overlapping and support the presence of a greater deletion of β-like genes in HPFH than in δβ thalassemia. It has been suggested that there is a region between the γ and δ regions which may be important in regulating the relative amount of γ globin gene activity (Figure 1).[13,83] It is assumed that δ structural gene DNA will interact with β cDNA. These results suggest that the lesser hybridization of HPFH DNA with β cDNA is due to a greater deletion of DNA sequences in this region adjacent to the δ structural genes than

in δβ thalassemia. The greater hybridization of β cDNA with δβ thalassemia DNA may indicate the persistence of a regulatory gene region including at least part of the δ structural gene. The persistence of this region may be responsible for the relatively decreased γ globin compensation in δβ thalassemia as compared to that in HPFH.[13] The persistence of the nucleotide sequences between the δ and β globin structural genes (Figure 1) may further suppress the production of γ globin expression postnatally in normal individuals and those with β^+ and β^0 thalassemia.

Using the methods described above, it has also been possible to detect gene deletions in heterozygotes for δβ thalassemia and HPFH in cellular DNA from white blood cells, spleen, and lymphocyte cell lines (Figure 4).[13,81] In the experiments reported to date, 40 to 42% of β cDNA is hybridized to δβ thalassemia heterozygotes DNA, as compared to 50 to 55% using normal DNA. DNA from HPFH heterozygotes hybridizes to between 35 and 37%. These techniques have also been used to confirm the presence of α globin gene deletions in hydrops fetalis DNA.[11] The authors were able to show that while α cDNA hybridizes to 45 to 50% with normal cellular DNA, only 20 to 25% of the α cDNA hybridizes with cellular DNA from livers of patients with hydrops fetalis-α thalassemia (Figure 4). More recently, this technique has been used to detect globin gene deletions in amniocentesis fluid of pregnancies at risk for hydrops fetalis and have been shown to easily distinguish between hydrops fetalis, HbH, and α thalassemia trait.[84]

In the authors' initial experiments, the cDNAs used were heterogeneous in size, although a significant percent of the DNA was approximately 500 nucleotides in length.[11,13,78] The authors have recently re-examined the hybridization of cellular DNA from normal and thalassemia patients to full-length or nearly full-length α and β cDNAs.[85] By incorporating 4 m*M* sodium pyrophosphate in reaction mixtures,[86] larger size and more homogeneous cDNA can be synthesized. The remaining smaller cDNA is eliminated by subsequent alkaline sucrose gradient centrifugation (Figure 5). By analysis on acrylamide gels, the α and β cDNAs obtained are homogeneous in size and about 700 nucleotides in length. Rabbit globin

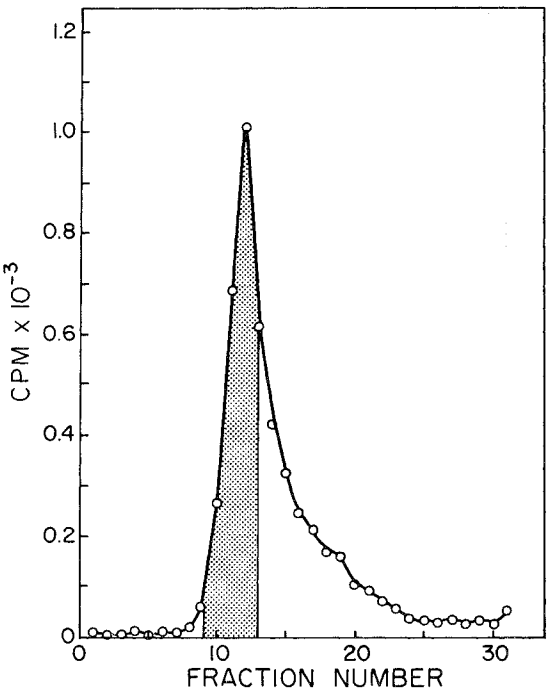

FIGURE 5. Alkaline sucrose density centrifugation of cDNA prepared in the presence of sodium pyrophosphate. The fractions in the shaded area were pooled and subsequently analyzed on a polyacrylamide gel and shown to contain approximately 700 nucleotides.

cDNA prepared in a similar fashion is capable of protecting over 95% of I^{125}-labeled rabbit globin mRNA from S_1 nuclease digestion at an equimolar ratio of cDNA to globin mRNA.

All of the experiments reported to date have utilized hydroxylapatite chromatography for evaluation of the exent of hybridization of globin cDNA to cellular DNA. With hydroxylapatite, the extent of globin gene deletion is difficult to ascertain, since partial hybrids as well as true hybrids between cDNA and cellular DNA are measured. Reports to date[11,16,17] indicate that 25 to 40% of the α cDNA is hybridized to hydrops fetalis DNA in both a vast excess of cellular DNA as well as in relative excess of cDNA. Thus, it is still unclear whether all or only a part of the α globin gene structural sequences are deleted in this disorder. To further explore this question, the authors have recently analyzed the hybridization of hydrops fetalis DNA with α cDNA by S_1 nuclease.[87] In these experiments, increasing amounts of cellular DNA have been added to a fixed amount of α and β cDNA and the percent of hybrid measured with S_1 nuclease. Using normal and hy-

drops DNA, approximately 40 to 45% of the β cDNA is hybridized at inputs of 3- to tenfold as much globin gene cellular DNA as compared to cDNA. There is a gradual increase in hybridization as increasing amounts of cellular DNA are added. Maximal hybridization (40 to 45%) is obtained at approximately a one-to-one ratio of cellular DNA gene to cDNA. Further excesses of cellular DNA do not lead to increased hybridization of the cDNA. This may be due to the preferential association of the two cellular DNA strands as compared to the competing hybridization between cDNA and its complementary strand in cellular DNA. Approximately 40% of the α cDNA is also protected from S_1 digestion in an excess of normal cellular DNA. When hydrops fetalis DNA is hybridized to α cDNA, only 5 to 8% of the α cDNA is hybridized, even in a vast excess of hydrops fetalis DNA. In addition, there is no gradual increase in the percent of hybridization using hydrops fetalis DNA as the amount of cellular DNA is increased. This result indicates that the hybridization between α cDNA and hydrops fetalis DNA probably represents background hybridization of either β cDNA contaminating the α cDNA or other nonspecific hybridization between the cDNA and hydrops fetalis DNA. The background on mixing cellular DNA and cDNA without incubation or mixing α cDNA with Escherichia coli DNA is approximately 2 to 3%. These results suggest that the deletion of α globin genes in hydrops fetalis-α thalassemia is complete or nearly complete. Similar results have been obtained using both α cDNA of heterogeneous size and full-length or nearly full-length α cDNA.[87]

The γ globin gene content of normal and thalassemia DNA has also been evaluated using γ cDNA determined as described previously.[64] In these experiments in relative cDNA excess, between two and four γ globin genes per haploid genome have been measured. Similar numbers of γ globin genes have been found in both β^+ thalassemia DNA and normal cellular DNA, indicating that the diminished amount of γ globin synthesis in β^+ thalassemia cannot be due to deletion of γ globin genes in this disorder. These and other measurements of γ globin gene content in erythroid and nonerythroid tissues[88] indicate that γ globin genes are not excised from human genomes during erythroid development.

Excess cDNA Hybridization

It has been reported that use of a vast excess of cDNA results in more accurate quantitation of globin genes and more accurate detection of globin gene deletions than can be obtained with other techniques.[89] In these experiments, increasing amounts of cDNA are added to a fixed amount of cellular DNA, and saturation levels were measured at high C_0t values. The potential advantage of vast excess cDNA hybridization is that the excess of cDNA will avoid the competition of one strand of cellular DNA with the other, since the cDNA is present in such large excess. A plateau value of hybridization is expected when all of the globin gene sequences in cellular DNA have been hybridized to the cDNA. However, the use of large amounts of cDNA as compared to cellular DNA has significant potential disadvantages. Background hybridization of the cDNA increases as the cDNA input is increased, and contamination of one cDNA with another (e.g., α with β) increases the hybridization as more cDNA is added, and no hybridization plateau is obtained. In most experiments performed to date, no plateau value has been obtained.[21,82,89,90] It has also been stated that excess cDNA hybridizations give more precise measurements of the number of globin genes in human DNA, since only a single saturation value is obtained.[89] However, this occurs only because a single value is designated as the presumed saturation value despite the fact that no single plateau value is obtained. Since only one value is used to calculate the globin gene number, the variability in these experiments is not adequately considered.

In addition, excess cDNA hybridizations have been shown to be relatively insensitive to detection of deletions of globin genes in heterozygotes, as compared to studies using a relative excess of cDNA in which competition of cDNA and cellular DNA occurs. In the case of heterozygotes for δβ thalassemia, no difference from normal in the percentage of cDNA hybridized was obtained in one study using excess cDNA.[90] By contrast, a significant decrease in the percent of hybridization of β cDNA from that of normal has been obtained in several δβ thalassemia heterozygotes using the relative excess of cDNA (Figure 4).[13,81]

From experiments to date, it can be seen that the relative purity of α, β, and γ cDNAs are suf-

ficient to detect deletions of α, β, or γ globin genes in cellular DNA in homozygotes for HPFH, $\delta\beta$, and α thalassemia, and in heterozygotes with these disorders. However, the variations in globin gene number obtained in these studies is significant and it is difficult to utilize this technology at present to determine accurately the absolute numbers of α, β, and γ globin genes. In addition, the cross-hybridization of δ globin genes to β cDNA and the possible presence of other partial or complete globin gene sequences which have significant homology with α, β, and γ cDNA make these measurements imprecise. These considerations also pose problems in pursuing the possibility of partial deletions of the β globin genes in β^+ or β^0 thalassemia, since the sensitivity using currently available cDNA probes may be insufficient to detect small changes. The availability of specific cDNA probes that are purer and more homogeneous in size may increase the sensitivity of these measurements of gene deletion and gene number.

Restriction Enzyme Analysis of Cellular DNA

The discovery of a class of enzymes called restriction endonucleases, which specifically cleave DNA at designated nucleotide sequences between three and six bases long, has led to further analysis of globin genes in cellular DNA fragments.[91,92] Cellular DNA can be cleaved into an array of specific fragments of different sizes and sequences with these enzymes. The specificity of cleavage sites of the restriction enzymes result in identical fragments being obtained from all the cellular DNA molecules. The restricted DNA fragments can then be separted by agarose gels and transferred without loss of resolution between fragments from these gels to nitrocellulose filters (blotting).[93] The nitrocellulose filters are then hybridized to specific highly radioactive cDNAs or mRNAs to localize fragments of cellular DNA containing the specific genes complementary to the specific probes used.[93-96] Using this method, the number of restricted rabbit cellular DNA fragments complementary to rabbit β globin cDNA has been determined.[96] The change in the size of cellular DNA fragments containing the globin genes following subsequent digestion of the DNA with other restriction enzymes can be used to determine the organization of cellular gene fragments with relation to each other and to sequences adjacent to the structural globin genes.[96] Using this technology, it may be possible to determine if there are differences between normal β globin structural genes and those of patients with β^+ and β^0 thalassemia. The authors have already used this technique to determine the extent of deletion and the structural nucleotide changes in HPFH and $\delta\beta$ thalassemia and in the α thalassemias.[97] DNA is prepared from either spleens or lymphocyte cell lines. Sixty μg samples of unfractionated human cellular DNA restricted with the enzyme EcoRI are run on agarose gels in alkali. After electrophoresis, the cellular DNA fragments are transferred to nitrocellulose filters and hybridized to ^{32}P-labeled human cDNA. Five to six discrete bands of radioactivity are detectable by subsequent radioautography corresponding to between three and five kilobase cellular globin DNA fragments.[97] Between 1 and 10 pg of human globin gene can be detected with this technique. The results indicate the sensitivity of this system in analyzing human cellular DNA fragments despite the complexity of the human genome and the presence of about 10^6 EcoRI fragments.

The authors have found changes in the size and number of bands between normal, HPFH, and $\delta\beta$ thalassemia DNA consistent with deletion of β-like genes in the latter two conditions and with a greater deletion in HPFH than in $\delta\beta$ thalassemia.[97] Subsequent restriction enzyme analysis of these cellular DNAs with other restriction endonucleases and with purified α,β, and γ cDNA probes should provide a detailed map of the changes in DNA in $\delta\beta$ thalassemia and HPFH, as compared to normal DNA.

Fractionation of the cellular DNA by C_0t analysis prior to agarose gel electrophoresis and blotting should provide information about the reiteration frequency of nucleotide sequences adjacent to the human globin structural genes similar to that obtained for the rabbit β globin gene.[96] It may be possible using this methodology to determine if there are differences in the DNA sequence either within or adjacent to the β structural gene between normal cells and those of patients with β^+ and β^0 thalassemia. These studies could lead to new techniques for prenatal diagnosis. Amniocentesis fluid containing fetal cells could be grown in tissue culture, and if reproducible differences in the restriction enzyme pattern of β globin genes are found in β thalassemia DNA as compared to normal DNA, this information could be used

to establish the prenatal diagnosis of homozygous β thalassemia using amniocentesis fluid cells instead of using the techniques of fetal blood sampling presently required.

SUMMARY OF GENE DEFECTS IN THE THALASSEMIAS

In δβ thalassemia and HPFH, deletions of β globin genes as well as variable amounts of δ structural genes appear to be responsible for the absence of β and δ globin mRNAs and globin. (See Table 3 for a summary of molecular defects in β thalassemia and related disorders.) In β^+ and β^0 thalassemia, no detectable deletion of β globin structural genes has been observed to date. In some cases of β^0 thalassemia, there are detectable β globin mRNA sequences, indicating that abnormal untranslatable β globin mRNA sequences may be present. The gene defect in these cases may be a nucleotide change in the DNA leading to a β mRNA incapable of normal binding to ribosomes or normal initiation. Globin mRNA has a sequence of approximately 50 nucleotides at its 5′ untranslated end and a methylated cap structure. In addition, there are approximately 100 untranslated nucleotides at the 3′ prime end of the β globin mRNA prior to the 100 to 150 poly A residues. It is possible that defects in either of these untranslated sequences could lead to abnormal mRNAs incapable of translation in the cytoplasm of cells.

In β^+ thalassemia, the amount of β globin mRNA sequences as measured by hybridization to cDNA is roughly proportional to the amount of β globin relative to α globin synthesized in both intact cells and by isolated mRNA translated in cell-free systems. The two possible defects in β^+ thalassemia at the gene level are decreased transcription of a normal β globin structural gene (defined here as the sequences in DNA transcribed into β globin mRNA and its precursors in the nucleus) and decreased stability of β globin mRNA or its precursors due to nucleotide changes in the β globin structural gene. If decreased transcription is present, the genetic defect in β^+ thalassemia most likely involves an abnormality in a regulatory gene sequence modulating β globin mRNA production by the β globin gene. By contrast, if decreased stability of β globin mRNA or its precursors exists, no such defect in regulatory gene activity need be postulated.

TABLE 3

Molecular Defects in β Thalassemia and Related Disorders

Type	β Globin synthesis	β Globin mRNA	β Globin genes
β^+	Decreased	Decreased	Present
β^0	Absent	Absent or abnormal	Present
δβ	Absent	Absent	Deleted
HPFH	Absent	Absent	More deleted

Recent findings by Leder[98] and Flavell[99] indicate that both mouse and rabbit β globin cellular genes have a 600 to 1000 nucleotide sequence inserted within the structural gene between the 5′ and 3′ nucleotide sequences represented in mature β globin mRNA. The authors have recently found a similar intragenic intervening sequence approximately 1 kb length within the δ, β, and Lepore structural genes. This inserted nucleotide sequence may be transcribed into globin mRNA precursors in the nucleus which are larger in size than cytoplasmic globin mRNA. If such normal β globin mRNA precursors are required for proper nuclear processing (nuclear stability) of β globin mRNA sequences, then a nucleotide defect in this inserted midstructural gene sequence in β^+ thalassemia nuclear RNA could result in decreased stability of β globin mRNA precursors in this disorder and lead to decreased β globin mRNA in the cytoplasm and decreased β globin. If, as postulated, this abnormal inserted midgene sequence is subsequently excised during the formation of mature globin mRNA, the presence of structurally normal β globin mRNA and β globin in β^+ thalassemia would be explained. A recent report is consistent with decreased stability of β globin mRNA in β^+ thalassemia.[100]

HEMOGLOBINOPATHIES RESULTING IN THALASSEMIA-LIKE DISORDERS

Several abnormal hemoglobins have been described in which there is decreased biosynthesis of the affected mutant globin chain similar to

that of α or β globin in the thalassemias. Two of these hemoglobins are associated with the clinical picture of thalassemia: the Lepore hemoglobins and hemoglobin Constant Spring.

The Lepore Hemoglobins

Several abnormal homoglobins known as the Lepore hemoglobins have been described in which the abnormal globin chain contains a δ-like sequence at its amino terminal end and a β-like globin sequence at its carboxyl terminal end.[3] These abnormal globin gene fusion products are thought to result from unequal crossing over between the δ and β globin structural genes during meiosis. The relative representation of δ-like sequence and β-like sequence varies in different types of Lepore hemoglobin and either the δ- or β-like component is the major structural feature in different Lepore hemoglobins. The amount of hemoglobin Lepore in heterozygotes for this disorder varies between 5 and 15% of the total hemoglobin. In homozygous hemoglobin Lepore, there is a marked excess of α globin over non-α globin synthesis similar to that seen in homozygous thalassemia,[66] and the clinical severity of the two disorders is comparable. Since Lepore globin has the same amino terminal amino acid sequence as δ globin, it has been postulated that Lepore globin synthesis is under the same genetic control as δ globin and is therefore synthesized in reduced amounts.[74] The reduction in δ globin synthesis could be ascribed to either decreased δ globin mRNA production or decreased stability of δ globin mRNA precursors, either in the nucleus or as mature δ globin mRNA. Low or undetectable δ globin synthesis is found in reticulocytes as compared to bone marrow cells and favors the concept of decreased δ globin mRNA stability.[74,75] In recent studies, the authors have found that there is a reduced amount of Lepore globin mRNA in reticulocytes of a pateint homozygous for hemoglobin Lepore by hybridization to α and β globin cDNA (Figure 3C).[66] The decrease in Lepore globin mRNA content by this method is proportional to the decrease in Lepore globin synthesis as compared to α globin synthesis. Hemoglobin Miyada is an abnormal globin chain which is β-like at its amino terminal end and δ-like at its carboxyl terminal end, and is believed to be the anti-Lepore product resulting from an unequal crossover between the δ and β globin genes. It has been found that hemoglobin Miyada is synthesized and present in decreased amounts in heterozygotes for this disorder.[101] This finding suggests that the δ-like end of this abnormal globin chain has also influenced either the transcription or processing of Miyada mRNA. However, Miyada globin mRNA biosynthesis should be under the control of β globin regulatory genes adjacent to it; therefore, the decreased synthesis of this globin chain does not fit with the concept of δ-like regulatory sequences being responsible for decreased Lepore and Miyada globin biosynthesis, although it is possible that both the 5′ and 3′ end of δ-m RNA or its precursors leads to decreased stability of δ mRNA.

The recent finding of sequences inserted within the β globin genes of humans as well as of rabbits[98,99] suggests another explanation for the reduced content of δ, Lepore, and Miyada globins by a single mechanism. It is possible that the δ globin structural gene has an altered inserted sequence as compared to that present in the β globin gene, which results in decreased stability of δ globin mRNA and its precursors. The Lepore and Miyada genes may also contain part or all of this abnormal δ inserted sequence as part of the fusion gene product (Figure 6). The presence of this altered inserted gene sequence could result in unstable globin mRNA precursors for δ, Lepore, and Miyada globins and could explain the decreased production of these globins by a common mechanism. Differences in the relative amount of δ, Miyada,and Lepore globins present in heterozygotes could be explained by a variability in the relative amount of δ vs. β inserted sequences present in each of the mRNA precursors for these chains.

Hemoglobin Constant Spring

Hemoglobin Constant Spring (HbCS, $\alpha_2^{cs}\beta_2$) is an elongated α globin chain containing 31 extra amino acids at its carboxyl terminal end. This abnormal α globin appears to result from a single base change in the normal termination for α globin.[102] Hemoglobin Constant Spring represents between 1 and 2% of the total hemoglobin present in cells and acts like an α thalassemia gene. Studies of globin chain synthesis in patients with hemoglobin CS indicate that α CS production is reduced proportionally to its content in intact cells.[103] There also appears to be no decreased rate of translation of α CS.[103] Al-

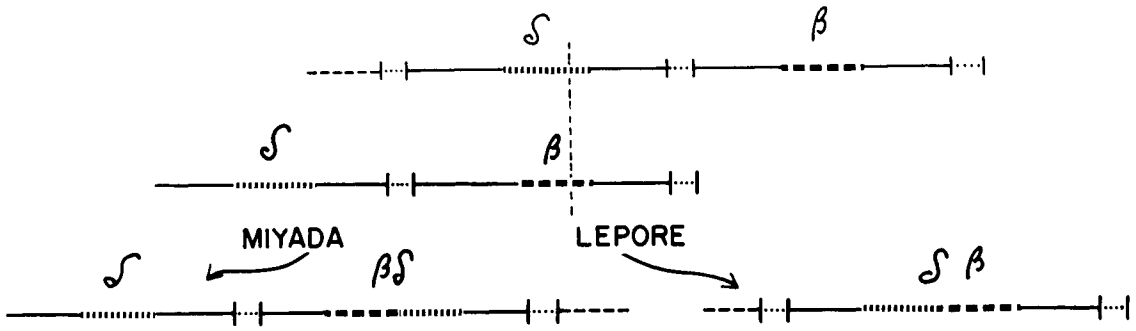

FIGURE 6. Formation of Lepore and anti-Lepore (Miyada) globin genes. The (▪▪▪▪▪▪▪) region of the δ sequence represents a postulated mid-gene nucleotide sequence; the (▬ ▪) region of the β sequence, the mid-gene sequence in the β structural gene. The crossovers leading to the formation of both Miyada and Lepore genes may include part or all of the δ mid-gene untranslated inserted sequence. This latter sequence may lead to instability of δ, Lepore, and Miyada nuclear RNA sequences.

pha Constant Spring may function as an α thalassemia gene either because of decreased transcription of α CS globin mRNA or abnormal processing of α CS mRNA precursors in the nucleus by virtue of an altered nucleotide sequence present in this elongated mRNA and its precursors.

HETEROCELLULAR HEMOGLOBIN F

Evidence has recently been presented which indicates there may be a genetic basis for increasing the percent of HbF producing cells in human erythroid populations.[104-106] The gene involved, termed the gene for heterocellular HPFH, has been found to be linked to either the β thalassemia or sickle cell gene in several cases. Detection of the presence of the heterocellular HPFH gene relies on immunofluorescence measurements of hemoglobin F in cells using an anti-HbF antibody. Individuals with this gene who are heterozygous for β thalassemia or sickle cell hemoglobin show no significant increase in the content of HbF-containing cells; however, homozygotes with sickle cell anemia, β thalassemia, or sickle β-thalassemia, who have also inherited the heterocellular HPFH gene, appear to be capable of markedly increasing their level of production of HbF.[104,105] It has recently been demonstrated in man that three β-like globins (β^s, β^c, and γ) can all be activated in a clone of cells derived from a single cell. These results suggest that the control of γ globin biosynthesis in early erythroid precursor cells may be regulated by genes that are normally inactivated at certain stages of differentiation but may be reactivated under certain conditions. The potential ability to increase the number of erythroid precursor cells that can produce HbF raises the possibility of alleviating the deficit in β chains in β thalassemia and decreasing the relative amount of HbS in sickle cell disease. The genetic mechanisms and control of the heterocellular HPFH genes remains to be determined.

FUTURE RESEARCH ON THE MOLECULAR BIOLOGY OF THALASSEMIAS

Several recent advances in molecular biology can potentially revolutionize research on the molecular basis of the thalassemia syndromes. Over the past 2 years, several groups have prepared full-length or nearly full-length rabbit, mouse, and human cDNAs and have then synthesized the complementary strand.[108-112] These have been inserted into either bacterial plasmids or phages. The insertion of even mixed cDNAs into bacterial plasmids or phages has resulted in essentially complete purification of the cDNAs, since only a single cDNA species is inserted into a single plasmid or phage molecule. This technology has permitted two advances. First, completely pure α, β, and γ cDNAs can be isolated, since a single plasmid will only contain a single type of cDNA even when a mixture of cDNAs is used. Secondly, large amounts of these purified cDNAs can be obtained since the plasmid or phage cDNA can be grown to large volumes in E. coli. The plasmid or phage DNA can be separated from the bacterial cellular DNA. The cDNA can then be reisolated and la-

beled to highly radioactive-specific activity using the technique of "nick translation" in which ^{32}p deoxynucleotides are incorporated into DNA which has been nicked with DNAse I.[113] The availability of large amounts of purified cDNAs should provide increased sensitivity for the measurement of specific globin DNA sequences. These purified cDNAs can also be used to isolate specific cellular globin gene fragments and globin mRNA precursors.

Incorporation of specific cellular DNA fragments into bacterial plasmids or phages may permit the cloning of large amounts of individual globin genes and determination of their structure. Leder has already demonstrated that the mouse globin genes can be purified 1000-fold using RPC-5 chromatography and agarose gel electrophoresis and has subsequently cloned these fragments in defective phages.[98] The colonies are assayed for their globin gene content by using either radioactively-labeled globin cDNA or globin mRNA. For similar experiments with human gene isolation, it is probable that a 1000-fold enrichment of the cellular DNA for globin genes must be accomplished. Recent rapid techniques of DNA sequencing provide another strong tool for the analysis of purified DNA sequences and hold promise for determination of the structure of nucleotide sequences adjacent to the human α, β, and γ globin genes.[114,115] The nucleotide structure of human β globin mRNA has recently been reported using a combination of restriction enzyme analysis of globin cDNA and RNA sequencing.[116-118] These data should be of use in research into the structure of nucleotide sequences inserted within globin genes, as well as those adjacent to globin structural genes. They will also be beneficial for analyzing restriction enzyme patterns obtained using cellular DNA.

In summary, investigation of the molecular biology of the thalassemia syndromes has already indicated that certain of these disorders are caused by gene deletions; others are associated with the presence of abnormal mRNAs. The occurrence of β^+ thalassemia is due either to a regulatory defect in transcription of β globin genes or abnormal processing of β mRNA. The precise nature of the molecular defects in the thalassemias should become apparent with the detailed analysis of the nucleotide sequences in DNA in normal cells and in those of patients with thalassemia. It is hoped that the results obtained from these experiments will provide clues to the molecular defects in other genetic diseases of man and lead to insights into more general principles involved in the regulation of specific gene expression in eukaryotic cells.

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