THE MOLECULAR BASIS OF THALASSEMIA

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I. INTRODUCTION*

Thalassemia[1] is the term used to refer to a heterogeneous group of hereditary disorders of human hemoglobin synthesis, which are characterized by absent or decreased synthesis of the alpha (α) or beta (β) globin chain of human adult hemoglobin [Hb A: $\alpha_2\beta_2$]. In those cases where some of the affected globin chain is synthesized, there is no evidence of an amino acid substitution.[2-4] However, in all cases where genetic evidence is available, the thalassemia gene appears to be allelic to the structural gene for the α or β globin chain.[1]

The major hematological manifestations of thalassemia are related to the imbalance of globin chain synthesis: when the synthesis of one chain is decreased, the chain which is synthesized in normal amounts accumulates in relative excess. Free, unmatched globin chains (especially free α chains) are unstable; they precipitate in the red cells forming inclusion bodies which damage the red cell membrane and lead to premature destruction of the red cell.[1,41,42]

The precise molecular mechanisms responsible for the imbalance of globin chain synthesis in thalassemia are unknown. When synthesis of one globin chain is totally absent, one may think of thalassemia as perhaps being the result of a gene deletion. However, one must invoke other molecular mechanisms for the more common forms of thalassemia in which a structurally normal globin chain is synthesized in decreased amounts.

In recent years, scientific advances in the fields of biochemistry and molecular biology have permitted the application of sophisticated techniques to the study of hemoglobin synthesis in normal and thalassemic human erythroid cells. As a result, there has emerged a more precise concept of the nature of the molecular defect(s) in thalassemia.

*The following abbreviations are used in this review: Hb: hemoglobin; mRNA: messenger RNA; AMV: avian myeloblastosis virus; cDNA: DNA copy mRNA synthesized by AMV DNA polymerase; cRNA: RNA copy of cDNA synthesized by *E. coli* RNA polymerase; RNase: ribonuclease; tRNA: transfer RNA; CS: Constant Spring; β^+-thalassemia: *β-thalassemia with present but decreased β-chain synthesis*; β^0-thalassemia: *β-thalassemia with absent β-chain synthesis*; N-terminal: amino-terminal; C-terminal: carboxy-terminal; TCA: trichloro-acetic acid.

In summary, it has been found that thalassemic erythroid cells lack normal amounts of globin messenger RNA (mRNA) for the affected globin chain. The cause of this deficiency is unknown but a limited number of possibilities exist.

In this review, we shall first describe the earlier studies of globin synthesis which provided indirect evidence for involvement of globin mRNA in the thalassemic defect. We shall then analyze in more detail the more recent studies of globin mRNA structure and function in thalassemia.

II. STUDIES OF GLOBIN CHAIN SYNTHESIS IN INTACT CELLS

A. Peripheral Blood Reticulocytes

The imbalance of globin chain synthesis, which is characteristic of thalassemia, was first directly demonstrated in thalassemic cells by three different laboratories between 1964 and 1966.[5-8] The technique that was used consists of incubating peripheral blood reticulocytes for 1 to 2 hr in the presence of a radioactive amino acid precursor, usually leucine or valine. Globin is then prepared from the total cell lysate or from the Hb A purified from the lysate by column chromatography. The globin is fractionated by carboxymethylcellulose column chromatography in the presence of 8 *m* urea, which separates the γ, β, and α globin chains. A chromatogram obtained from normal, nonthalassemic, peripheral reticulocytes is shown in Figure 1A. It can be seen that equal amounts of radioactive, newly synthesized α and β globin chains are present under the α and β globin chain

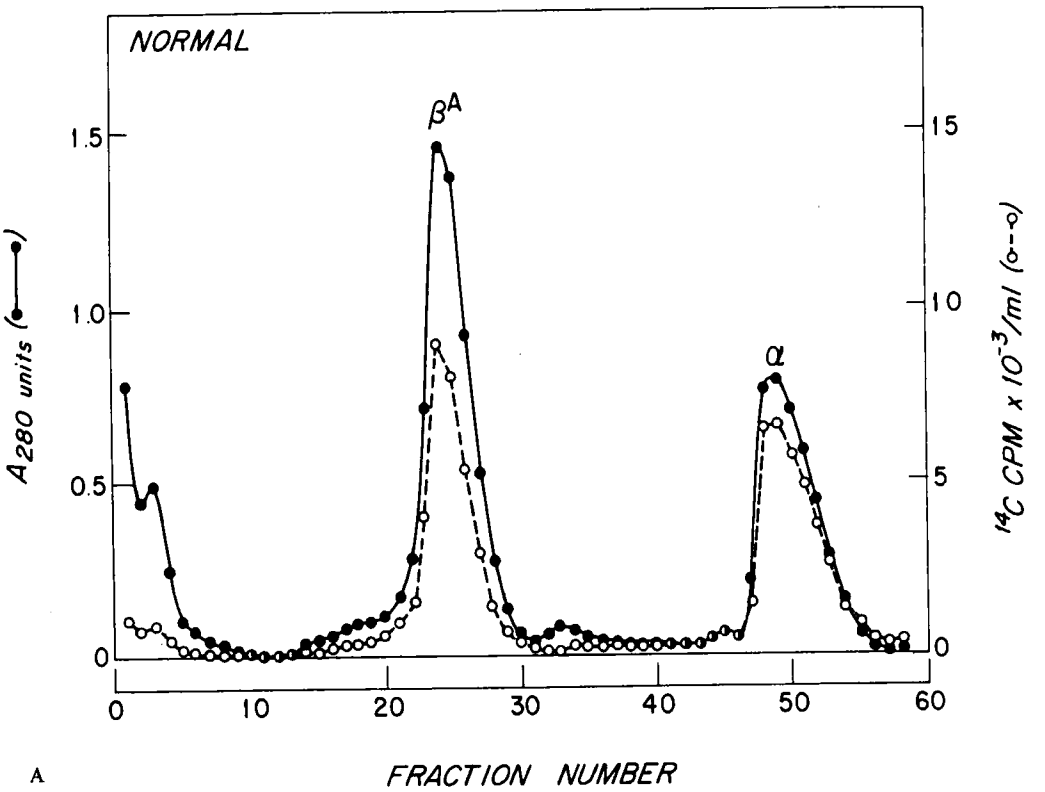
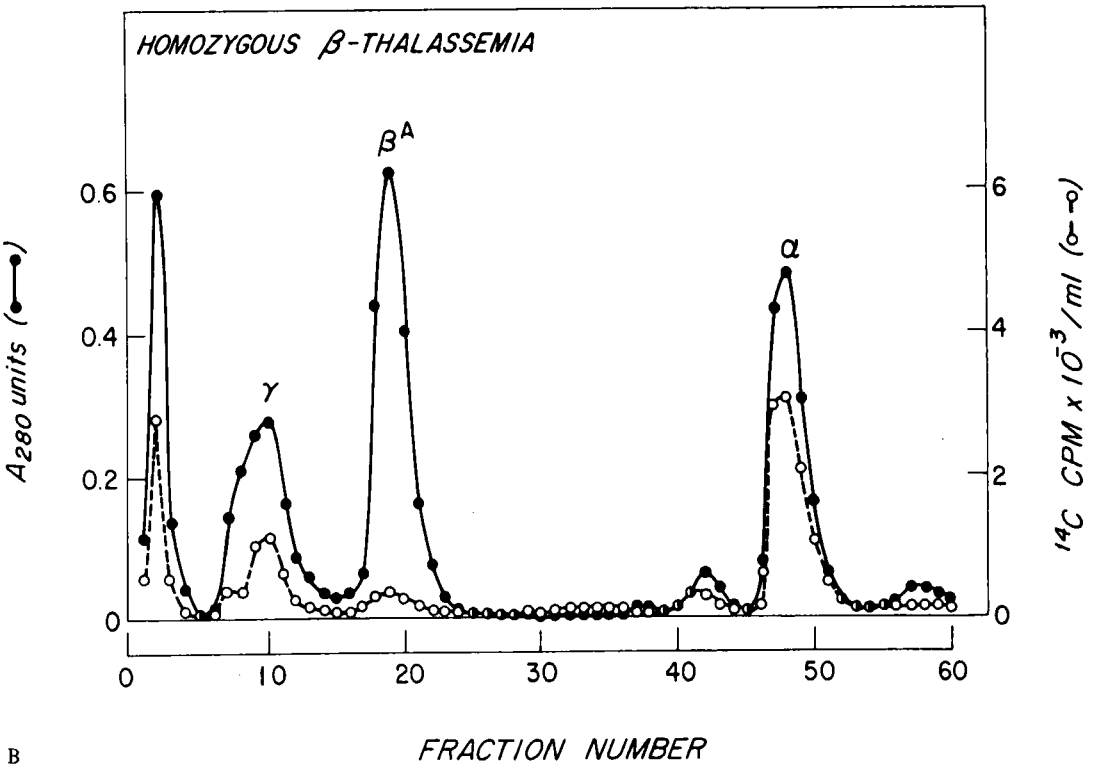
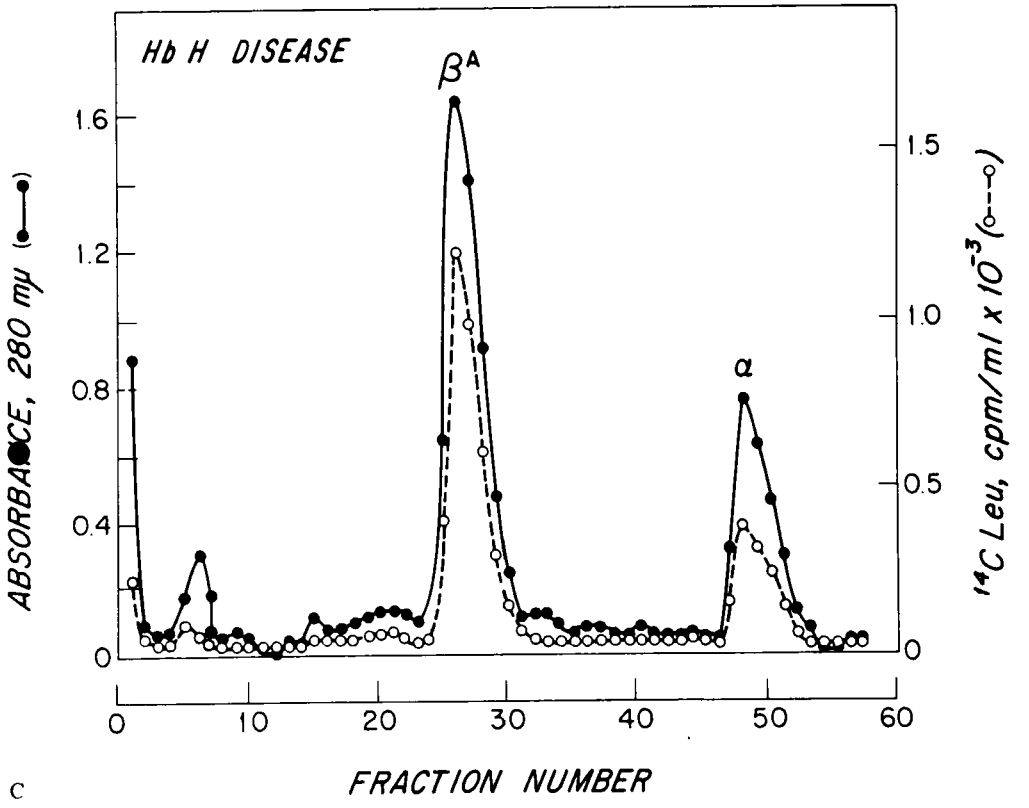


FIGURE 1. Globin synthesis by intact reticulocytes. Heparinized peripheral blood was incubated in the presence of [^{14}C]leucine for 2 hr at 37°C. The red cells were then washed and lysed and the lysate freed of membranes by centrifugation at 27,000 × g for 20 min. Globin was prepared from the lysate by acid acetone precipitation and fractionated by carboxymethylcellulose column chromatography.[18] ●——●: optical density of globin chains; ○---○: radioactivity incorporated into newly synthesized protein. A. Nonthalassemic patient with hemolytic anemia and Hb A; there is virtually equal incorporation of leucine into α and β chains. B. Patient with homozygous β^+-thalassemia; there is markedly decreased incorporation of leucine into β chains relative to α chains. C. Patient with Hb H disease (double heterozygosity for two α thalassemia genes of different severity); there is decreased incorporation of leucine into α chains relative to β chains. (From Forget, B. G. and Kan, Y. W., in *Hematology of Infancy and Childhood*, Nathan, D. G. and Oski, F., Eds., W. B. Saunders, Philadelphia, 1974, in press. With permission.)



B

FIGURE 1B.



C

FIGURE 1C.

peaks. By this technique, one can thus obtain a quantitative ratio of β to α globin chain synthesis; in normal cells, this ratio is equal to 1.0. When applied to the study of β-thalassemic reticulocytes, the initial studies[5-8] all demonstrated a decreased incorporation of radioactivity into β chains of Hb A compared to α chain incorporation. Similar studies have since been repeated in a number of laboratories, and the results of these studies all indicate marked decrease of β chain synthesis relative to α chain synthesis in β-thalassemia.[9-12,19,24]

In the usual heterozygotes for β-thalassemia, approximately half as much radioactivity is incorporated into β chains as into α chains; however, for unexplained reasons, in American Negroes with heterozygous β-thalassemia, the β/α ratio can be normal.[13-15] In homozygotes for β-thalassemia, there is either absent β chain radioactivity or marked decrease in β chain radioactivity, resulting in a β to α ratio of 0.1 to 0.3 (Figure 1B). It should be pointed out that this technique does not measure absolute rates of α and β chain synthesis but only expresses a ratio of one to the other. An attempt has been made to express the data in terms of absolute rates,[16] and the findings are consistent with a normal rate of α chain synthesis in β-thalassemia.

The same techniques were applied to the study of α-thalassemia.[7,17-19,24] In Hb H disease (double heterozygosity for two α-thalassemia genes of different severity) the globin synthesis profile reveals decreased incorporation of radioactivity into α chains when compared to β chain synthesis. The α to β ratio in this instance is 0.3 to 0.6 with a mean of 0.4 (Figure 1C). Recently, a hydropic infant with severe homozygous α-thalassemia (Hb Bart's syndrome) was studied by this technique, and total absence of α chain synthesis was demonstrated.[20] When α-thalassemia heterozygotes are studied by this technique a less striking imbalance in globin synthesis is observed in comparison to the β-thalassemia heterozygotes.[18,24] In heterozygotes for "severe" α-thalassemia, (α-thalassemia$_1$ trait), the α/β ratio is 0.7 to 0.8; in heterozygotes for "mild" α-thalassemia ("silent carrier state" or α-thalassemia$_2$ trait), it is 0.8 to 0.95.[18,24] Alpha thalassemia in the American Negro does not demonstrate as marked or as consistent an imbalance in globin chain synthesis.[21]

B. Bone Marrow

When the technique of intact cell globin synthesis was applied to the study of marrow from patients with β-thalassemia, interesting observations were noted. In homozygous β-thalassemia the β to α synthetic ratio was still abnormal but the value was closer to 1.0 than in the peripheral blood.[15,22] These findings suggested that the thalassemic defect in β chain synthesis may be more severe in peripheral blood than in marrow. Other workers, however, have not been able to demonstrate a significant difference between marrow and peripheral blood globin synthesis in homozygous β-thalassemia.[9,19,23] A major difficulty in interpreting these various results arises from the fact that the red cell population in homozygous β-thalassemia is heterogeneous and the peripheral blood red cells constitute a selected population of cells which may have survived longer than others because of greater synthesis of γ chains of Hb F, even though they have little β chain synthesis.

In heterozygous β-thalassemia an even more interesting observation has been made by Schwartz. In the marrow of such heterozygotes, the β to α synthetic ratio is very close to 1.0.[25] This observation has been confirmed in a number of different laboratories in simple β-thalassemia trait[19,23,26,27] and in Hb Lepore trait,[28,29] which resembles β-thalassemia trait (see Section III.E.l, "Hemoglobin Lepore"). The findings are difficult to interpret. It is unlikely that there exists quantitatively normal α and β chain synthesis in the marrow of these patients because over 90% of globin synthesis occurs in the marrow, and if decreased β chain synthesis occurred only at the reticulocyte stage, then the β-thalassemic heterozygotes should not be anemic and the red cells should not be hypochromic (i.e., have a subnormal cellular hemoglobin concentration). Another possible explanation for hypochromia despite relatively equal amounts of α and β chain synthesis is that the α chain synthesis as well as β chain synthesis is decreased in marrow cells of patients with heterozygous β-thalassemia, whereas in the reticulocytes the imbalance becomes obvious. There is as yet no experimental evidence to support this type of presumed feedback inhibition of synthesis of the nonaffected globin chain in thalassemia. Studies by Clegg and Weatherall on globin synthesis in heterozygous β-thalassemia marrow[27] indicate that despite the experimental

synthetic ratio of 1.0 there is indeed imbalance between β and α globin synthesis, as evidenced by the finding of a free pool of α globin chains in such marrow cells. The authors postulate two explanations for the equal ratio. First, in marrow cells, nonglobin peptides may co-chromatograph with the β chain, thereby falsely increasing the radioactivity in the β chain region. Second, the authors also provide some evidence that, in vitro, α chains are more unstable in marrow as compared to peripheral blood and may undergo proteolysis in time, thereby falsely lowering the radioactivity found in the α chain peak. The question is still not entirely resolved and remains the subject of intense debate. More recently, cases of heterozygous β-thalassemia of unusual severity have been described in which the peripheral blood β/α globin chain synthetic ratio is 0.5, whereas the marrow β/α ratio is less than 1.0, ranging between 0.7 and 0.8.[30,31]

C. Summary

Studies of globin synthesis in intact erythroid cells of individuals with α- and β-thalassemia indicate that there exists in these syndromes imbalance of globin chain synthesis, with absent or decreased synthesis of β globin chains in β-thalassemia and absent or decreased synthesis of α globin chains in α-thalassemia. These studies, however, do not shed any light on the possible molecular mechanisms for the observed imbalance in globin chain synthesis.

III. STUDIES ON MOLECULAR MECHANISMS IN THALASSEMIA

In the following descriptions of experimental work on molecular mechanisms in thalassemia we will assume that the reader is acquainted, in general, with the different processes involved in the synthesis of a protein in animal cells: transcription of the genetic information of the cell's DNA into mRNA; transport of the mRNA from nucleus to cytoplasm where it binds to ribosomal subunits in the presence of specific initiation factors and initiator transfer RNA (tRNA) (process of initiation); polypeptide chain synthesis by sequential peptide bond formation between tRNA-bound amino acids in a specific sequence as the ribosome moves along the mRNA as a "tickertape," allowing the amino acid-bearing tRNA's to bind to their specific recognition sequence (codon) on the mRNA (process of translation); and, finally, release of the completed polypeptide chain (chain termination) from the ribosome into the cell sap where it combines with other peptide chains (or other cellular components) and asssumes its functional conformation.

A. Ribosome Function

The earliest studies on molecular mechanisms in thalassemia involved studies of ribosome function in β-thalassemic reticulocytes as compared to that of nonthalassemic reticulocytes. These studies [32-36] were carried out by a group at Columbia University under the direction of Dr. P. Marks.

In summary, these studies revealed that ribosome number[32] and function was similar in β-thalassemic reticulocytes and nonthalassemic reticulocytes. Although specific radioactivity of ribosomes of β-thalassemic reticulocytes incubated with a radioactive amino acid was reduced,[32,33] this reduction could be accounted for by the decreased synthesis of Hb A (and presumably β chains); radioactive profile of polysomes,[32] ability to synthesize fetal hemoglobin (Hb F),[32] and capacity of ribosomes to synthesize polyphenylalanine in response to the artificial mRNA poly U were all normal in β-thalassemic as compared to nonthalassemic ribosomes.[35] Addition of reticulocyte supernatant fraction from nonthalassemic humans or rabbits to β-thalassemic ribosomes in a cell-free system did not stimulate protein synthesis by the thalassemic ribosomes, and thalassemic supernatant fraction did not depress protein synthesis by normal ribosomes.[35] In a later study[36] by this group, it was shown that the number of free ribosomal 60s and 40s subunits was increased by 50% in thalassemic reticulocytes compared to normal cells, but addition of either of these thalassemic or other normal ribosomal subunits to a nonthalassemic cell-free system resulted in equal stimulation of protein synthesis, indicating that the ribosomal subunits themselves were not functionally abnormal.

In conclusion, these studies revealed no significant differences between β-thalassemic and nonthalassemic ribosomes, except for the decreased amount of Hb A and β chain synthesized by the former. The results of these experiments led to the conclusion that the defect in β-thalassemia must reside in the globin mRNA or the mRNA-ribosome complex.[35]

B. Rate of Globin Chain Assembly

One of the early hypotheses on the molecular defect in thalassemia stated that the globin mRNA might contain a base substitution resulting in a codon that called for the same amino acid as the normal codon (thus no synthesis of an abnormal globin chain). However, if the new codon required a tRNA for the amino acid, which was normally in short supply in the cell, then the rate of translation and the total amount of synthesis of the affected globin chain would be diminished. There exist, in fact, experimental models for translational control ("modulation") of protein synthesis by tRNA.[37,38]

This hypothesis has been tested by studying the rate of assembly of the β chain in β-thalassemia. The technique employed ("Dintzis plots") consists of pulse labeling reticulocytes for different short periods of time using a radioactive amino acid, then examining the appearance of the label in specific peptides at various positions of the globin chain. If there were a slowdown of β chain synthesis in β-thalassemia due to an abnormal codon (or other abnormality), then there should be a different pattern or rate of peptide labeling when compared to the pattern obtained from nonthalassemic reticulocytes.

The experiment was first done by Clegg et al.[39] using reticulocytes of Oriental patients with β-thalassemia. The results indicated normal assembly time of the β chain that was synthesized by these β-thalassemic patients. There was no evidence of a slower rate of β chain mRNA translation compared to nonthalassemic controls. The authors concluded from their studies that there was no defect in β chain translation or termination in β-thalassemia.

Rieder performed similar studies using peripheral blood reticulocytes (and nucleated red cells) from five different patients of Italian ancestry and one Black patient with homozygous β-thalassemia.[40] Results similar to the previous study were obtained. However, not only was there no slowing of β-mRNA translation in these β-thalassemic cells, the rate of translation was faster in β-thalassemia than in the nonthalassemic controls. The presence of large numbers of nucleated red blood cells in the blood may be the cause for this unexpected finding, which was not observed by Clegg et al. The radioactive amino acid used by Rieder was valine rather than leucine; these studies, therefore, provided data on the first (amino terminal) amino acid of the β chain and indicated that the normal rate of β chain translation in β-thalassemia applies to the earliest measurable amino acid in the β globin chain.

The rate of assembly of globin in thalassemia has been also studied in a different manner by the technique of analyzing the distribution of nascent α and β globin chains on polysomes of normal and thalassemic reticulocytes. Polysomes are groups of various numbers of ribosomes simultaneously involved in the translation of a single strand of mRNA. The number of ribosomes attached to a strand of mRNA at a given time will depend on a number of factors: rate of initiation, rate of translation, and rate of chain termination and release. In normal reticulocytes, in man[43,44] as well as in rabbits,[45] the nascent α and β globin chains are heterogeneously distributed on the polysomes. The larger, more rapidly sedimenting polysomes carry predominantly β chains, whereas the smaller, more slowly sedimenting polysomes carry predominantly α chains. It is generally agreed that the rates of translation (and of termination) of the α and β chains are similar;[46] therefore, the predominance of β chains on larger polysomes is taken to reflect a normally faster rate of initiation of β chains than α chains.[46,47]

If there were a slowing of the rate of translation (or of initiation, or of termination) in thalassemia, then it should be reflected in the profile of nascent globin chains on thalassemic polysomes. This has been studied both in thalassemic reticulocytes[43] and bone marrow cells.[48] The technique involves sucrose density gradient centrifugation of lysates of human erythroid cells previously incubated for a short period of time in the presence of [^{35}S] methionine which becomes incorporated into the globin chains being synthesized by the cells. The polysomes of various sizes are then isolated, [^{3}H] methionine globin is added as a standard, and the mixture is digested with trypsin. The digests are then fractionated by high voltage electrophoresis to separate α from β chain specific methionine peptides; a β/α ratio of nascent globin chains on the various sized polysomes is thus obtained.

The studies performed with thalassemic cells revealed the same pattern as in nonthalassemic cells: the nascent β chains in β-thalassemia are preferentially associated with the same sized polysomes as in nonthalassemic cells.[43,48] If the rate of translation of β chains in β-thalassemia were abnormally slow (while initiation was normal),

then the nascent β chains would be associated with larger polysomes than normal. Similar findings in bone marrow cells of patients with β-thalassemia[47] indicate that normal rates of translation (and initiation) occur in β-thalassemic cells at early as well as late stages of maturation. In α-thalassemia (Hb H disease) similar studies were performed and revealed similar findings: a normal distribution of nascent chains, with α chains predominating on smaller polysomes.[48] Again, this normal distribution implies normal rates of globin chain translation and initiation in α-thalassemia as well as in β-thalassemia.

C. Initiation of Globin Chain Synthesis

As just described in the previous section, analysis of the distribution of nascent globin chains on polysomes can provide information about the rate of initiation of α and β globin synthesis. If α and β chain translation and termination are equal in normal reticulocytes[46] and are normal in thalassemic cells,[39,40] then the size of polysomes bearing α and β nascent chains is directly proportional to the rate of initiation of these chains.[43] Thus, in normal cells, the finding of β chains predominantly on larger polysomes implies that β chain synthesis is initiated more rapidly than α chain synthesis; the cell must, therefore, achieve balanced synthesis by having more α-mRNA than β-mRNA.[46,47] In β-thalassemia, if β chain initiation were abnormally slow, then the nascent β chains would be found associated with smaller rather than larger polysomes. Since that is not the case,[43,48] one must conclude that those β chains which are being synthesized in β-thalassemia are being initiated at a normal rate.

More direct studies of initiation have also failed to reveal a defect of chain initiation in thalassemia. These studies were performed by the group at the National Institutes of Health under the direction of Dr. W. F. Anderson. These workers have devised a cell-free protein synthesizing system[49-51] which is very active and efficient in translating exogenously added mRNA and which has a number of advantages. The system utilizes primarily rabbit reticulocyte components, but human components are also effective; the background protein synthesis from endogenous mRNA is virtually absent because the rabbit ribosomes are treated with low doses of ribonuclease to destroy the endogenous mRNA. Finally the system is entirely fractionated so that the effect of various components can be studied individually. These same workers have also purified and characterized the major protein factors (initiation factors) that are specifically involved in the initiation of globin chain synthesis.[52-54]

The initial studies performed involved the incubation of β-thalassemic ribosomes in the cell-free system to study the effect of added normal initiation factors on the translation of endogenous mRNA by the thalassemic ribosomes.[55] These experiments showed that added initiation factors (rabbit or human) do increase total protein synthetic activity by the β-thalassemic ribosomes in the cell-free system, but analysis of the products synthesized revealed that α chains were still synthesized in a great excess over β chains.[55] The decreased β chain synthesis in β-thalassemia therefore did not appear to be associated with a deficiency of initiation factors.

The ability of β-thalassemic ribosomes to initiate and translate exogenously added globin mRNA was next tested in this cell-free system.[56] Thalassemic and nonthalassemic ribosomes were incubated in the cell-free system in the presence of rabbit globin mRNA. Both types of ribosomes were equally effective in translating the rabbit mRNA, and the relative amounts of rabbit α and β chains synthesized were similar with both types of ribosomes.[56] These studies confirmed the previously described studies of Bank and Marks[35] on the functional ability of β-thalassemic ribosomes but added the important new dimension of showing normal translation of a natural mRNA (instead of simply a synthetic mRNA:Poly U) by thalassemic ribosomes. These results again implied that the defect in β-thalassemia must reside in the globin mRNA itself.

These workers also studied the fine details of the initiation process in β-thalassemia.[57] In eukaryotic protein synthesis, the process of peptide chain initiation involves the binding, to mRNA and ribosomes, of a specific methionine-bearing tRNA, methionyl $tRNA_f$, which is different from the tRNA (methionyl $tRNA_m$) that inserts methionine residues at other sites of a peptide chain. The initial peptide bond is therefore formed between methionine and the amino acid that is situated at the amino terminus of the completed chain; the methionine residue that initially was situated at the amino terminus is normally cleaved from the growing chain after it has a length of 20 to 30 amino acids. The study of this initial

dipeptide bond formation can shed light on the process of chain initiation.

A possible defect in thalassemia could be abnormal synthesis of the initial dipeptide (perhaps associated with a base substitution at or near the initiator codon of the mRNA). Such a defect would be missed in the previously described studies because the initial methionine residue escapes scrutiny by the techniques used. The normal initial dipeptide for human α and β globin chains would be expected to be methionine-valine, and for the γ chain of Hb F, methionine-glycine. The initial dipeptides synthesized in the fractionated cell-free system in the presence of thalassemic and normal human globin mRNA were studied and found to be similar with both types of mRNA: methionine-valine for the α and β chains, and methionine-glycine for the γ chain.[57] In addition, the specific initiator tRNA (methionyl $tRNA_f$) donated the methionine for this initial dipeptide in both the normal and β-thalassemic β chains synthesized in the cell-free system in the presence of the appropriate mRNA.[57] It was concluded that the mechanism of globin chain initiation is normal in β-thalassemia.

All of these studies indicate that when some β chain is synthesized in β-thalassemia, it is initiated and translated normally. The deficient synthesis of β chains in β-thalassemia is therefore due to less frequent initiation of β chains. This could be caused by quantitative deficiency of β chain mRNA or to a β mRNA that is abnormal in its ability to bind to the ribosome in the process immediately preceding the specific biochemical events of chain initiation.

D. Chain Termination

In those cases of thalassemia where some of the affected globin chain is synthesized, there is no evidence of an abnormality of globin chain termination. If such an abnormality existed, the studies of Clegg et al.[39] and of Rieder[40] on the rate of globin chain assembly in thalassemia would have revealed a slow rate of appearance of completed β chains in lysates and in the postribosomal supernatant fraction of β-thalassemic reticulocytes. Such was not the case.

In those cases of thalassemia where there is absence of completed globin chains, one possible explanation could be premature chain termination due to a nonsense mutation of the mRNA: a base substitution changing a normal codon to a chain termination codon. If this were the case, one would expect to find incomplete fragments of the affected globin chain.[58] Experimental systems have been devised which suggest that this hypothesis can be tested: incomplete globin chains, which are initiated at the normal N terminus, but permaturely terminated, are not immediately degraded in reticulocytes but can be detected as such.[58]

A search for incomplete β globin chain fragments was carried out in reticulocytes of β-thalassemic patients from the Ferrara region of Italy, in which there was totally absent synthesis of completed β chains (so-called β^0-thalassemia).[59] The study was performed with a great deal of technical care and detail, but no incomplete β chain fragments could be identified.[59]

In the severe form of homozygous α-thalassemia (hydrops fetalis with Hb Bart's) which is usually fatal in utero or within hours of birth, there is total absence of completed α chains. In such a case a search was made for N terminal peptides longer than 11 amino acid residues.[20] None were found, indicating that if nonsense mutation were present in the α chain mRNA it would have to be located within the first 33 nucleotide residues following the initiation codon.[20]

In summary, then, there is no evidence to support the hypothesis of abnormal chain termination in the thalassemia syndromes.

E. Thalassemia-like Disorders Associated with Structurally Abnormal Globin Chains

1. Hemoglobin Lepore

We have previously stated that in thalassemia the globin chain which is synthesized in decreased amounts is structurally normal, i.e., shows no evidence of an amino acid substitution. There are two rare thalassemia-like disorders, however, in which a structurally abnormal globin chain is in fact synthesized. The first is the hemoglobin Lepore syndrome[60] in which the abnormal globin chain appears to be a hybrid chain, having the N terminal amino acid sequence of the normal δ chain of Hb A_2, and the C terminal amino acid sequence of the normal β chain.[61] This defect appears to have resulted from nonhomologous crossing over of the chromosomes at meiosis in the region of the adjacent δ and β loci.[61] As illustrated in Figure 2, this phenomenon would yield on

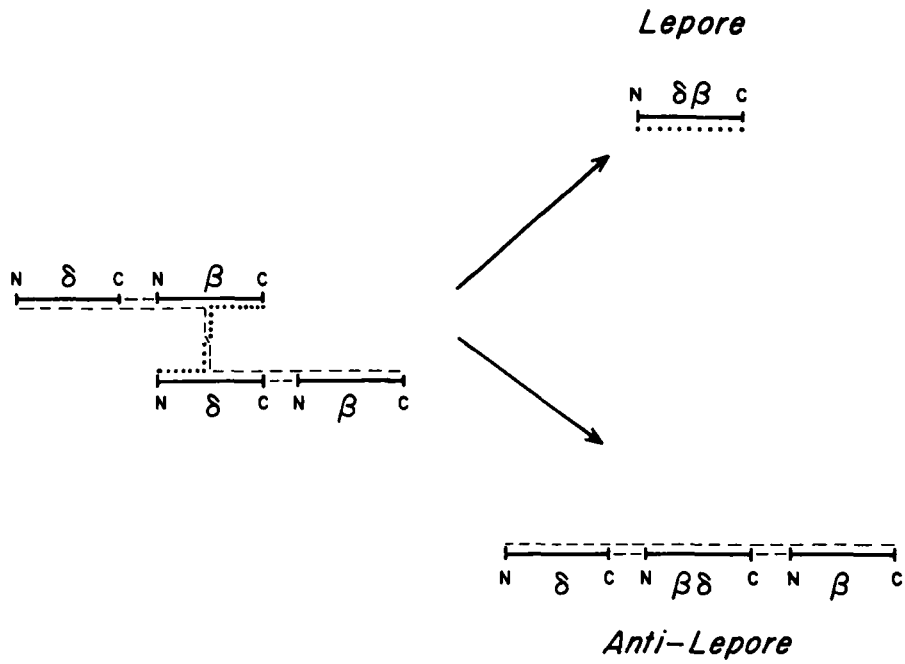


FIGURE 2. Diagrammatic representation of origin of Hb Lepore and anti-Lepore by nonhomologous crossing over between β and δ globin chain genes. The N and C indicate the extremities of the gene coding for the amino (N) and carboxy (C) terminal amino acids of the globin chains. (From Forget, B. G. and Kan, Y. W., in *Hematology of Infancy and Childhood*, Nation, D. G. and Oski, F., Eds., W. B. Saunders, Philadelphia, 1974, in press. With permission.)

one chromosome a fused δ-β (Lepore) locus only, and on the other chromosome a hybrid (anti-Lepore) locus containing the N terminal amino acid sequence of the β chain and the C terminal sequence of the δ chain, situated between the normal δ chain and β chain loci. The Hb Lepore gene resembles β-thalassemia because there is a marked decrease in synthesis of the gene product and presumably absence of the normal β (and δ) chain loci of the affected chromosome. This latter assumption is confirmed by the findings in patients homozygous for Hb Lepore, in which there is total absence of normal Hb A and Hb A_2.[1] Clinically, heterozygous and homozygous Hb Lepore resemble heterozygous and homozygous β-thalassemia. In addition, the doubly heterozygous state of Hb Lepore and β-thalassemia is clinically similar to homozygous β-thalassemia.

The condition in which the δ, β-δ, and β loci all occur on one chromosome is called the anti-Lepore syndrome, and its demonstration is important in confirming the Lepore cross over hypothesis. Two instances of such an abnormal hybrid N β-C δ chain have recently been demonstrated, in Hb P Nilotic[62,63] and Hb Miyada.[64] The anti-Lepore syndrome does not resemble thalassemia because the affected chromosome carries, in addition to the mutant gene, normal β and δ genes with normal output.

In both the heterozygous Lepore and anti-Lepore syndromes, the abnormal hemoglobin comprises approximately 15% of the total hemoglobin. It is not immediately obvious why these abnormal hemoglobins should be present in such low amounts. Biosynthetic studies in intact cells indicate that in both instances there is decreased synthesis of these hemoglobins rather than increased destruction, and that the synthesis of the abnormal chains, similar to the δ chain of Hb A_2, occurs primarily in the bone marrow cells and is virtually absent in the peripheral blood reticulocyte.[65,66,105,106] These findings are probably easier to understand in the case of Hb Lepore; one would expect the Lepore chain to be under the same control as the δ chain, because the Lepore gene must include the genetic information (control gene or operator) adjacent to the N terminus of the δ chain gene. However, if the Lepore gene were strictly under the same control as the normal δ chain gene, then it should be expressed only up to 5% (and not 15%) of the total hemoglobin.

The situation is even more confused with the

anti-Lepore globin chain. In theory, one would expect this chain to be expressed as well as the normal β chain, if it is under control of the normal β chain control gene expected to be adjacent to the N terminus of the β chain gene (the latter supposition is, however, purely speculative). In fact, the anti-Lepore gene is expressed less well than the β chain.

One possible explanation for these observations is that both the pre-initiation and post-termination regions of the mRNA are important for mRNA stability and its effective translation. The characteristics of δ chain synthesis may be in great part due to instability and rapid degradation of the δ chain mRNA because of its secondary structure. The latter may be influenced by the nature of the 3′ and 5′ untranslated sequences of the δ-mRNA, which are situated at the carboxy- and amino-terminal side, respectively, of the structural gene information of the mRNA. Since both the Lepore and anti-Lepore globin chain mRNA's must contain either the pre-initiation (5′) or post-termination (3′) untranslated sequences of the δ chain mRNA, these sequences may render these mutant mRNA's less stable than normal β chain mRNA; these mRNA's should, however, be more stable than the δ chain mRNA because they contain either the 3′ or 5′ terminal untranslated sequences of the β chain mRNA.[106]

2. Hemoglobin Constant Spring

The second thalassemia-like condition in which a structurally abnormal globin chain is synthesized is the hemoglobin Constant Spring (Hb CS) syndrome.[67,68] Hb CS is an abnormal hemoglobin that consists of two β chains and two abnormally long α chains, which are elongated at their C terminal end by an additional 31 amino acids. This globin chain is produced in only small amounts (1 to 3% of the total hemoglobin) and therefore the gene for this abnormal globin chain resembles an α-thalassemia gene. Clinically, the simple heterozygous state for Hb CS has no hematologic manifestations except for the presence of 1% or less of the abnormal hemoglobin.

Hb CS was first detected in association with the α-thalassemia syndrome of Hb H disease, which can be considered as a mild form of "homozygous" α-thalassemia in which some α chains and Hb A are synthesized (see Section II.A, "Peripheral Blood Reticulocytes," and Figure 1C). The affected patient was clinically similar to other patients with Hb H disease but hemoglobin electrophoresis revealed 3% Hb CS.[67] Small amounts of Hb CS have been subsequently observed in a number of patients (mostly of Oriental ancestry) with Hb H disease. Family study in these cases usually reveals that one parent has the hematological findings of typical heterozygous α-thalassemia (hypochromia and microcytosis with normal levels of Hb A_2 and Hb F) whereas the other parent is hematologically normal except for the finding of 1% Hb CS. It should be pointed out that in the usual case of Hb H disease without Hb CS, one parent has typical heterozygous α-thalassemia (so-called α-thalassemia$_1$ trait) whereas the other parent appears hematologically normal. The latter is referred to as having the silent carrier state of α-thalassemia (or α-thalassemia$_2$ trait), because the α-thalassemia gene carried by this person is so mild that it is undetected in the heterozygote. It therefore appears that the gene for Hb CS behaves as a mild α-thalassemia gene, similar to that of the silent carrier state (or α-thalassemia$_2$ trait).

The fact that some normal α chains and Hb A are produced in patients with Hb H disease and Hb CS is considered to be evidence that the normal α chain loci (or genes) are duplicated.[1,69] The gene for α-thalassemia$_1$ is thought to be completely inactive in directing α chain synthesis: infants homozygous for α-thalassemia$_1$ have hydrops fetalis with Hb Bart's, and total absence of α chain synthesis.[20] Therefore, the α chain synthesis seen in patients with Hb H disease and Hb CS could not arise from the activity of the thalassemic α chain gene inherited from the parent with α-thalassemia$_1$ trait, but must result from the activity of the α chain gene inherited from the parent with Hb CS trait. The latter parent must therefore transmit, *in cis*, both a gene for the structurally abnormal CS α chain and a gene for a normal α chain. Duplication of the α chain locus has therefore been invoked, and there is other evidence as well to support such a hypothesis.[69]

Parenthetically, it is much easier to explain the genetics of α-thalassemia if one assumes duplication of the α chain locus: heterozygous α-thalassemia$_2$ (silent carrier state) would result from inactivation of one of the four α chain loci; heterozygous α-thalassemia$_1$ would be the result of inactivation of two α chain loci *in cis*; Hb H disease would represent inactivation of three α

chain loci; and hydrops fetalis with Hb Bart's, absence of activity from all four α chain loci.

The reason for the finding of only small amounts of Hb CS in the red cells of affected individuals is not known. There is evidence that the mutant hemoglobin is synthesized in decreased amounts rather than rapidly destroyed.[70] The pattern of synthesis of the CS globin chain is similar to that of the Lepore chain (and δ chain): there is much less synthesis in reticulocytes than in bone marrow cells,[70] suggesting instability of the mRNA as one reasonable possibility for this finding.

The precise molecular basis for the origin of Hb CS is also uncertain, but there is convincing evidence to suggest that the α CS chain is synthesized because of a chain termination mutation which allows translation of usually untranslated portions of the α chain mRNA.[53] This was initially suggested[53] because of the nature of the amino acid in position 142 (adjacent to the usual α chain C terminal amino acid, number 141) of the CS α chain; this amino acid is glutamine, and the codons for glutamine are CAA and CAG, which could arise by single base substitution of the chain termination codons UAA or UAG.

A second abnormally long human α chain has been identified in Hb Wayne.[71] Hb Wayne is presumed to have arisen by frameshift mutation, at position 139 of the α chain, resulting in the synthesis of an α chain that contains 146 amino acids instead of the normal 141. However, the amino acids in positions 139, 140, and 141 of the Wayne α chain are not the same as in the normal α chain. A frameshift mutation results from the loss (deletion) of one (or two) of the three nucleotides in a codon of the mRNA. This phenomenon changes the normal reading phase or triplet punctuation of the mRNA. The translational apparatus cannot skip over the deletion and it will therefore read and decode the mRNA sequence in a new arrangement of successive triplet codons. The result will be the synthesis of a polypeptide chain with a different amino acid sequence and different length from normal: the normal chain termination codon will not be read as such because it is out of phase, and translation will continue until a new chain termination codon (in phase) is encountered by chance association of adjacent nucleotides. One can derive a single hypothetical α chain mRNA nucleotide sequence to account for both the Hb Wayne and Hb CS α chain amino acid sequences if one supposes that the former is due to a frameshift mutation and the latter due to a chain termination mutation.[71,72] More recently, studies of the nucleotide sequence of normal globin mRNA have given added support to the hypothesis of chain termination mutation to explain Hb CS. Nucleotide sequences have been identified in normal globin mRNA, which match the proposed sequence of the chain termination region and match other amino acid sequences in the α CS chain (see Table 1). Furthermore, these sequences are deficient in α-thalassemia mRNA (see Figure 11 and Section VI, "Structural Studies of Globin Messenger RNA"). All of these findings make chain termination mutation a very likely cause for the origin of Hb CS.

Other mechanisms that have been proposed[1,53] for the origin of the α CS chain but which seem less likely are (1) a suppressor tRNA mutation giving rise to a tRNA that reads the chain termination codon as a codon for a specific amino acid and (2) a Lepore-type cross over between the α chain gene and the gene for another protein.

If the only abnormality in the α CS gene is the chain termination mutation, it is not clear why this alone should give rise to so little gene product. It is difficult to conceive how this single base substitution should lead to either decreased transcription of mRNA from the DNA or instability and rapid destruction of the mRNA. A single base substitution in the termination codon should not significantly alter the secondary structure of the mRNA in solution. However, it is possible that during the translation of the normally untranslated portions of the mRNA the conformation of the mRNA changes in such a manner that it becomes very susceptible to attack by various nucleases and is rapidly degraded. This latter explanation is pure speculation.

Another possible explanation for the low amount of Hb CS is that it results from a chain termination mutation in a so-called "silent" α chain locus which normally produces only small amounts of gene product.[1] There is good evidence for such silent loci in higher apes.[75] If this were the case, however, one would also have to postulate the co-existence of another α-thalassemia$_2$ gene *in cis* with the Hb CS gene to explain the genetics of Hb H disease associated with Hb CS.

TABLE 1

Nucleotide Sequences of Human Globin Messenger RNA

Spot number	Sequence	Chain
3†	136 137 138 139 140 141 leu thr ser lys tyr arg NN(G) – ACC – UCC – AAA – UAC – CG(U)	Alpha
7*	79 80 81 82 83 asp asn leu lys gly (G)AC – AAC – CUC – AAG – (G)NN	Beta
11*	115 116 117 118 119 ala his his phe gly (G)CC – CAU – CAC – UUU – G(G)N	Beta
12*	144 145 146 lys tyr his Term NN(G) – UAU – CAC – UAA – G(C)N	Beta
13*	56 57 58 59 60 gly asn pro lys val (G)GC – AAC – CCU – AAG – (G)NN	Beta
21*	101 102 103 104 glu asn phe arg NN(G) – AAC – UUC – AGN	Beta
22†	123 124 125 126 ala ser leu asp (G)CC – UCA – CUU – GNN	Alpha
28*	83 84 85 86 gly thr phe ala N(G)C – ACC – UUU – GNN	Beta
30*	86 87 88 89 ala thr leu ser (G)CC – ACA – CUG – (A)NN	Beta
44*	138 139 140 ala asn ala (G)CU – AAU – GNN	Beta
6*	(G)U[AAU,AU,U_{3-5},C_{2-3}]AAAG(G)	No match
25*	(G)U[AU_{1-2},U_{1-2}]AUG(A)	No match
14†	155 156 157 158 ala ser gln arg (G)CC – UCC – CAA – CG(G)	Alpha CS
51†	145 146 147 ala ser val (G)CC – UCG – (G)NN	Alpha CS
56†	141 142 143 arg term ala N(G)U – UAA – G(C)N	Alpha + Alpha CS

TABLE 1 (continued)

The spot numbers refer to Figure 11. The nucleotide sequences are based on the composite information obtained by enzymatic digestions of oligonucleotides from cRNA (differentially labeled with the 4 triphosphates) and from the natural 10S mRNA labeled in vitro by various techniques.[73,74] Listed over the nucleotide sequence is the amino acid sequence matched (by computer analysis) to the nucleotide sequence in the indicated codon grouping. The nucleotides in parentheses at the 3′ terminus (right) and 5′ terminus (left) of the hyphenated nucleotide sequence are not contained in the numbered oligonucleotide but are known to be present in the indicated position by "nearest neighbor" analysis[73,74] and by the nature of the nuclease (T_1 RNase) digest, respectively. The exact sequence of the nucleotides in brackets cannot yet be determined from the sequence information alone. N indicates an unknown nucleotide in a codon. The numbers over the sequences refer to the position of the indicated amino acid in either the α or β globin chain. The sequence listed for spot number 51 also matches the amino acid sequence of α chain position 130–131. The sequence listed for spot number 56 also matches (in a different codon grouping: NN[G]-UUA-AG[C] = y-leu-ser) the following amino acid sequences: β 87–89, α 1–3, α 79–81, α 82–84, and α 100–102. *: indicates that the oligonucleotide is prominent in the fingerprint of α-thalassemia cRNA. †: indicates that the oligonucleotide is faint or absent in the fingerprint of α-thalassemia cRNA.

Modified from References 73 and 74.

IV. FUNCTION OF GLOBIN MESSENGER RNA IN THALASSEMIA

The main conclusion that one can draw from all of the studies described in the previous section on molecular mechanisms is that there must be a deficiency of functional mRNA for α and β chain synthesis in α- and β-thalassemia, respectively. By ruling out other molecular mechanisms, all of the previous studies provide indirect evidence for the involvement of mRNA in the biosynthetic abnormality of globin chain synthesis in thalassemia. The globin mRNA may be deficient quantitatively or structurally abnormal (in its untranslated regions) and thereby less efficient functionally (i.e., in binding to ribosomes). Involvement of mRNA in the thalassemia defect was first demonstrated directly by translation of mRNA, isolated from thalassemic reticulocytes, in heterologous cell-free protein-synthesizing systems capable of translating exogenously added mRNA.

A. Reticulocyte mRNA in Homozygous β^+-Thalassemia

Involvement of globin mRNA in the thalassemia defect was first demonstrated directly by Nienhuis and Anderson[76] and Benz and Forget[77] who succeeded in isolating functional globin mRNA from β-thalassemic reticulocytes and having it translated in a heterologous cell-free protein-synthesizing system. In both studies the imbalance of human globin chain synthesis characteristic of the intact thalassemic reticulocytes was duplicated in the cell-free system in the presence of β-thalassemic globin mRNA, which was the only human or thalassemic reticulocyte component present in the incubation mixture.

The cell-free system used by Nienhuis and Anderson[76] was the fractionated rabbit reticulocyte cell-free system previously mentioned which contained RNase-treated rabbit reticulocyte ribosomes (to eliminate endogenous mRNA), rabbit reticulocyte initiation factors (ribosomal salt wash), tRNA, and supernatant enzyme fraction. The thalassemic mRNA was isolated by sodium dodecyl sulfate treatment of ribosomes of two siblings with homozygous β-thalassemia whose reticulocytes produced some β chains (β^+-thalassemia) but in markedly decreased amounts. The cell-free system used by Benz and Forget[77] was the Krebs II mouse ascites tumor cell system, which consists of a membrane-free (S30) lysate of disrupted tumor cells, preincubated in protein-synthesizing conditions to decrease endogenous mRNA activity. No added initiation factors were used in the initial experiments. The thalassemic mRNA was obtained by phenol extraction of total reticulocyte lysates of two unrelated patients with homozygous β^+-thalassemia.

In control experiments using reticulocyte

mRNA from nonthalassemic patients, both cell-free systems synthesized approximately equal amounts of α and β chains (Figure 3). However, in the presence of β-thalassemic mRNA, much more α than β chain was synthesized and the β/α synthetic ratio obtained was similar to that of the patients' intact cells[76,77] (Figure 3).

These experiments, then, conclusively demonstrated that the thalassemic imbalance of globin chain synthesis is mediated via the cell's globin mRNA; there must be either true quantitative deficiency of the mRNA for the affected chain or, at least, deficiency of functional mRNA capable of participating in protein synthesis. It should also be noted that mRNA isolated from total reticulocyte lysate[77] gave the same result as polysome-derived mRNA;[76] this finding indicates that there does not exist in thalassemic reticulocytes a pool of supernatant mRNA which is untranslated in the thalassemic cells but capable of being translated in a heterologous cell-free system (and presumably in a normal cell).

These initial experiments have now been confirmed in a number of different individual patients with homozygous β-thalassemia. Dow et al.[78] translated polysomal mRNA from reticulocytes of five patients with homozygous β^+-thalassemia in the Krebs II ascites cell-free system, supplemented by rabbit reticulocyte ribosomal salt wash, which increases the activity of the system.[82] In all five cases the α/β synthetic ratios obtained in the cell-free system were similar to those obtained in intact cells of the same patients. Forget et al.[79] also reported results of cell-free translation of reticulocyte mRNA from a series of ten individual patients with homozygous β^+-thalassemia using the Krebs II cell-free system (with added rabbit ribosomal salt wash). In all cases tested there was deficiency of functional mRNA for the β globin chain, and the degree of imbalance between α and β chain synthesis was similar in the cell-free system and intact cells (Figures 1B and 5B).

B. Bone Marrow mRNA in Homozygous β^+-Thalassemia

Because the reticulocyte is an end-stage cell and the thalassemic reticulocyte is probably less healthy than a normal reticulocyte, it is conceivable (although unlikely) that the deficiency of mRNA in the thalassemic reticulocyte is a "preterminal" event, not representative of the situation in less mature erythroid cells in thalassemia. For this reason, globin mRNA isolated from thalassemic marrow has been tested in the cell-free systems. Nienhuis et al.[23] tested individual marrow and reticulocyte mRNA preparations from the same two siblings initially studied.[76] Natta et al.[81] studied marrow mRNA of one case of homozygous β^+-thalassemia and one case of β-thalassemia intermedia; Forget et al.[79] also studied marrow mRNA of one patient with β-thalassemia intermedia. In all cases, cell-free translation of the mRNA revealed deficiency of functional mRNA for β globin chains. All of these studies, however, used total unfractionated marrow, which necessarily contained considerable numbers of reticulocytes. Although the results are convincing and probably valid, the conclusions drawn from the marrow studies would be more firm if the mRNA had been isolated from nucleated marrow cells that had been previously purified of contaminating reticulocytes.

C. Reticulocyte and Marrow mRNA in Heterozygous β-Thalassemia

Function of reticulocyte and marrow globin mRNA has been tested in heterozygous β-thalassemia in order to study the role of mRNA in the phenomenon of apparently balanced globin chain synthesis in intact marrow cells of patients with heterozygous β-thalassemia.[23,81] In summary, these studies have shown that, in the two cases studied,[23,81] both the reticulocyte and marrow globin mRNA of patients with heterozygous β-thalassemia direct the synthesis of less β^A chain than α chain when translated in a cell-free system, even though the intact marrow cells demonstrate balanced globin chain synthesis. In three patients doubly heterozygous for β^0-thalassemia (absent β^A chain synthesis) and sickle hemoglobin ($\alpha_2\beta_2{}^S$), similar observations have been made;[23,81] these patients manifest in their intact cells the same phenomenon as simple β-thalassemia heterozygotes: imbalanced β^S/α chain synthesis in reticulocytes but balanced β^S/α synthesis in marrow cells. Cell-free translation of both reticulocyte and marrow globin mRNA from these patients resulted in decreased β^S chain synthesis compared to α chain synthesis.[23,81] The authors concluded that, despite the presence of balanced globin chain synthesis in the marrow cells of these patients with heterozygous β-thalassemia, there was evidence for decreased functional globin mRNA for β chains in these marrow cells. The implication is that the

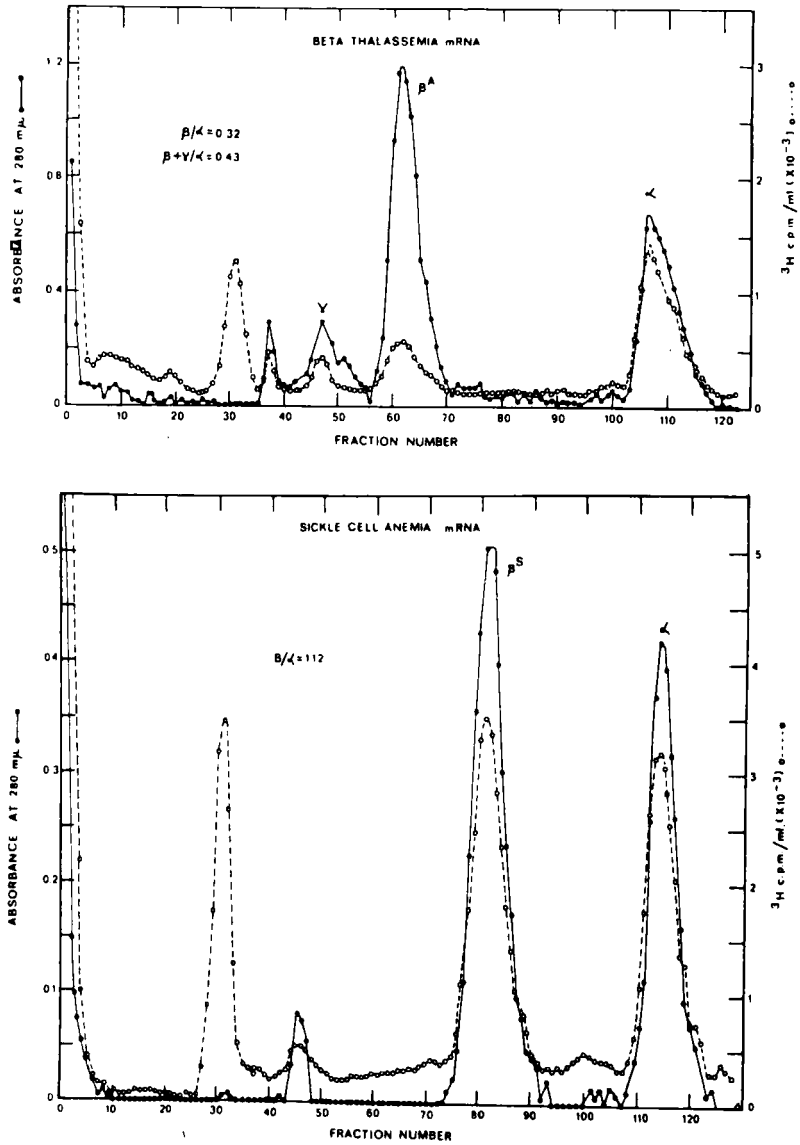


FIGURE 3. Translation of human globin messenger RNA in the Krebs II cell-free system. Total cellular RNA was prepared from reticulocyte lysates by detergent-phenol-cresol extraction and fractionated by sucrose gradient centrifugation.[77] The fractions containing RNA sedimenting between 4S and 18S were pooled, and the RNA was precipitated with ethanol and added to the Krebs II cell-free system.[77] After incubation for 1 hr at 37°C in the presence of [^{3}H]leucine, nonradioactive hemoglobin was added to the reaction mixture in the form of membrane-free red cell lysate; globin was then prepared from the mixture by acid acetone precipitation and chromatographed as in Figure 1. ●——●: optical density of globin chain markers. ○----○: radioactivity incorporated into protein by the cell-free system. *Top*: pooled mRNA from two patients with homozygous β-thalassemia; there is decreased incorporation of leucine into β chains relative to α chains (β/α: 0.3). *Bottom*: pooled mRNA from patients with homozygous sickle cell anemia; there is virtually equal incorporation of leucine into β and α chains (β/α: 1.1). (From Benz, E. J., Jr. and Forget, B. G., *J. Clin. Invest.*, 50, 2755, 1971. With permission.)

balanced globin chain synthesis observed in intact marrow cells is due to either an artifact of the analytical method or to translational control (depression) of α chain synthesis.

With regard to these studies, one can again point out that the mRNA was obtained from unfractionated marrow cells containing variable numbers of reticulocytes, the mRNA of which could "dilute" the mRNA activity of the nucleated marrow cells. Ideally, one would like to see the studies repeated using mRNA isolated from relatively pure populations of nucleated marrow cells. Another difficulty in the analysis of these results arises from the fact that both of the cell-free systems used in these studies give variable β/α synthetic ratios depending on the amount of mRNA used.[23,82,83] At limiting concentrations of mRNA, α and β chain synthesis are roughly equal, but total activity of the system is submaximal. At high concentrations of mRNA giving maximal protein synthetic activity, two to three times as much β as α chain is synthesized.[23,82,83] In fact, using higher concentrations of mRNA, marrow mRNA from patients with heterozygous β-thalassemia resulted in equal synthesis of α and β chains in the cell-free system.[80] In conclusion, cell-free translation of mRNA from unfractionated marrow cells is probably not a sensitive enough assay to definitively resolve the issue relating to the finding of balanced α and β globin chain synthesis in marrow cells of patients with heterozygous β-thalassemia.

D. Reticulocyte and Marrow mRNA in β^0-Thalassemia

Globin mRNA function in β-thalassemia with absent rather than decreased β^A chain synthesis (so-called β^0-thalassemia) is of particular interest because of observations in β^0-thalassemic patients of Ferrara, Italy (see next section), which are interpreted as showing the presence of substantial amounts of β chain mRNA in these patients which is not translated because of the supposed lack of a factor present in the supernatant fraction of nonthalassemic reticulocytes. Globin mRNA from a number of β^0-thalassemia syndromes (other than Ferrara thalassemia) has been studied in the cell-free systems: marrow and reticulocyte mRNA from two patients with Hb S-β^0-thalassemia,[23] marrow and reticulocyte mRNA from another case of Hb S-β^0-thalassemia and one case of homozygous β^0-thalassemia,[78,81] reticulocyte mRNA from two Chinese siblings with homozygous β^0-thalassemia,[80] reticulocyte mRNA from three cases of Hb S-β^0-thalassemia, two cases of homozygous β^0-thalassemia, and one case of Hb Lepore-β^0-thalassemia.[79,88] In all cases there was undetectable synthesis of β^A chains in the cell-free system (Figure 4) despite use of saturating amounts of the thalassemic mRNA in some cases, which should favor translation of β chain mRNA in the cell-free systems used. These results indicate that in non-Ferrara type β^0-thalassemia there is absence of functional mRNA for β^A globin chains and no evidence of deficiency of another factor specifically required for β chain synthesis.

E. Ferrara-type β^0-Thalassemia

One possible exception to the rule of deficient mRNA in thalassemia may be the (homozygous) β^0-thalassemia of the Ferrara region of northern Italy. Intact reticulocytes from these patients synthesize no β chains;[9,84] in addition, there is no evidence in these patients for the synthesis of incomplete β globin chains.[59]

Conconi et al.[84] have reported that ribosomes isolated from patients with homozygous Ferrara β^0-thalassemia will synthesize β^A globin chains when added to a human reticulocyte cell-free system that contains nonthalassemic reticulocyte supernatant fraction. The same ribosomes incubated in the same cell-free system but in the presence of thalassemic reticulocyte supernatant synthesize no β chains but do synthesize α and γ chains. To prove that the normal supernatant was not providing β^A chain mRNA to the Ferrara ribosomes, supernatant from reticulocytes of patients with sickle cell anemia ($\alpha_2\beta^S_2$) was substituted and gave similar results: synthesis of β^A chain (but not β^S chain) was observed after incubation of the Ferrara ribosomes in the cell-free system. Furthermore, the "induction" of β^A chain synthesis by the nonthalassemic supernatant was not abolished by pretreatment of the latter with ribonuclease but was abolished by pretreatment with trypsin.[86] The "induced" β^A chain was identified as such not only by its chromatographic properties on carboxymethylcellulose columns but by analysis of tryptic peptides. The newly synthesized radioactive peak which cochromatographed with the β chain optical density marker contained radioactive β^A chain-specific peptides (although total radioactivity was frequently quite low);[84] when Hb S supernatant was used, there

reconstituted cell-free system containing Ferrara ribosomes and nonthalassemic supernatant.[86] However, there was unexplained absence of β^A chain synthesis and the appearance of γ chain synthesis when adult nonthalassemic ribosomes were incubated with the Ferrara thalassemic supernatant.[86]

One of the difficulties with these studies is that they rely on a cell-free system that has very low activity and which contains some surprising components such as 9.5 m*M* Mg^{++} (6.5 μmol in 0.68 ml)[85] (which is much higher than that used in all other functional mammalian cell-free systems) and thalassemic ribosomes obtained in part from sonicated lysates[84] (this treatment would be expected to dissociate mRNA from ribosomes). For these reasons the results obtained from this type of cell-free system have been viewed with some skepticism.

It should also be pointed out that when β chain is synthesized by Ferrara ribosomes in the cell-free system, less β than α chain is synthesized,[84] so that the β/α synthetic ratio appears somewhat similar to that of β^+-thalassemia. Conceivably, these patients could have a defect similar to patients with β^+-thalassemia, and an additional defect causing suppression of their already reduced capacity to synthesize β chains.

An even more remarkable observation has been made by Conconi and his colleagues.[86] When Ferrara thalassemia patients receive blood transfusions it is possible to detect small amounts of β chain synthesis in their circulating red blood cells if approximately 10 to 15 days after the transfusion intact peripheral blood cells are incubated in the presence of a radioactive amino acid.[86] This finding is interpreted as indicating that the "factor" that Ferrara thalassemics lack can be provided by normal intact red cells and somehow transferred to the patients' own cells to stimulate the synthesis of β chains in these cells by translation of their usually inactive β chain mRNA.

However, it has not yet been unequivocally established that the observed β chain synthesis occurs in the patients' own cells and not in transfused cells (i.e., reticulocytes originating from stem cells infused with the transfused blood). Further developments in this fascinating condition are awaited with great interest. It is certainly conceivable that Ferrara-type β^0-thalassemia is a distinctly different genetic variant of β-thalassemia in which deficiency of functional mRNA is not the factor (or not the only factor) responsible for absence of β chain synthesis.

F. Reticulocyte mRNA in α-Thalassemia

Reticulocyte mRNA from patients with the α-thalassemia syndrome of Hb H disease has also been tested in the Krebs II cell-free system.[83,87] Similar results were obtained in the three cases examined in the two studies. The Hb H disease mRNA directed the synthesis of a great excess of β chains over α chains (Figure 5A). The imbalance of β to α chain synthesis observed in the cell-free system was much greater than in the intact cells of the same patient (Figure 1C). Some of this difference might be attributed to the property of the Krebs II cell-free system to synthesize more β than α chain in the presence of saturating amounts of mRNA;[82,87] however, the same exaggerated imbalance of globin chain synthesis was observed in the cell-free system when Hb H disease mRNA was used in rate limiting amounts.[83,87] Therefore, it appears that in Hb H disease reticulocytes there is a greater deficiency of functional α chain mRNA than suggested by the ratio of β to α chain globin synthesis in the intact cells. This exaggerated deficiency of α chain mRNA has also been confirmed by mRNA-DNA hybridization studies (see next section). These findings suggest the presence of some translational control in the Hb H disease reticulocyte by which the decreased α chain synthesis limits the synthesis of β chains, leading to less β to α chain imbalance than would be expected from the β to α chain mRNA content of the cells. A possible role for α chains in the control of β chain synthesis has been suggested by a number of workers.[89-93] In rabbit reticulocytes, one finds a small pool of soluble, free α chains (not bound to β chains),[89,90] as well as a small quantity of completed α chains bound to ribosomes.[89] It has been proposed that these α chains bind to β chains while the latter are still being synthesized on the ribosome, and aid in the release of the β chains from ribosomes in the form of $\alpha\beta$ dimers. This hypothesis is supported by results of experiments that have examined the effect of added free globin chains on the synthesis of hemoglobin in cell-free reticulocyte lysates.[91-93]

G. Summary and Conclusions

With the possible exception of Ferrara-type β^0 thalassemia, cell-free translation of thalassemic globin mRNA, in all cases tested, has resulted in

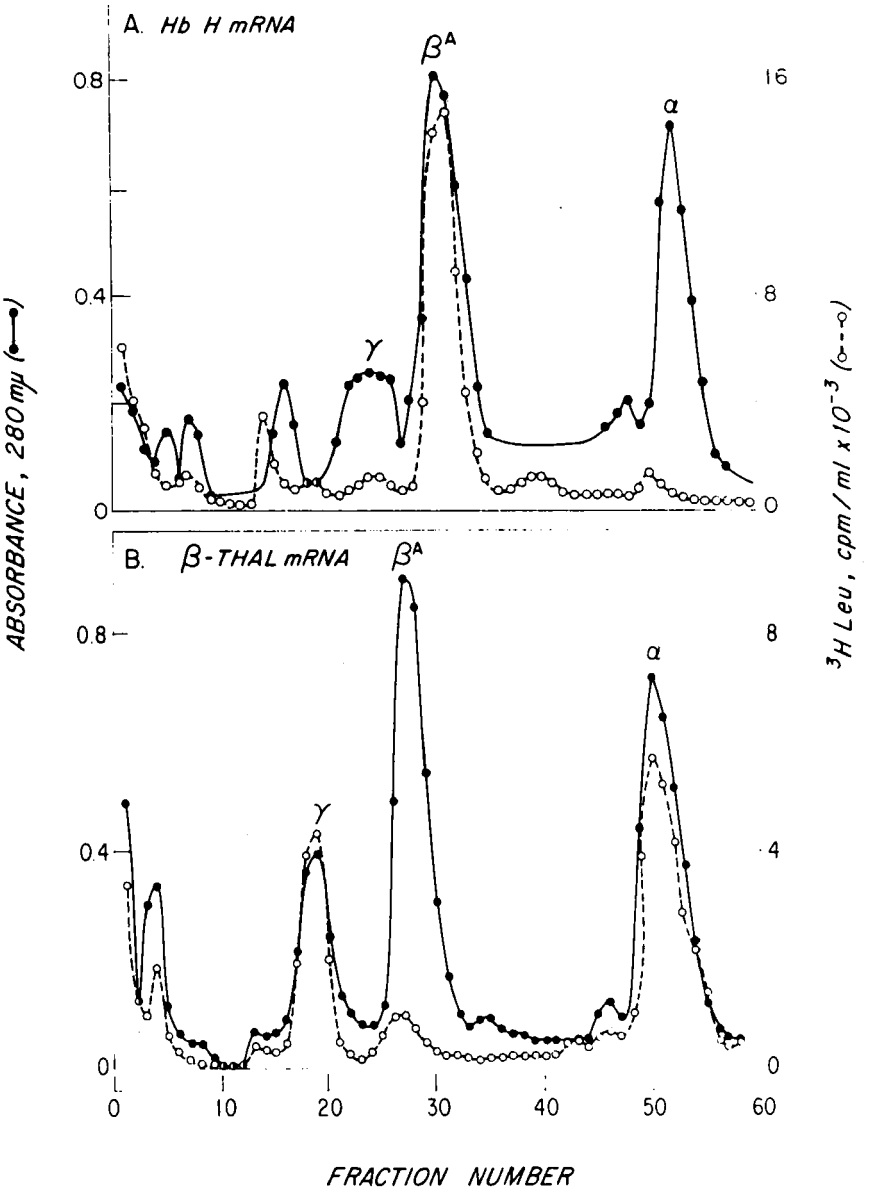


FIGURE 5. Cell-free translation of thalassemic globin messenger RNA. Globin mRNA was isolated from reticulocyte lysates and translated in the Krebs II cell-free system supplemented by ribosomal salt wash fraction, as in Figure 4B. A. Patient with Hb H disease (same patient as in Figure 1C). In the cell-free system there is a much greater excess of β chain synthesis, relative to α chain synthesis, than in the same patient's intact reticulocytes (Figure 1C). B. Patient with homozygous β^+-thalassemia (same patient as in Figure 1B). In the cell-free system the β/α globin chain synthetic ratio is similar to that in intact reticulocytes of the same patient (Figure 1B) but there is somewhat more γ chain synthesis relative to α chain synthesis in the cell-free system than in intact cells. ●——●: optical density of marker globin chains. ○- - -○: radioactivity incorporated into newly synthesized protein in the cell-free system.

the same type of imbalance of globin chain synthesis as is observed in intact thalassemic cells. These studies provide direct evidence for a deficiency of functional mRNA for α or β globin chain in α and β thalassemia, respectively. These studies, however, only demonstrate deficiency of biological activity and do not necessarily indicate that the affected globin mRNA is deficient in absolute

chemical amounts. The studies still leave open the possibility that there exist in thalassemia substantial amounts of an abnormal mRNA which is unable, both in the intact thalassemic cells and in heterologous cell-free systems, to function normally in protein synthesis.

V. QUANTITATIVE STUDIES OF GLOBIN MESSENGER RNA IN THALASSEMIA BY MOLECULAR HYBRIDIZATION

A. Principles of the Hybridization Assay

Ideally, one would like to assay the relative amounts of α and β chain mRNA in thalassemic cells by a direct chemical quantitative assay that does not rely on the ability of the mRNA to function in a cell-free system. Such an assay is essential to definitively settle the issue of whether or not there exists in thalassemia a substantial amount of an mRNA (for α or β globin chains) that is unable to function normally in protein synthesis, either because it is structurally abnormal (in a segment other than its strict structural gene information) or because of some other abnormality in the cells' protein synthesis "machinery." Such an assay has recently become possible.

A technique often used for the identification and/or quantitation of a specific mRNA present in a mixture of RNA's is that of molecular hybridization of the RNA mixture with DNA complementary to the specific mRNA in question. Until recently the use of this technique has been limited, in eukaryotic systems, by the lack of availability of specific DNA probes. However, a number of important research contributions have made possible the acquisition of separate radioactive DNA probes specific for α and β globin mRNA. The RNA-dependent DNA polymerase of avian myeloblastosis (also referred to as reverse transcriptase and as AMV DNA polymerase) can synthesize in vitro a radioactive DNA copy (cDNA) of a portion of globin mRNA.[94-96] The reaction requires oligo dT as a primer, which presumably acts by binding to the poly A sequence situated at the 3′-terminus of the globin mRNA. The size of the cDNA[94-96] and the nature of the RNA sequences transcribed from it[74] suggest that the cDNA may be the copy of approximately 75% of the mRNA. The radioactive cDNA that is synthesized will hybridize almost completely to purified globin mRNA,[94-96] but with the use of total reticulocyte mRNA the probe must consist of a mixture of α and β chain-specific sequences. It is, however, possible to obtain purified rabbit globin mRNA that is 80 to 90% enriched in α or β chain-specific mRNA. The α chain mRNA is obtained from a ribonucleoprotein particle which is found in the postribosomal supernatant fraction of rabbit reticulocytes[97] – a serendipitous observation; the β chain mRNA is obtained from the largest polysomes of *O*-methyl-threonine-treated reticulocytes.[98] *O*-Methyl-threonine is an isoleucine analogue and the rabbit α and β chains contain isoleucine in different positions; in the β chain isoleucine is first found only at position 112 of the sequence, whereas in the α chain isoleucine is first encountered at position 10. In the presence of *O*-methyl-threonine, polysomes bearing α and β mRNA will be "blocked" at the site of the first isoleucine codon on the mRNA. In the case of β mRNA the polysomes will be large because many other ribosomes will have started to translate the mRNA in the time it takes the first ribosome to reach position 112; on the other hand, the α chain polysomes will be blocked early in the translation process and will accumulate as small polysomes. This technique can also serve as a means to obtain relatively purified α chain mRNA. In the *O*-methyl-threonine procedure one gets an exaggeration of the normal asymmetrical distribution of α and β chain synthesizing polysomes with β chains on more heavily sedimenting polysomes and the α chains on more slowly sedimenting polysomes.[43-45]

The purified α and β mRNA's are efficient substrates for AMV DNA polymerase and the resulting cDNA's are specific for α and β chain-specific sequences, as evidenced by cross-hybridization experiments carried out between the two types of mRNA's and cDNA's[99-101] (Figure 6). Sufficient homology exists between human and rabbit globin chain amino acid (and mRNA) sequences to permit the use of this type of hybridization assay to detect (and quantitate) relative amounts of human α and β chain mRNA sequences in human reticulocyte RNA.[99,100] An outline of the general procedure is shown in Figure 7, parts A and B. The amount of hybridization is detected by digesting the reaction mixture, at the end of the hybridization period, with the S_1 nuclease of *Aspergillus oryzae* (or another

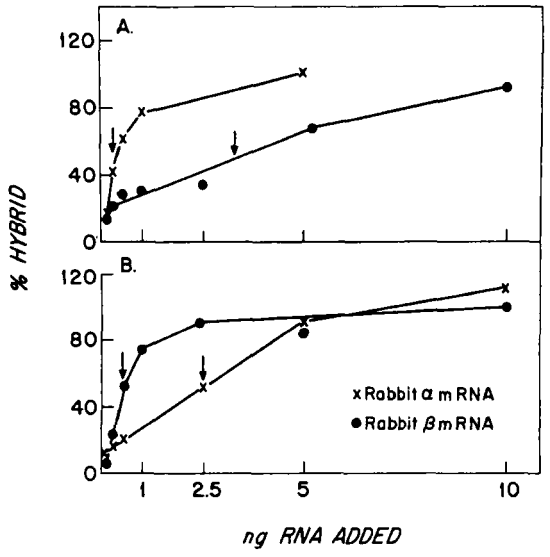


FIGURE 6. Molecular hybridization of chain-specific rabbit globin messenger RNA's to their DNA copies. Saturation hybridization curves were obtained using a constant amount of radioactive DNA copy (cDNA) obtained by incubating rabbit α or β globin mRNA with the RNA-dependent DNA polymerase of avian myeloblastosis virus (AMV DNA polymerase). Increasing amounts of α or β globin mRNA were added to individual reaction mixtures and hybridization allowed to proceed for 40 hr at 65°C – a time sufficient to allow hybridization of all the RNA to the cDNA.[99] The amount of hybridization was then determined by digestion of the reaction mixture with the S_1 nuclease of *Aspergillus oryzae* (Figure 7),[99] which degrades single-stranded nucleic acids but leaves intact DNA-RNA hybrids. A. Hybridization using constant amount of rabbit α cDNA. B. Hybridization using constant amount of rabbit β cDNA. X: rabbit α mRNA derived from ribosome free supernatant.[97] ●: rabbit β mRNA derived from largest polysomes of *O*-methyl-threonine-treated reticulocytes.[98] The arrows indicate the points of half maximal (saturation) hybridization: eight times as much α mRNA is required to achieve half saturation with β cDNA than is required to achieve half saturation with its own α cDNA. The "α mRNA" therefore contains α:β mRNA sequences in the ratio of 8:1. Conversely, six times more β mRNA is required to obtain half saturation with α cDNA than is required to achieve half saturation with the β cDNA; the "β mRNA" therefore contains α:β mRNA sequences in the ratio of 1:6.[99] (From Housman, D., Forget, B. G., Skoultchi, A., and Benz, E. J., Jr., *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1574, 1972. With permission.)

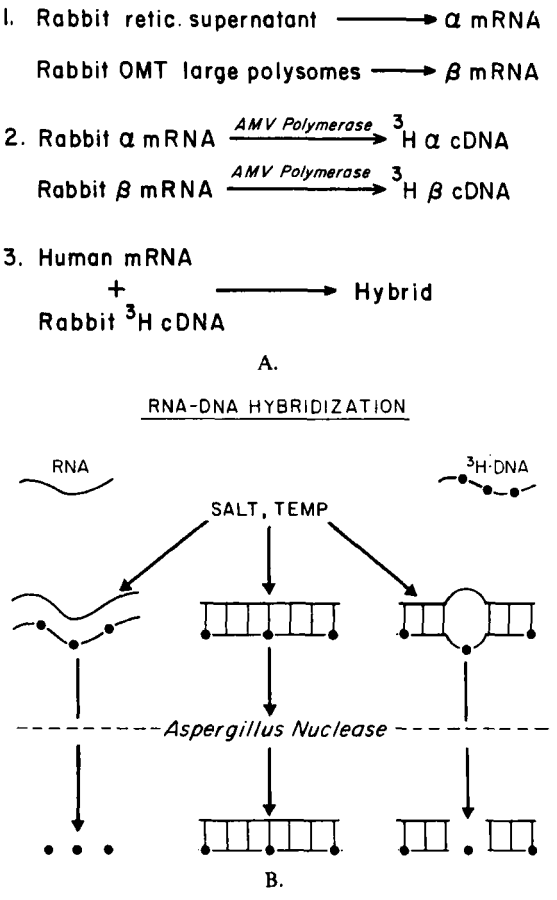


FIGURE 7. Hybridization procedure. A. Outline of the general approach. Sufficient homology exists between rabbit and human mRNA's and cDNA's that cross hybridization will occur between human and rabbit nucleic acids. AMV polymerase refers to the RNA-dependent DNA polymerase of AMV. B. Schematic representation of the hybridization assay. Three possible results are illustrated. On the left there is no homology between mRNA and cDNA and therefore no hybrid formation. The S_1 nuclease of *Aspergillus oryzae* will completely degrade the radioactive cDNA and no TCA-precipitable counts will be recovered. In the center there is complete homology between mRNA and cDNA, and formation of a perfect hybrid; if sufficient mRNA is present to bind all of the cDNA, 100% of the labeled cDNA will be resistant to S_1 nuclease and be recovered as TCA-precipitable counts (see text). Finally, on the right there is partial homology between mRNA and cDNA; an imperfect hybrid is formed with redundant loops of cDNA and mRNA in the nonhomologous regions. These loops will be degraded by the S_1 nuclease and less than 100% of the labeled cDNA will be recovered as TCA-precipitable counts even if the mRNA is present in a great excess over the cDNA. This last situation is representative of the conditions when human mRNA is hybridized to rabbit cDNA; even at saturating amounts of mRNA, less than 100% hybridization will occur because there is only partial homology between the species. Therefore, the final percent hybridization will be proportional to the degree of homology between mRNA and cDNA.

enzyme) which specifically degrades single-stranded nucleic acids and leaves intact double-stranded hybrids (Figure 7B). Therefore, even if there is not perfect homology between RNA and cDNA (as in human-rabbit cross-hybridization experiments), the portions of the hybrid complex in which there is good homology will be protected from nuclease digestion, and these nondegraded hybrid complexes will be precipitated by 10% TCA and retained on Millipore® filters. In such cases, however, one never recovers 100% of the counts of the radioactive cDNA probe. The non-homologous (nonhybridized) regions of the cDNA are degraded by the nuclease and therefore remain soluble in the presence of TCA and pass through the filters. The maximum amount, or percentage, of the probe recovered is proportional to the degree of homology between mRNA and cDNA. When normal (nonthalassemic) human globin mRNA is hybridized to the rabbit α and β cDNA probes (Figure 8), it can be seen that there is more homology between human and rabbit α chain mRNA sequences than between the β chain mRNA sequences. This finding is somewhat surprising, since by amino acid composition alone one would expect the opposite: the amino acid sequences of the human and rabbit globin chains differ by 26 out of 141 amino acids for the α chain, and by only 16 out of 146 amino acids for the β chain. Amino acid composition alone, however, does not reflect differences in mRNA sequences in the third or "wobble" position of the mRNA codons, which may be more or less homologous between human and rabbit α and β chain mRNA's. The untranslated sequences of these mRNA's may also be more or less homologous.

The hybridization reaction can be carried out in two different ways. The first procedure involves performing saturation hybridization curves,[99,101] as in Figures 6 and 8; a constant amount of cDNA is hybridized with variable amounts of mRNA ranging from limiting to saturating amounts. This method has the advantage that the results are expressed on a linear scale; differences ranging from one- to tenfold can, therefore, be more easily measured than on a logarithmic scale. The ratio of the amounts of input RNA required to achieve half maximum (saturation) hybridization against β and α cDNA is the ratio of α to β mRNA sequences in the RNA. The second procedure involves performing Cot curves;[100] the amounts of DNA and RNA are constant (the RNA usually being present in excess), and the time of hybridization is varied in a number of replicate samples. Achievement of maximum hybridization is a function of two factors: the time (t) of the reaction, and the concentration (C) of the nucleic acid being tested (Cxt, thus Cot); percentage of hybridization is plotted against the log of the Cot. The curve obtained is an expression of the rate at which hybridization occurs, which in turn is affected by the amount of mRNA present. The Cot curve is the classical tool used to study the frequency of specific sequences in DNA.[102] Use of a logarithmic scale, however, makes it difficult to precisely quantitate differences between 1 and 10. Both techniques have been applied to the quantitation of α to β mRNA sequences in thalassemia.[99,100]

B. Hybridization Studies in α- and β^+-Thalassemia

Housman et al.[99] have studied thalassemic mRNA by saturation hybridization. When α -thalassemia (Hb H disease) mRNA is hybridized with rabbit α cDNA (Figure 8A), five to ten times more RNA is required to achieve maximum hybridization than is necessary for the nonthalassemic control. On the other hand, both α--thalassemia and control mRNA achieve maximum hybridization with the rabbit β cDNA (Figure 8B) at roughly the same RNA input. The α to β mRNA content (on the basis of the ratio of the half saturation values) was 1:6. In Hb H disease, therefore, one observes a true quantitative deficiency of α mRNA sequences relative to β mRNA sequences, and this deficiency is greater than expected from the intact cell β/α globin chain synthetic ratio. Similar observations were made in a second case of Hb H disease,[99] and the mRNA preparations of these two patients were the same which stimulated a great excess of β chain synthesis over α chain synthesis when translated in the Krebs II cell-free protein-synthesizing system (Figure 5A).[88] By similar hybridization experiments, total absence of α mRNA has been demonstrated by Kan et al. in reticulocytes of an infant with homozygous α-thalassemia (hydrops fetalis with Hb Bart's).[114]

When β-thalassemia mRNA was hybridized with rabbit α and β cDNA's (Figure 8)[99] there was only a relatively small difference between the half saturation values, a β to α mRNA ratio of 1 to 2, whereas the β to α synthetic ratio in the cell-free system and intact cells was 1 to 10. This unexpected finding was attributed to cross

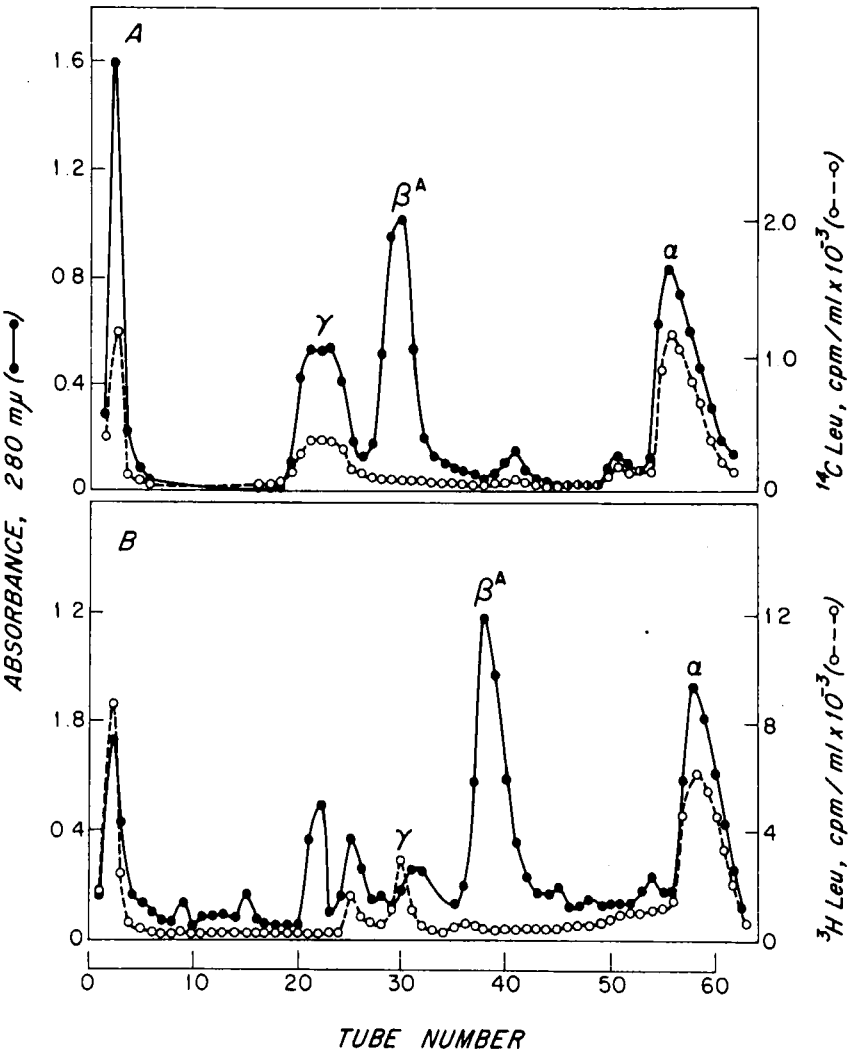


FIGURE 4. Globin synthesis in homozygous β^0-thalassemia. A. Globin synthesis by intact reticulocytes of a patient (of southern Italian ancestry) with homozygous β^0-thalassemia. Experimental conditions are similar to those described in Figure 1. There is absent β chain synthesis and excess synthesis of α chains relative to γ chains (of Hb F). The optical density of β chains was contributed by transfused red cells. B. Translation of reticulocyte globin mRNA from the same patient in the Krebs II cell-free system. Experimental conditions are similar to those described in Figure 3 except that the cell-free system was supplemented by rabbit reticulocyte salt wash fraction.[83] The pattern of globin synthesis is similar to that seen in intact reticulocytes; there is absent β chain synthesis. ●——●: optical density of marker globin chains. o---o: radioactivity incorporated into newly synthesized protein.

was no radioactivity in the peptide specific for the β^S globin chain, β^S-T_1. In a subsequent study[85] it was shown that patients of southern Italian ancestry with β^+-thalassemia do not manifest this phenomenon; in the cell-free system the amount of β^A chain synthesis is similar whether thalassemic or nonthalassemic supernatant is used.

These results were interpreted as indicating that in Ferrara thalassemia substantial amounts of β globin mRNA are present but not translated because of the absence of a specific (protein) factor present in nonthalassemic supernatant. The authors did not believe that a specific inhibitor was present in the Ferrara thalassemic cells because the Ferrara supernatant did not inhibit the β^A chain "induction" when it was added to the

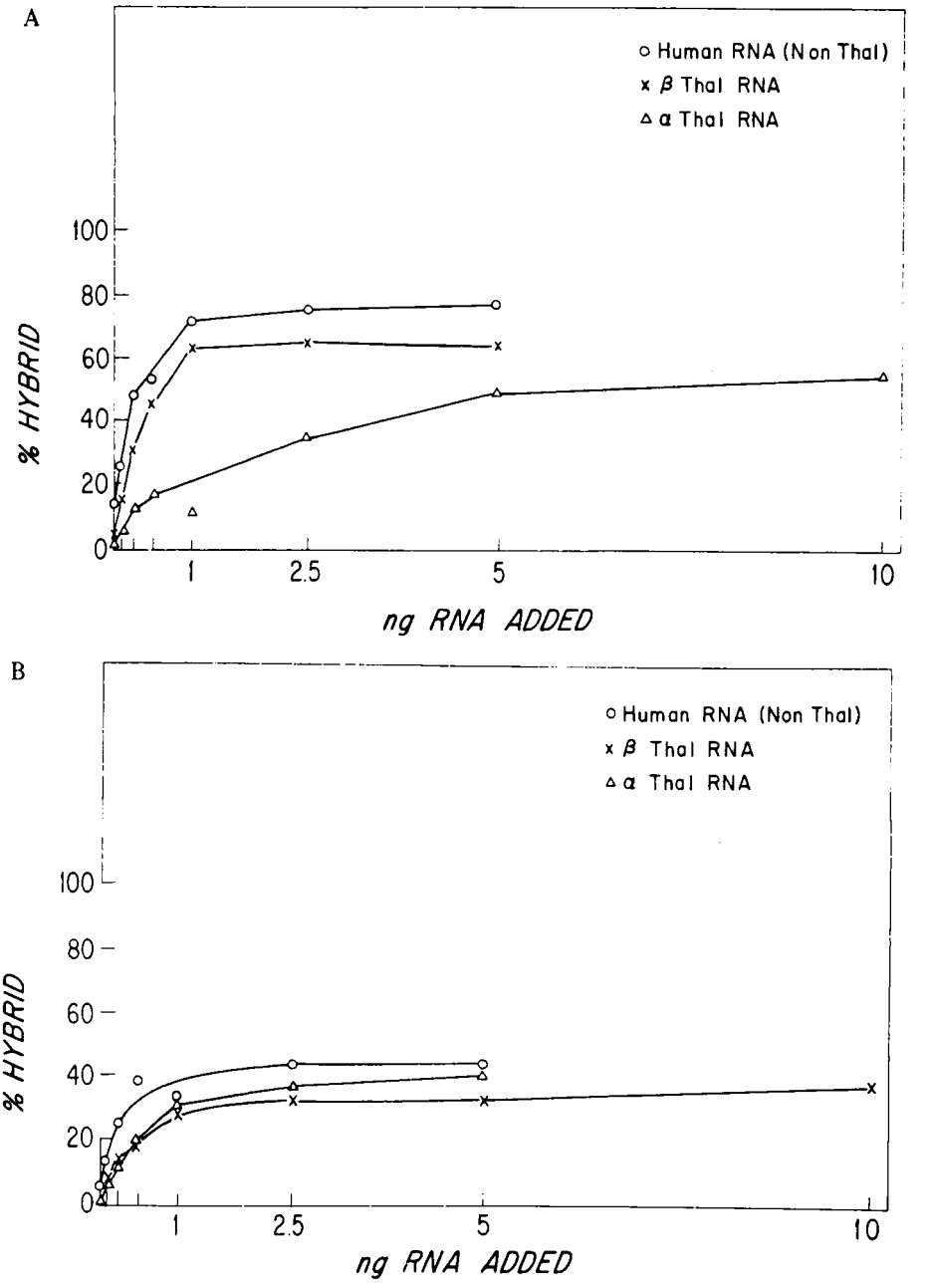


FIGURE 8. Hybridization of human globin messenger RNA's with rabbit α and β cDNA. Globin mRNA was isolated from total reticulocyte lysates of patients with nonthalassemic hemolytic anemia and Hb A (○), homozygous β^+-thalassemia (x), and Hb H disease (Δ). Various amounts of the RNA's were hybridized with a fixed amount of rabbit cDNA (65°C for 40 hr)[99] to obtain saturation curves (see Figure 6 caption). A. Hybridization with rabbit α cDNA. B. Hybridization with rabbit β cDNA. In the case of the Hb H disease mRNA (Δ) (same mRNA as in Figure 5A), six times more RNA is required to achieve half saturation with the α cDNA than with the β cDNA; this mRNA therefore contains α:β mRNA sequences in the ratio of 1:6. The nonthalassemic mRNA achieves half saturation with both cDNA's at roughly the same RNA input and therefore contains α:β mRNA sequences in the ratio of roughly 1:1. In the case of the β thalassemic mRNA (same mRNA as in Figure 5B), there is only a 1-to-2 difference in the half saturation values, indicating an α to β mRNA ratio of 2:1 whereas the intact cell and cell-free α/β synthetic ratio of this patient was 10:1 (Figures 1B and 5B). The maximum percentage of hybridization achieved by human mRNA with rabbit cDNA is higher with the α cDNA (60 to 70%) than with the β cDNA (35 to 40%), indicating greater homology between human and rabbit α chain mRNA's than between human and rabbit β chain mRNA's.

hybridization of γ chain mRNA (of Hb F) with the β cDNA; the mRNA of the patient in question had substantial amounts of γ chain activity[99] (Figure 5B), and a fair degree of homology exists between β and γ chain amino acid sequences (39 amino acid differences in 146 residues). A more specific assay was therefore devised; the α-thalassemia mRNA that was shown to contain α to β mRNA sequences in a ratio of at least 1:6 (Figure 8) was transcribed into cDNA and used as a probe which was perfectly complementary to human β mRNA. By performing melting curves of hybrids between this cDNA and mRNA containing either β mRNA alone or β plus γ chain mRNA activity, it was determined that increasing hybridization temperature from 65 to 78 to 80°C left the β mRNA-β cDNA hybrid intact but dissociated the γ mRNA-β cDNA hybrid. The β-thalassemic mRNA was then tested under these conditions (Figure 9) and showed a marked deficiency of β chain-specific sequences: β to α mRNA ratio of 1:10,[99] similar to the synthetic ratio obtained in the intact cell. Reticulocyte RNA from three other patients with β^*-thalassemia was studied, and in all cases specific hybridization assays revealed deficiency of β chain-specific mRNA sequences, consistent with the degree of deficient β chain synthesis observed in the patients' intact cells.[79,103] The RNA used in these studies was obtained from total reticulocyte lysates rather than polysomes; the demonstrated deficiency of α and β mRNA therefore represents total cellular deficiency and not simply deficiency of polysome-bound mRNA. These findings rule out the existence in thalassemic reticulocytes of large amounts of an mRNA that is unable to bind to ribosomes and therefore remains inactive and free in the postribosomal supernatant.

Kacian et al.[100] studied hybridization of mRNA by Cot curves. The mRNA was incubated in the same reaction mixture with [^{3}H]labeled rabbit β cDNA and [^{32}P]labeled rabbit α cDNA.

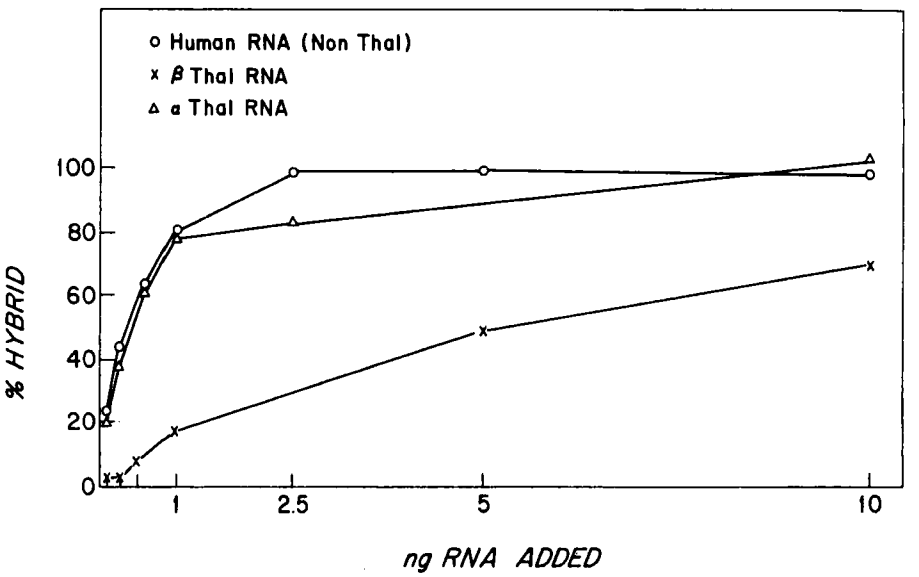


FIGURE 9. Hybridization of human globin messenger RNA's with cDNA of Hb H disease mRNA. Hybridization was performed as in Figure 8, but using as a source of human β cDNA the cDNA of the Hb H disease mRNA which was shown in Figure 8 to contain α:β mRNA sequences in the ratio of 1:6. Hybridization temperature was increased from 65 to 78°C to render unstable (melt) imperfect hybrids between γ chain mRNA and β cDNA. The symbols and the specific mRNA's are the same as in Figure 8. In these conditions the hybridization pattern of the nonthalassemic and Hb H disease mRNA is not altered; half saturation of hybridization is achieved at approximately the same RNA input as with the rabbit β cDNA, but now 100% hybridization is achieved, indicating formation of a perfectly homologous hybrid. Now, however, ten times more β thalassemic mRNA is required to achieve half saturation with the β cDNA than was required to achieve half saturation with the rabbit α cDNA (Figure 8A); the α·β mRNA content of this RNA is therefore 10:1. The previous ratio of 2:1 (Figure 8b) was probably due to hybridization of the patients's γ chain mRNA (Figure 5B) with the rabbit β cDNA, giving a falsely high value for β chain mRNA content.

The ratio of α to β mRNA in a sample of RNA was expressed as the ratio $\beta\,Cot_{1/2}/\alpha\,Cot_{1/2}$, i.e., the Cot values at the midpoint between minimum and maximum hybridization of the RNA with β and α cDNA's, respectively. Nonthalassemic RNA gave ratios ranging from 0.8 to 1.3 in five of six samples, but one sample gave a ratio of 0.5; the mean was 0.9. RNA from two patients with Hb H disease gave α/β mRNA ratios of 0.6 and 0.7 (intact cell α/β synthetic ratios were 0.45 and 0.55). RNA from seven patients with β-thalassemia gave α/β mRNA ratios ranging from 1.4 to 4.9. In five of the seven cases, the α/β ratio obtained by hybridization (3.3 to 4.9) was considered to correlate well with the α/β synthetic ratios obtained by intact cell incubation (3.8 to >20) and cell-free translation of the mRNA (2.2 to 6.2). In the other two cases, hybridization revealed only slightly decreased β mRNA (α/β: 1.4 and 1.7) whereas a greater deficiency was expected on the basis of intact cell incubation (α/β: 3.5 and >20) and cell-free translation (α/β: 4.5 and >20). The latter discrepancy may have been due to the previously mentioned problem of cross hybridization between γ mRNA and β cDNA. Because rabbit (and not human) β cDNA was used in this study, stringent conditions to eliminate this cross hybridization could not be employed. The study used mRNA obtained both from polysomal RNA and total lysate RNA, and similar results were obtained with both types of RNA, providing additional evidence against the existence of a nonfunctional supernatant pool of mRNA in thalassemia.

C. Hybridization Studies in β^0-Thalassemia

In view of the findings in Ferrara-type β^0-thalassemia, much attention has been focused on other types of β^0-thalassemia. There have not yet been any reports of hybridization of Ferrara β-thalassemia RNA, but Forget et al. have reported studies using RNA from southern Italian and Sicilian patients with β^0-thalassemia.[104] The most striking demonstration of β mRNA deficiency was provided by the study of RNA from three siblings with homozygous δβ-thalassemia, a rare condition in which there is total absence of the minor hemoglobin Hb A_2 as well as Hb A (the patients have 100% Hb F), in contrast to the usual forms of β-thalassemia in which Hb A_2 is usually elevated. When RNA from these patients is hybridized with human β cDNA under stringent conditions to eliminate γ chain cross hybridization, there is no

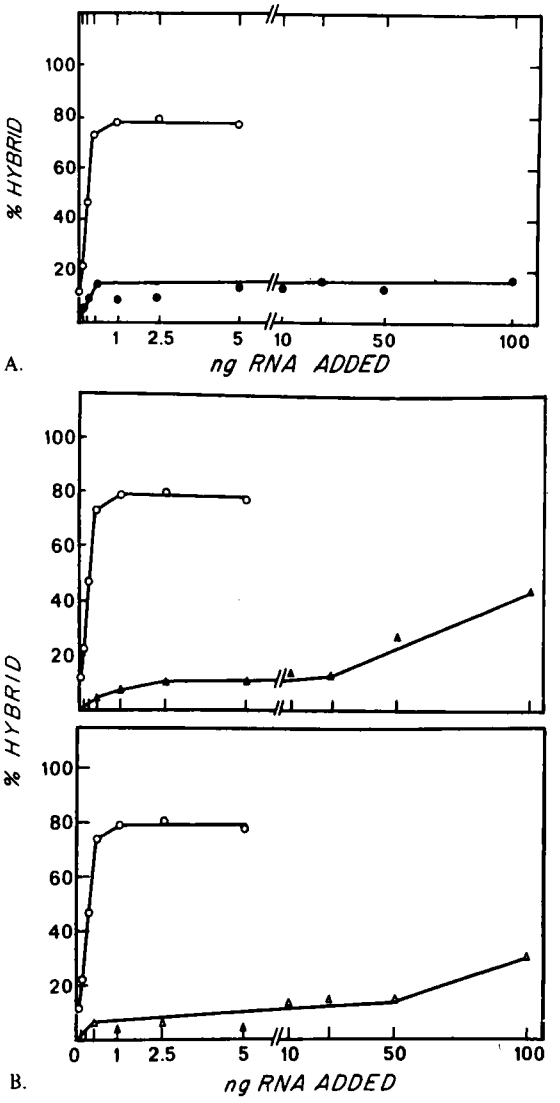


FIGURE 10. Hybridization of messenger RNA from patients with β^0-thalassemia. mRNA was hybridized as in Figure 9 with cDNA of Hb H disease mRNA as a probe for human β chain mRNA; the cDNA, however, had two to three times the specific radioactivity as the cDNA used in Figure 9.[104] A. Pooled reticulocyte mRNA from three siblings with homozygous δβ-thalassemia, whose red cells totally lack Hb A and Hb A_2[104] (●); nonthalassemic control (○). B. Reticulocyte RNA from two unrelated patients (○ and ▲) with homozygous β^0-thalassemia of the high Hb A_2 variety[104] (▲: same patient as in Figure 4); nonthalassemic control (○). In the case of homozygous δβ-thalassemia, (A), there is total absence of RNA that will hybridize with the β cDNA (above the 15% hybridization baseline contributed by α chain-specific sequences in the Hb H disease cDNA), even at extremely high RNA inputs. In the case of the two patients with homozygous β^0-thalassemia (B), some hybridization is seen but only at an extremely high RNA input; this hybridization represents less than 1% of the sequences in these RNA preparations which hybridized with α chain cDNA, and is presumably due to hybridization of δ chain mRNA with β cDNA.

significant hybridization even though 200 times more RNA is added than is necessary to achieve maximum hybridization with nonthalassemic RNA (Figure 10A).[104] A small but constant amount of hybridization is observed at a level of approximately 15%. This probably represents hybridization of α chain-specific mRNA in the RNA preparation to α cDNA sequences present in the Hb H disease cDNA. As previously discussed (Section V.B, "Hybridization Studies in α- and β^+-Thalassemia," and Figure 8), the Hb H disease mRNA has been shown to have α and β mRNA sequences in the ratio of 1 to 6 (roughly 15% α mRNA and 85% β mRNA). The cDNA of this mRNA would be expected to have roughly the same relative content of α- and β-specific sequences. Hybridization of RNA from two unrelated patients with A_2-β^0-thalassemia revealed a small amount of hybridization above the baseline (Figure 10B), but only at RNA inputs 100 times greater than required for maximum hybridization of nonthalassemic RNA. This small amount of hybridizable material (less than 1% of the α chain mRNA in the samples) could be δ chain mRNA which would be expected to hybridize with β cDNA even under the stringent conditions utilized because of the very close homology between δ and β chains (only 10 amino acids different in 146 residues). These studies were interpreted as indicating absence of β chain mRNA in these three instances of β^0-thalassemia of southern Italian and Sicilian origin.

D. Summary and Conclusions

Hybridization studies of thalassemic mRNA to α and β chain-specific cDNA's indicate that in Hb H disease and β^+-thalassemia there is an absolute decrease in the amount of α and β chain mRNA, respectively, compared to the amount of mRNA present for the other chain.[99,100] In α^0-thalassemia of Oriental origin and β^0-thalassemia of southern Italian origin, there appears to be absence of α and β chain mRNA, respectively.[104,114] These demonstrations of true quantitative deficiency of chain-specific mRNA's in thalassemia still leave unresolved the precise molecular basis for this deficiency. The following processes can all be considered as feasible explanations and remain to be investigated:

1. Absent (with or without gene deletions) or decreased transcription of the globin gene into mRNA
2. Abnormal processing of the mRNA precursor molecules transcribed from the gene, and/or abnormal transport of the mRNA from nucleus to cytoplasm
3. Synthesis of an mRNA that is structurally abnormal in its untranslated regions and thus rendered unstable, and susceptible to early degradation in immature erythroid precursor cells, either because of its secondary structure alone or because it is unable to bind normally to ribosomes.

In fact, two different groups have recently reported, evidence for α chain gene deletion in homozygous α-thalassemia.[115,116] These workers have hybridized α cDNA with total DNA extracted from the liver of stillborn infants with hydrops fetalis and Hb Bart's. No significant hybridization was demonstrated by Cot curves using α cDNA and the DNA from the hydropic infants, whereas normal Cot curves were obtained using the same cDNA and liver DNA from nonthalassemic infants.

VI. STRUCTURAL STUDIES OF GLOBIN MESSENGER RNA

A. Nonthalassemic Globin mRNA

Another approach to the study of qualitative or quantitative differences in α and β globin mRNA is the direct analysis of the primary structure or nucleotide sequence of the mRNA. A number of different techniques have been used for this purpose,[73] but probably the most successful has been the study of the [^{32}P]labeled RNA synthesized from globin cDNA by *E. coli* RNA polymerase.[74] The labeled synthetic RNA (cRNA) can be digested with specific nucleases and fractionated in two dimensions to give a characteristic "fingerprint" pattern (Figure 11A) analogous to peptide fingerprints of proteins. The individual spots can then be eluted and their sequence analyzed by further enzymatic degradation. A number of sequences of human globin mRNA have been determined by this procedure (Table 1). Most of the sequences match unique amino acid sequences in either α or β globin chains, but others do not, and presumably represent untranslated sequences of the globin mRNA. Certain of these

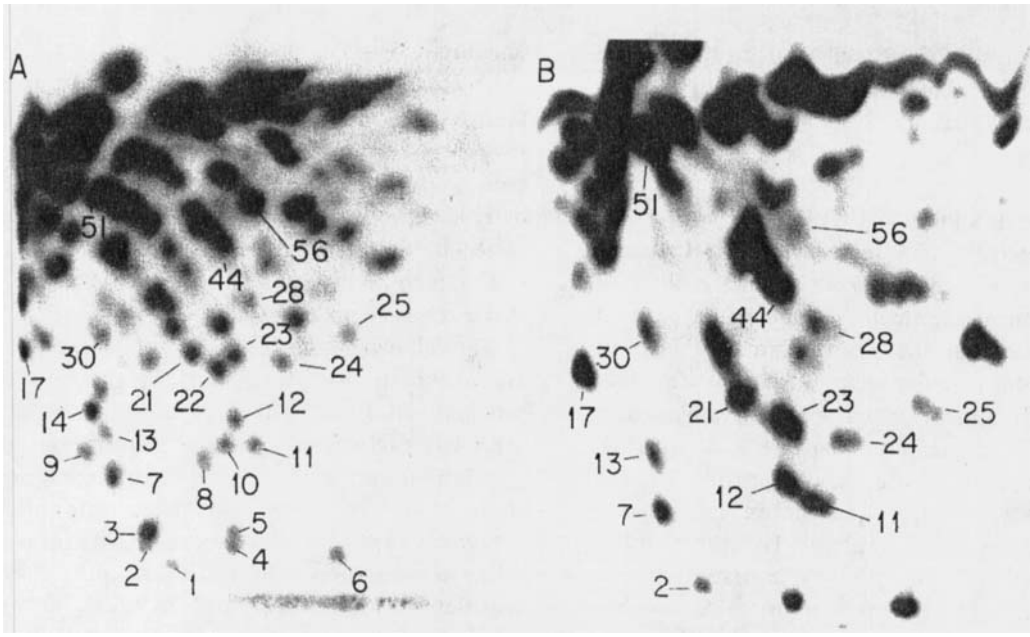


FIGURE 11. Nucleotide sequence analysis of human globin messenger RNA. Radioactive ([^{32}P] labeled) RNA (cRNA) was synthesized by incubating human globin cDNA with *E. coli* RNA polymerase.[73,74] The RNA was then digested with T_1 RNase and the digest fractionated in two dimensions: (1) from left to right by high voltage electrophoresis on urea-Cellogel® strips at pH 3.5; (2) from bottom to top by displacement chromatography ("homochromatography") on thin-layer plate of DEAE cellulose, using a solution of partially hydrolyzed nonradioactive yeast RNA in 7 *M* urea. The fingerprint pattern was revealed by radioautography. A. Fingerprint of RNase T_1 digest of nonthalassemic (sickle cell anemia) cRNA labeled with α[^{32}P]GTP. B. Fingerprint of RNase T_1 digest of Hb H disease cRNA (same patient as in Figures 5 and 8), labeled with α[^{32}P]GTP. The sequences of the numbered spots are listed in Table 1. The Hb H disease fingerprint is deficient in a number of spots which are prominent in the nonthalassemic RNA and which match α chain amino acid sequences. This technique therefore confirms the deficiency, in Hb H disease, of α chain mRNA, previously demonstrated by cell-free translation (Figure 5) and hybridization (FIgure 8) techniques. The sequences also confirm the hypothesis of a chain termination mutation as the cause of the abnormally long α chain in Hb Constant Spring,[67,68] (spots number 14, 51, 56; see Table 1).

sequences, however, do match amino acid sequences in the abnormally long segment of the Hb Constant Spring α chain, and provide support for the origin of this abnormal chain by chain termination mutation. The evidence for this conclusion is as follows. The sequence of spot number 56 (Table 1) contains the codon NGU (which could be a codon for arginine, the last amino acid [number 141] of the α chain), followed by the chain termination codon UAA. A single base substitution in this codon, UAA to CAA, would give the codon for glutamine which is the amino acid in position 142 of the α CS chain. More importantly, however, the nucleotide codon sequence following the UAA of spot number 56 is GCN, which is the codon for alanine, and alanine is precisely the amino acid found in position 143 of the α CS chain. In addition, the sequences of spot numbers 51 and 14, isolated from normal mRNA, also match other amino acid sequences in the abnormally long segment of the α CS chain. The sequences of spot numbers 51 and 56 were predicted, on theoretical grounds, to be the likely sequences for this region of the α chain mRNA by comparing the amino acid sequence of Hb CS to that of Hb Wayne[71,72] (see Section III.E.2, "Hemoglobin Constant Spring"). However, because these two nucleotide sequences are rather short, considered as isolated sequences, they can also be matched to other di- and tri-peptide sequences in normal α or β globin chains.[73,74] Spot number 14, on the other hand, is longer, and its sequence cannot be matched to any amino acid sequences in normal α and β globin chains. Spot number 14 must therefore be a normally untranslated nucleotide sequence of normal mRNA and its sequence can code for amino acids 155 to 158 of the α CS chain. The assignment of spots 51, 56, and 14 to α chain mRNA is reinforced by the fact that these sequences are deficient in fingerprints of mRNA from our patient with Hb H disease (see next section). In summary, then, nucleotide se-

quence analysis of normal and α thalassemic mRNA provides convincing evidence to confirm the hypothesis that Hb CS arose by chain termination mutation.

B. Thalassemic Globin mRNA

The fingerprint of α-thalassemia (Hb H disease) cRNA (Figure 11B) shows a distinctly different pattern from the normal. A number of spots that are prominent in the normal are very faint or absent in the α thalassemic cRNA; the sequences that are prominent match β chain amino acid sequences, whereas those that are faint match α chain sequences. These results provide another direct demonstration of quantitative deficiency of α chain mRNA in α-thalassemia. The fingerprint of β-thalassemia cRNA also demonstrated quantitative deficiency of many spots.[73] Among the prominent spots in the β-thalassemia cRNA were numbers 3, 14, 22, and 56 (Table 1), which match amino acid sequences in the α and α CS chains, whereas the many spots that match β chain sequences were faint or absent (B. G. Forget and S. M. Weissman, unpublished observations).

Nucleotide sequence analysis of thalassemic globin mRNA therefore confirms the previous demonstrations by hybridization studies of quantitative deficiency of α and β globin mRNA in α- and β-thalassemia, respectively.

VII. CONCLUSIONS AND FUTURE PROSPECTS

The cumulative results of all the various studies described in the preceding pages lead to the final conclusion that chain-specific globin mRNA is deficient in the thalassemia syndromes. One possible exception to this generalization is the β^0-thalassemia of Ferrara, in which there may be deficiency of a factor specifically required for translation of β mRNA. However, there still remain a number of unanswered questions: Is the deficiency of mRNA as great in the immature erythroid cells as it is in reticulocytes? Is the affected globin mRNA initially transcribed from the DNA in decreased amounts, or in normal amounts with subsequent degradation? Is the processing of globin mRNA precursors normal in thalassemia? Is there abnormal transport of mRNA from nucleus to cytoplasm? Why are Hb Lepore and Hb Constant Spring synthesized in decreased amounts? What is the role of untranslated sequences in globin gene transcription and globin mRNA stability? Are β^0- and α^0-thalassemia due to gene deletions? Obviously, research in thalassemia must focus on these questions in order to determine more precisely the nature of the specific molecular defect that is responsible for the abnormality in quantity of globin mRNA observed in thalassemic reticulocytes and marrow cells.

The following areas of study in thalassemic cells are likely to lead to further insight into the problem: study of globin synthesis and globin mRNA (structure and quantity) in relatively pure populations of immature erythroid precursors, study of transcription of globin gene into mRNA precursors (heterogeneous nuclear RNA) in intact cells, transcription of chromatin into globin-specific RNA sequences in vitro using reconstituted cell-free transcription systems, search for gene deletions by vast DNA excess hybridization, and, eventually, gene isolation and DNA sequence analysis of thalassemic globin genes.

Technology is advancing rapidly and certain specific procedures are already available to permit some of these studies. It is possible to obtain relatively pure populations of immature erythroid cells by antibody lysis of mature cells[107,108] and cell separation techniques.[109] Cell-free systems have been devised that carry out transcription of chromatin from erythroid cells into globin-specific RNA sequences.[110-113] As hybridization techniques improve, it should be feasible to isolate the DNA of the globin genes by preparative hybridization of total cellular DNA to purified globin mRNA; then one could study directly the structure of the globin gene DNA. Finally, one technique that would be most valuable, but which has thus far been elusive, is the establishment of a permanent human cell culture line of hemoglobin-synthesizing cells to permit the study in vitro of the factors that affect expression of the human globin genes; work with hybrid cell lines may accomplish this goal.

Research in thalassemia is now entering a new phase; the emphasis is shifting from the cytoplasm and globin mRNA to the nucleus and the globin gene or DNA. The next few years should see exciting developments in this virgin area of thalassemia research and, perhaps, at long last, the pinpointing of the precise molecular genetic defect(s) in the thalassemia syndromes.

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REFERENCES

1. **Weatherall, D. J. and Clegg, J. B.,** *The Thalassaemia Syndromes,* 2nd ed., Blackwell Scientific Publications, Oxford, 1972.
2. **Guidotti, G.,** Thalassemia, in *Conference on Hemoglobin,* Arden House, Columbia University, New York, 1962.
3. **Baglioni, C.,** Correlations between genetic and chemistry of human hemoglobins, in *Molecular Genetics,* Part I, Taylor, J. H., Ed., Academic Press, New York, 1963, 405.
4. **Jones, R. T. and Schroeder, W. A.,** Chemical characterization and subunit hybridization of human hemoglobin H and associated compounds, *Biochemistry,* 2, 1357, 1963.
5. **Heywood, J. D., Karon, M., and Weissman, S.,** Amino acids: incorporation into alpha- and beta-chains of hemoglobin by normal and thalassemic reticulocytes, *Science,* 146, 530, 1964.
6. **Heywood, D., Karon, M., and Weissman, S.,** Asymmetrical incorporation of amino acids into the alpha and beta chains of hemoglobin synthesized in thalassemic reticulocytes, *J. Lab. Clin. Med.,* 66, 476, 1965.
7. **Weatherall, D. J., Clegg, J. B., and Naughton, M. A.,** Globin synthesis in thalassemia: an *in vitro* study, *Nature,* 208, 1061, 1965.
8. **Bank, A. and Marks, P. A.,** Excess alpha chain synthesis relative to beta chain synthesis in thalassemia major and minor, *Nature,* 212, 1198, 1966.
9. **Bargellesi, A., Pontremoli, S., and Conconi, F.,** Absence of beta-globin synthesis and excess of alpha-globin synthesis in homozygous beta-thalassemia, *Eur. J. Biochem.,* 1, 73, 1967.
10. **Modell, C. B., Lotter, A., Steadman, J. H., and Huehns, E. R.,** Haemoglobin synthesis in β-thalassemia, *Br. J. Haematol.,* 17, 485, 1969.
11. **Weatherall, D. J., Clegg, J. B., Na-Nakorn, S., and Wasi, P.,** The pattern of disordered haemoglobin synthesis in homozygous and heterozygous β-thalassemia, *Br. J. Haematol.,* 16, 251, 1969.
12. **Conconi, F., Bargellesi, A., Del Senno, L., Menegatti, E., Pontremoli, S., and Russo, G.,** Globin chain synthesis in Sicilian thalassemic subjects, *Br. J. Haematol.,* 19, 469, 1970.
13. **Braverman, A. and McCurdy, P. R.,** Mild homozygous β-thalassemia in Negroes, *Clin. Res.,* 19, 37, 1971.
14. **Friedman, S., Hamilton, R. W., and Schwartz, E.,** β-Thalassemia in the American Negro, *J. Clin. Invest.,* 52, 1453, 1973.
15. **Friedman, S., Oski, F. A., and Schwartz, E.,** Bone marrow and peripheral blood globin synthesis in an American black family with beta thalassemia, *Blood,* 39, 785, 1972.
16. **Bank, A., Braverman, S., O'Donnell, J. V., and Marks, P. A.,** Absolute rates of globin chain synthesis in thalassemia, *Blood,* 31, 226, 1968.
17. **Clegg, J. B. and Weatherall, D. J.,** Hemoglobin synthesis in α-thalassemia (hemoglobin H disease), *Nature,* 215, 1241, 1967.
18. **Kan, Y. W., Schwartz, E., and Nathan, D. G.,** Globin chain synthesis in alpha thalassemia syndromes, *J. Clin. Invest.,* 47, 2515, 1968.
19. **Shchory, M. and Ramot, B.,** Globin chain synthesis in the marrow and reticulocytes of beta thalassemia, hemoglobin H disease, and beta-delta thalassemia, *Blood,* 40, 105, 1972.
20. **Weatherall, D. J., Clegg, J. B., and Wong Hock Boon,** The hemoglobin constitution of infants with the hemoglobin Bart's hydrops fetalis syndrome, *Br. J. Haematol.,* 18, 357, 1970.
21. **Schwartz, E. and Atwater, J.,** α-Thalassemia in the American Negro, *J. Clin. Invest.,* 51, 412, 1972.
22. **Braverman, A. S. and Bank, A.,** Changing rates of globin chain synthesis during erythroid cell maturation in thalassemia, *J. Mol. Biol.,* 42, 57, 1969.
23. **Nienhuis, A. W., Canfield, P. H., and Anderson, W. F.,** Hemoglobin messenger RNA from human bone marrow: isolation and translation in homozygous and heterozygous β-thalassemia, *J. Clin. Invest.,* 52, 1735, 1973.
24. **Ramot, B., Ben-Bassat, I., Mozel, M., and Shacked, N.,** Globin synthesis in α and β-thalassemia, *Isr. J. Med. Sci.,* 9, 1469, 1973.
25. **Schwartz, E.,** Heterozygous beta thalassemia: balanced globin synthesis in bone marrow cells, *Science,* 167, 1513, 1970.

26. Kan, Y. W., Nathan, D. G., and Lodish, H. F., Equal synthesis of α- and β-globin chains in erythyroid precursors in heterozygous β-thalassemia, *J. Clin. Invest.*, 51, 1906, 1972.
27. Clegg, J. B. and Weatherall, D. J., Hemoglobin synthesis during erythroid maturation in β-thalassemia, *Nat. New Biol.*, 240, 190, 1972.
28. Gill, F., Atwater, J., and Schwartz, E., Hemoglobin Lepore trait: globin synthesis in bone marrow and peripheral blood, *Science*, 178, 623, 1972.
29. White, J. M., Long, A., and Lehmann, H., Compensation of β chain synthesis by the single β chain gene in Hb Lepore trait, *Nat. New Biol.*, 240, 271, 1972.
30. Friedman, S., Ozsoylu, S., Luddy, R., and Schwartz, E., A new form of β-thalassemia trait of unusual severity, *Blood*, 42, 990, 1973.
31. Stamatoyannopoulos, G., Woodson, R., Papayannopoulou, Th., Heywood, D., and Kurachi, S., Inclusion-body β-thalassemia trait: a new form of β-thalassemia producing clinical manifestations in simple heterozygotes, *N. Engl. J. Med.*, 290, 939, 1974.
32. Burka, E. R. and Marks, P. A., Ribosomes active in protein synthesis in human reticulocytes: a defect in thalassemia major, *Nature*, 199, 706, 1963.
33. Marks, P. A. and Burka, E. R., Hemoglobins A and F: formation in thalassemia and other hemolytic anemias, *Science*, 144, 552, 1964.
34. Marks, P. A., Burka, E. R., and Rifkind, R. A., Control of protein synthesis in reticulocytes and the formation of hemoglobins A and F in thalassemia syndromes and other hemolytic anemias, *Medicine*, (Baltimore), 43, 769, 1964.
35. Bank, A. and Marks, P. A., Protein synthesis in a cell-free human reticulocyte system: ribosome function in thalassemia, *J. Clin. Invest.*, 45, 330, 1966.
36. Fuhr, J., Natta, C., Marks, P. A., and Bank, A., Protein synthesis in cell-free systems from reticulocytes of thalassemic patients, *Nature*, 224, 1305, 1969.
37. Anderson, W. F. and Gilbert, J. M., tRNA dependent translational control of *in vitro* hemoglobin synthesis, *Biochem. Biophys. Res. Commun.*, 36, 456, 1969.
38. Weiss, G. B., Translational control of protein synthesis by tRNA unrelated to changes in tRNA concentration, *J. Mol. Evol.*, 2, 199, 1973.
39. Clegg, J. B., Weatherall, D. J., Na-Nakorn, S., and Wasi, P., Hemoglobin synthesis in beta thalassemia, *Nature*, 220, 664, 1968.
40. Rieder, R. F., Translation of β-globin mRNA in β-thalassemia and the S and C hemoglobinopathies, *J. Clin. Invest.*, 51, 364, 1972.
41. Nathan, D. G. and Gunn, R. B., Thalassemia: the consequences of unbalanced hemoglobin synthesis, *Am. J. Med.*, 41, 815, 1966.
42. Nathan, D. G., Stossel, T. B., Gunn, R. B., Zarkowsky, H. S., and Laforet, M. T., Influence of hemoglobin precipitation on erythrocyte metabolism in alpha and beta thalassemia, *J. Clin. Invest.*, 48, 33, 1969.
43. Nathan, D. G., Lodish, H., Kan, Y. W., and Housman, D., Beta thalassemia and translation of globin messenger RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 68, 2514, 1971.
44. Clegg, J. B., Weatherall, D. J., and Eunson, C. E., The distribution of nascent globin chains on human reticulocyte polysomes, *Biochim. Biophys. Acta*, 247, 109, 1971.
45. Hunt, R. T., Munro, A. J., and Hunter, A. R., Control of hemoglobin synthesis: a difference in the size of polysomes making α and β chains, *Nature*, 220, 481, 1968.
46. Lodish, H. F. and Jacobsen, M., Regulation of hemoglobin synthesis: equal rates of translation and termination of α and β-globin chains, *J. Biol. Chem.*, 247, 3622, 1972.
47. Lodish, H. F., Alpha and beta globin messenger ribonucleic acid: different amounts and rates of initiation of translation, *J. Biol. Chem.*, 246, 7131, 1971.
48. Cividalli, G., Nathan, D. G., and Lodish, H. F., Translational control of hemoglobin synthesis in thalassemic bone marrow, *J. Clin. Invest.*, 53, 955, 1974.
49. Gilbert, J. M. and Anderson, W. F., Cell-free hemoglobin synthesis. II. Characteristics of the transfer ribonucleic acid-dependent assay system, *J. Biol. Chem.*, 245, 2342, 1970.
50. Crystal, R. G., Nienhuis, A. W., Elson, N. A., and Anderson, W. F., Initiation of globin synthesis: preparation and use of reticulocyte ribosomes retaining inhibition region messenger ribonucleic acid fragments, *J. Biol. Chem.*, 247, 5357, 1972.
51. Crystal, R. G. and Anderson, W. F., Initiation of hemoglobin synthesis: comparison of model reactions that use artificial templates, with those using natural messenger RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 706, 1972.
52. Prichard, P. M., Gilbert, J. M., Shafritz, D. A., and Anderson, W. F., Factors for the initiation of haemoglobin synthesis by rabbit reticulocyte ribosomes, *Nature*, 226, 511, 1970.
53. Shafritz, D. A. and Anderson, W. F., Isolation and partial characterization of reticulocyte factors M_1 and M_2, *J. Biol. Chem.*, 245, 5553, 1970.
54. Crystal, R. G., Nienhuis, A. W., Prichard, P. M., Picciano, D., Elson, N. A., Merrick, W. C., Graf, H., Shafritz, D. A., Laycock, D. G., Last, J. A., and Anderson, W. F., Initiation of globin synthesis, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 24, 310, 1972.
55. Gilbert, J. M., Thornton, A. G., Nienhuis, A., and Anderson, W. F., Cell-free hemoglobin synthesis in beta thalassemia, *Proc. Natl. Acad. Sci. U.S.A.*, 67, 1854, 1970.

56. **Nienhuis, A. W., Laycock, D. G., and Anderson, W. F.,** Translation of rabbit hemoglobin messenger RNA by thalassemic and non-thalassemic ribosomes, *Nat. New Biol.*, 231, 205, 1971.
57. **Crystal, R. G., Elson, N. A., Nienhuis, A., Thornton, A. C., and Anderson, W. F.,** Initiation of globin synthesis in β-thalassemia, *N. Engl. J. Med.*, 288, 1091, 1973.
58. **Baglioni, C., Colombo, B., and Jacobs-Lorena, M.,** Chain termination: a test for a possible explanation of thalassemia, *Ann. N.Y. Acad. Sci.*, 165, 212, 1969.
59. **Dreyfus, J. C., Labie, D., Vibert, M., and Conconi, F.,** An attempt at demonstrating the existence of a nonsense mutation in β-thalassemia, *Eur. J. Biochem.*, 27, 291, 1972.
60. **Gerald, P. S. and Diamond, L. K.,** The diagnosis of thalassemia trait by starch block electrophoresis of the hemoglobin, *Blood*, 13, 61, 1958.
61. **Baglioni, C.,** The fusion of two peptide chains in hemoglobin Lepore and its interpretation as a genetic deletion, *Proc. Natl. Acad. Sci. U.S.A.*, 48, 1880, 1962.
62. **Lehmann, H. and Charlesworth, D.,** Observations on hemoglobin P (Congo type), *Biochem. J.*, 119, 43, 1970.
63. **Badr, F. M., Lorkin, P. A., and Lehmann, H.,** Haemoglobin P-Nilotic containing a β-δ chain, *Nat. New Biol.*, 242, 107, 1973.
64. **Ohta, Y., Yamaoka, K., Sumida, I., and Yanase, T.,** Hemoglobin Miyada, a β-δ fusion peptide (anti-Lepore) type discovered in a Japanese family, *Nat. New Biol.*, 234, 218, 1971.
65. **White, J. M., Lang, A., Lorkin, P. A., Lehmann, H., and Reeve, J.,** Synthesis of haemoglobin Lepore, *Nat. New Biol.*, 235, 208, 1972.
66. **Roberts, A. V., Weatherall, D. J., and Clegg, J. B.,** The synthesis of human haemoglobin A_2 during erythroid maturation, *Biochem. Biophys. Res. Commun.*, 47, 81, 1972.
67. **Milner, P. F., Clegg, J. B., and Weatherall, D. J.,** Haemoglobin H disease due to a unique haemoglobin variant with an elongated α-chain, *Lancet*, 1, 729, 1971.
68. **Clegg, J. B., Weatherall, D. J., and Milner, P. F.,** Hemoglobin Constant Spring – a chain termination mutant? *Nature*, 234, 337, 1971.
69. **Wasi, P.,** Annotation: is the human globin α-chain locus duplicated? *Br. J. Haematol.*, 24, 267, 1973.
70. **Kan, Y. W., Todd, D., and Dozy, A. M.,** Hemoglobin Constant Spring: possibly unstable mRNA and evidence for 2 locus theory for α chain production, *Clin. Res.*, 20, 471, 1972.
71. **Seid-Akhavan, M., Winter, W. P., Abramson, R. K., and Rucknagel, D. L.,** Hemoglobin Wayne: a frameshift variant occurring in two distinct forms, *Blood*, 40, 927, 1972.
72. **Laux, B., Dennis, D., White, H. B., III,** Human α-chain globin messenger: prediction of a nucleotide sequence, *Biochem. Biophys. Res. Commun.*, 54, 894, 1973.
73. **Forget, B. G., Marotta, C. A., Weissman, S. M., Verma, I. M., McCaffrey, R. P., and Baltimore, D.,** Nucleotide sequences of human globin messenger RNA, *Ann. N.Y. Acad. Sci.*, in press, 1974.
74. **Marotta, C. A., Forget, B. G., Weissman, S. M., Verma, I. M., McCaffrey, R. P., and Baltimore, D.,** Nucleotide sequences of human globin messenger RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 2300, 1974.
75. **Boyer, S. H., Noyes, A. N., Vrablik, G. R., Donaldson, L. J., and Schaeffer, E. W.,** Silent hemoglobin alpha genes in apes: potential source of thalassemia, *Science*, 171, 182, 1971.
76. **Nienhuis, A. W. and Anderson, W. F.,** Isolation and translation of hemoglobin messenger RNA from thalassemia, sickle cell anemia and normal human reticulocytes, *J. Clin. Invest.*, 50, 2458, 1971.
77. **Benz, E. J., Jr. and Forget, B. G.,** Defect in messenger RNA for human hemoglobin synthesis in beta thalassemia, *J. Clin. Invest.*, 50, 2755, 1971.
78. **Dow, L. W., Terada, M., Natta, C., Metafora, S., Grossbard, E., Marks, P. A., and Bank, A.,** Globin synthesis of intact cells and activity of isolated mRNA in β-thalassemia, *Nat. New Biol.*, 243, 114, 1973.
79. **Forget, B. G., Baltimore, D., Benz, E. J., Jr., Housman, D., Lebowitz, P., Marotta, C. A., McCaffrey, R. P., Skoultchi, A., Swerdlow, P. S., Verma, I. M., and Weissman, S. M.,** Globin messenger RNA in the thalassemia syndromes, *Ann. N.Y. Acad. Sci.*, 232, 76, 1974.
80. **Kan, Y. W., Dozy, A. M., and Holland, J. P.,** Absence of functional β-globin mRNA in homozygous β^0-thalassemia, *Blood*, 42, 991, 1973.
81. **Natta, C., Banks, J., Niazi, G., Marks, P. A., and Bank, A.,** Decreased β globin mRNA activity in bone marrow cells in homozygous and heterozygous β-thalassemia, *Nat. New Biol.*, 244, 280, 1973.
82. **Metafora, S., Terada, M., Dow, L. W., Marks, P. A., and Bank, A.,** Increased efficiency of exogenous messenger RNA translation in a Krebs ascites cell lysate, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1299, 1972.
83. **Benz, E. J., Jr., Swerdlow, P. S., and Forget, B. G.,** Globin messenger RNA in hemoglobin H disease, *Blood*, 42, 825, 1973.
84. **Conconi, F., Rowley, P. T., Del Senno, L., Pontremoli, S., and Volpato, S.,** Induction of β-globin synthesis in the β-thalassemia of Ferrara, *Nat. New Biol.*, 238, 83, 1972.
85. **Rowley, P. T. and Kosciolek, B.,** Distinction between two types of beta-thalassemia by inducibility of the cell-free synthesis of beta-chains by non-thalassemic soluble fraction, *Nat. New Biol.*, 239, 234, 1972.
86. **Conconi, F. and Del Senno, L.,** The molecular defect of the Ferrara β-thalassemia, *Ann. N.Y. Acad. Sci.*, 232, 54, 1974.

87. Grossbard, E., Terada, M., Dow, L. W., and Bank, A., Decreased α globin messenger RNA activity associated with polyribosomes in α thalassemia, *Nat. New Biol.*, 241, 209, 1973.
88. Benz, E. J., Jr., Swerdlow, P. S., and Forget, B. G., Absence of functional messenger RNA activity for beta globin chain synthesis in β^0-talassemia. *Blood*, in press, 1974.
89. Baglioni, C. and Campana, T., α-Chain and globin: intermediates in the synthesis of rabbit hemoglobin, *Eur. J. Biochem.*, 2, 480, 1967.
90. Schaeffer, J. R., Evidence for soluble α chains as intermediates in hemoglobin synthesis in the rabbit reticulocyte, *Biochem. Biophys. Res. Commun.*, 28, 647, 1967.
91. Schaeffer, J. R., Trostle, P. K., and Evans, R. F., Inhibition of the biosynthetic completion of rabbit hemoglobin by isolated human hemoglobin chains, *J. Biol. Chem.*, 244, 4284, 1969.
92. Blum, N., Maleknia, N., Schapira, G., Kneip, B., and Reibel, L., α Hémoglobine libre et biosynthèse de l'hémoglobine, *Biochim. Biophys. Acta*, 179, 448, 1969.
93. Blum, N., Maleknia, M., Schapira, G., Kneip, B., and Benrubi, M., α et β globines libres et biosynthèse de l'hémoglobine, *Biochim. Biophys. Acta*, 199, 236, 1970.
94. Verma, I. M., Temple, G. F., Fan, H., and Baltimore, D., *In vitro* synthesis of DNA complementary to rabbit reticulocyte 10S RNA, *Nature*, 235, 163, 1972.
95. Kacian, D. L., Spiegelman, S., Bank, A., Terada, M., Metafora, S., Dow, L., and Marks, P. A., *In vitro* synthesis of DNA components of human genes for globin, *Nature*, 235, 167, 1972.
96. Ross, J., Aviv, H., Scolnick, E., and Leder, P., *In vitro* synthesis of DNA complementary to purified rabbit globin mRNA, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 264, 1972.
97. Jacobs-Lorena, M. and Baglioni, C., Messenger RNA for globin in the postribosomal supernatant of rabbit reticulocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1425, 1972.
98. Temple, G. and Housman, D., Separation and translation of the mRNA's coding for α and β chains of rabbit globin, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1574, 1972.
99. Housman, D., Forget, B. G., Skoultchi, A., and Benz, E. J., Jr., Quantitative deficiency of chain specific globin mRNA in the thalassemia syndromes, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 1809, 1973.
100. Kacian, D. L., Gambino, R., Dow, L. W., Grossbard, E., Natta, C., Ramirez, F., Spiegelman, S., Marks, P. A., and Bank, A., Decreased globin messenger RNA in thalassemia detected by molecular hybridization, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 1886, 1973.
101. Housman, D., Skoultchi, A., Forget, B. G., and Benz, E. J., Jr., Use of globin cDNA as a hybridization probe for globin mRNA, *Ann. N.Y. Acad. Sci.*, in press, 1974.
102. Britten, R. J. and Kohne, D. E., Repeated sequences in DNA, *Science*, 161, 529, 1968.
103. Benz, E. J., Jr., Forget, B. G., and Housman, D., Molecular genetics of the β-thalassemia syndromes, *Pediatr. Res.*, 8, 387, 1974.
104. Forget, B. G., Benz, E. J., Jr., Skoultchi, A., Baglioni, C., and Housman, D., Absence of messenger RNA for beta globin chain in β^0-thalassemia, *Nature*, 247, 379, 1974.
105. Gill, F., Atwater, J., and Schwartz, E., Hemoglobin Lepore trait: globin synthesis in bone marrow and peripheral blood, *Science*, 178, 623, 1972.
106. Roberts, A. V., Clegg, J. B., Weatherall, D. J., and Ohta, Y., Synthesis *in vitro* of anti-Lepore haemoglobin, *Nat. New Biol.*, 245, 23, 1973.
107. Boorsook, H., Ratner, K., and Tattrie, B., Studies on erythropoiesis II: a method of separating immature forms from mature adult rabbit erythroblasts, *Blood*, 34, 32, 1969.
108. Cantor, L. N., Morris, A. J., Marks, P. A., and Rifkind, R. A., Purification of erythropoietin-responsive cells by immune hemolysis, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1337, 1972.
109. Denton, M. J. and Arnstein, H. R. V., Characterization of developing adult mammalian erythroid cells separated by velocity sedimentation, *Br. J. Haematol.*, 24, 7, 1973.
110. Axel, R., Cedar, H., and Felsenfeld, G., Synthesis of globin ribonucleic acid from duck reticulocyte chromatin *in vitro*, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 2029, 1973.
111. Gilmour, R. S. and Paul, J., Tissue-specific transcription of the globin gene in isolated chromatin, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 3440, 1973.
112. Anderson, W. F., Steggles, A., Wilson, G., Kantor, J., Picciano, D., Falvey, A., Velez, R., and Nienhuis, A., Cell-free transcription of human bone marrow chromatin, *Ann. N.Y. Acad. Sci.*, in press, 1974.
113. Steggles, A. W., Wilson, G. N., Kantor, J. A., Picciano, D. J., Falvey, A. K., and Anderson, W. F., Cell-free transcription of mammalian chromatin: transcription of globin messenger RNA sequences from bone marrow chromatin with mammalian RNA polymerase, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 1219, 1974.
114. Kan, Y. W., Todd, D., Holland, J., and Dozy, A., Absence of α globin mRNA in homozygous α-thalassemia, *J. Clin. Invest.*, 53, 37a, 1974.
115. Taylor, J. M., Dozy, A., Kan, Y. W., Varmus, H. E., Lie-Ingo, L. E., Ganeson, J., and Todd, D., Genetic lesion in homozygous α thalassemia (Hydrops faetalis), *Nature*, in press, 1974,
116. Ottolenghi, S., Lanyon, W., Paul, J., Williamson, R. W., Weatherall, D. J., Clegg, J., Pritchard, J., Pootrakul, S., and Wong, H., The severe form of α thalassemia is caused by a hemoglobin gene deletion, *Nature*, in press, 1974.