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Authentic β-globin mRNA sequences in homozygous β^0-thalassemia

(cDNA/hybridization/nucleotide sequences)

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ABSTRACT **In a patient with homozygous β^0-thalassemia in whom studies of reticulocyte hemoglobin synthesis showed no β-globin chain synthesis *in vivo* and *in vitro*, molecular hybridization studies revealed RNA sequences complementary to β-globin cDNA. The fact that these sequences were authentic β-globin mRNA was shown by fingerprint analysis of T_1 ribonuclease-digested mRNA and by sequencing of oligonucleotides unique to β-globin mRNA. The β-mRNA that failed to direct β-globin chain synthesis was not detectably shortened or degraded and contained poly(A) sequences.**

The common form of β-thalassemia that is associated with an increased Hb A_2 level can be classified into two types according to the presence or absence of β-globin chain synthesis in the homozygous state (1–4). In homozygous β^+-thalassemia, a decreased amount of β-globin mRNA consistent with the degree of reduction in β-globin chain synthesis has been demonstrated by several groups of investigators (5–8). In contrast, in β^0-thalassemia in which no β-globin chain is synthesized in the homozygous state, there is disagreement as to the presence or absence of β-globin mRNA. By using radioactive DNA (cDNA) complementary to globin mRNA as a probe for β-globin mRNA sequences, Forget *et al.* (9), Housman *et al.* (10), and Tolstoshev *et al.* (11) initially concluded that β-globin mRNA was absent in β^0-thalassemia. On the other hand, Kan *et al.* (12), also using hybridization techniques, described the presence of mRNA homologous to β-globin cDNA in tissues from two patients. These findings were substantiated by Ramirez *et al.* (13) who also detected mRNA that hybridized to a variable extent with β-globin cDNA. Recently, Benz *et al.* (14) and Forget *et al.* (15) also found, in some patients, variable amounts of mRNA that hybridized to β-globin cDNA. However, hybridization analysis only furnishes indirect evidence of the presence of β-globin mRNA sequences. As the extent of the diversity between β- and δ-globin mRNA sequences is not fully known, there is a theoretical possibility that the mRNA that hybridizes to β-globin cDNA is δ-globin mRNA. The large amount of β-mRNA sequences detected in some patients argues against this possibility, but it is conceivable that a compensatory increase in δ-mRNA synthesis may occur in homozygous β^0-thalassemia.

In this study, we isolated mRNA from a patient with homozygous β^0-thalassemia and determined the sequence of certain oligonucleotides specific for β-globin mRNA and not for γ- and δ-globin mRNA. The results show that oligonucleotide sequences specific for β-globin mRNA were present, thus demonstrating unequivocally that the mRNA that hybridized to the β-globin cDNA was β-globin mRNA. In addition, studies of the nonfunctional β^0-thalassemia mRNA showed that it was largely intact and contained poly(A) sequences.

MATERIALS AND METHODS

Patient Materials. A 28-year-old Chinese male with homozygous β^0-thalassemia was studied. This patient had not been transfused for more than 10 years because he had developed antibodies against multiple erythrocyte antigens. His hematocrit ranged from about 22 to 26%. Both his parents had the high A_2 type of heterozygous β-thalassemia. Electrophoresis studies of his blood showed hemoglobins F and A_2 and no hemoglobin A. β-Globin chains were not synthesized in his intact reticulocytes, and the mRNA isolated from the reticulocytes directed γ- and α- but no β-globin chain synthesis in a wheat germ cell-free system.

Blood used as a nonthalassemic control was obtained from a patient with sickle cell anemia. Blood from a fetus that died from homozygous α-thalassemia was used as a source of γ-mRNA because it contained predominantly γ- and a small amount of β-globin chain sequences but no α-globin sequences.

Preparation of RNA and DNA. Peripheral blood was washed three times with isotonic saline and the erythrocytes were lysed with the Orskov reaction according to the method of Boyer *et al.* (16). With this technique, only erythrocytes were lysed and 98% of the leukocytes remained intact. The latter were saved for the DNA preparation as described (17). Ribonucleoproteins from the erythrocytes lysate were precipitated at pH 5, and the RNA was extracted with phenol, chloroform, and isoamyl alcohol (12). Approximately 15 A_{260} units of total RNA were obtained per 50 ml of blood. (One unit is the amount of material that when dissolved in 1 ml of solvent gives an absorbance of 1 with a path length of 1 cm.) The RNA was twice passed through oligo(dT)-cellulose columns (18) and the poly(A)-containing RNA eluted with low-salt buffer was separated by centrifugation on a 15–30% (wt/vol) sucrose density gradient. Fractions between the 8S and 12S positions were pooled.

To prepare mRNA rich in β-globin sequences, we separated the sickle cell mRNA by electrophoresis on 5% polyacrylamide gels in 98% formamide (19). The slowly migrating band in the 10S region containing more β than α sequences was isolated and the RNA was eluted from the gel, extracted with phenol and purified on oligo(dT)-cellulose (20).

Hybridization Analysis. Radioactive DNAs complementary to α- and β-globin mRNA were synthesized according to the methods of Verma *et al.* (21), as described (17). α- and β-globin sequences in the mRNA were measured by hybridization with α- and β-globin cDNAs that were labeled with [α-^{32}P]dCTP (specific activity 120 Ci·mmol^{-1}) and [^{3}H]dCTP (specific activity 23 Ci·mmol^{-1}), respectively. The β-cDNA was a tran-

script of the mRNA from a patient with hemoglobin H disease and contained about 90% β- and 10% α-globin sequences. α-cDNA was prepared from a mixture of α- and β-cDNAs by selective removal of the β-cDNA-hydrops mRNA hybrids on hydroxylapatite columns. The purified α-cDNA contained about 80% α sequences. To quantitate β-globin structural genes, we incubated β-cDNA labeled with [α-^{32}P]dCTP and unique sequence DNA labeled with [^{3}H]CTP as internal controls together with the sheared leukocyte DNA by methods described (12, 17).

Fingerprint and Sequence Analysis of Globin mRNA. Purified mRNA (0.2 μg) was completely digested with ribonuclease T_1, the 3′ phosphate was removed with *Escherichia coli* alkaline phosphatase, and the 5′ position was labeled with ^{32}P using T_4 polynucleotide kinase, according to Simsek *et al.* (22). The [γ^{32}P]ATP used for 5′ labeling (specific activity of 2 to 3 × 10^6 cpm·pmol^{-1}) was prepared according to the method of Glynn and Chappell (23) as modified by Maxam and Gilbert (24). After the phosphorylation step, the reaction was made 20 mM in Na_2EDTA, pH 7, and heated to 100° for 2 min, and then 5 μg of KOH-digested yeast RNA (25) was added as carrier.

To reduce the amount of unutilized [γ^{32}P]ATP, we passed the labeled digest of thalassemia mRNA through a Sephadex G-25 column (0.8 × 20 cm) and the first two-thirds of the included radioactivity was collected. The sample was lyophilized, resuspended in 3 μl of water, applied to strips of cellulose acetate (2.5 × 55 cm) (Schleicher and Schuell), and electrophoresed in 5% pyridine acetate, pH 3.5, 7 M urea at 25° and 5 kV for 70 min. After electrophoresis, the sample was transferred to a DEAE-cellulose chromatography plate (20 × 40 cm) (Analtech: CEL HR/AVICEL/DEAE: 10/5/2, 250 μm). The plate was washed with 95% ethanol, dried, and run with water at 65° for 60 min. Chromatography in the second dimension was performed in a homochromatography solution containing 3% RNA that was previously digested with 0.03 M KOH according to Silberklang *et al.* (26).

Several oligonucleotides were then eluted from these fingerprints (25) and their sequences were determined by partial snake venom phosphodiesterase digestion. The digests were electrophoresed in 5% pyridine acetate, pH 3.5, 7 M urea and then chromatographed in a second dimension urea using a 3% RNA homochromatography solution [mixture C of Brownlee (25)] prepared by digestion with 1 M KOH for 15 min at 25° and titrated to pH 4.5 with acetic acid. To determine the 5′ end nucleotide, we digested aliquots of the eluted oligonucleotides to completion with snake venom phosphodiesterase and separated the 5′ mononucleotides by electrophoresis in 5% pyridine acetate, pH 3.5, with authentic absorbance markers (25).

Analysis of Size of β-Globin mRNA in β^0-Thalassemia. The mRNA from the patient with β^0-thalassemia was separated on 5% acrylamide gel electrophoresis in formamide, according to the method of Maniatis *et al.* (19) with these modifications: 70 μl of ethylene diacrylate (instead of *N,N′*-methylene-bisacrylamide) and 70 μl of *N,N,N′,N′*-tetramethylenediamine were added per 7.5 ml of gel solution. The cylindrical gels (0.6 × 7.0 cm) were sliced into 43 fractions, each of which was about 40 μl in volume. To each slice, we added 50 μg of yeast tRNA, 1000 cpm of β-globin [^{3}H]cDNA, NaCl to a final concentration of 0.06 M, sodium dodecyl sulfate to 0.5%, and water to a final volume of 100 μl. (The final concentration of formamide was 40%.) The reaction mixture was covered with 300 μl of mineral oil and incubated at 68° for 16 hr to dissolve the gel. The temperature was then changed to 40° for 48 hr to allow annealing, and the percentage of cDNA annealed to mRNA determined with the S_1 assay (12).

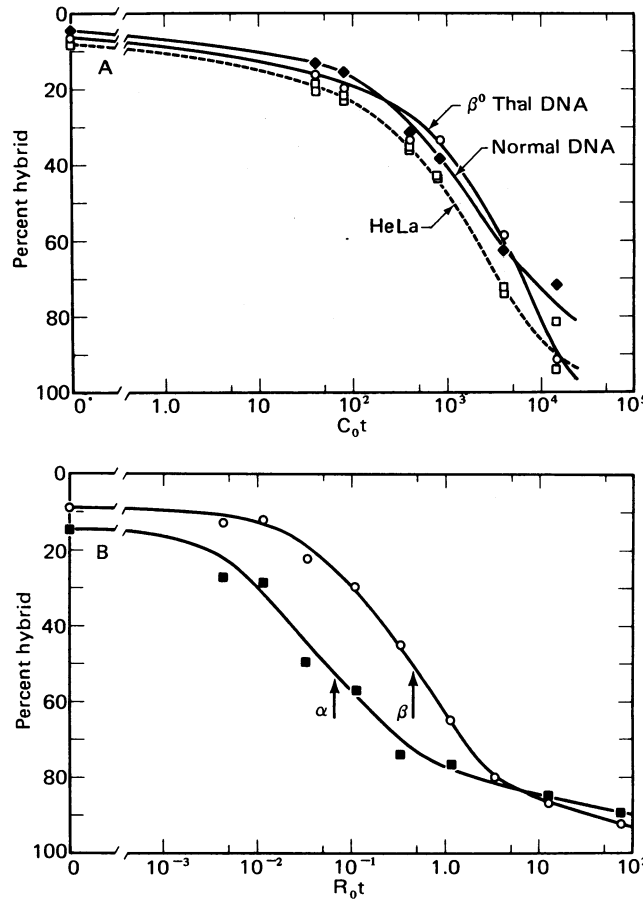

FIG. 1. (*A*) Rates of annealing of ^{32}P-labeled β-cDNA and unique-sequence HeLa [^{3}H]DNA to normal and β^0-thalassemia DNA. The results of two experiments were plotted together. In one, β-cDNA (♦) and unique-sequence HeLa DNA (□) were incubated with normal DNA; in the other, β-cDNA (○) and unique HeLa DNA (□) were incubated with β^0-thalassemia DNA. C_0t, initial concentration of total DNA (moles of nucleotide/liter) × time (sec). (*B*) Rates of annealing of ^{32}P-labeled α-cDNA and ^{3}H-labeled β-cDNA to total reticulocyte RNA from the patient with β^0-thalassemia. Arrows indicate the ½ R_0t values [½ value of initial concentration of total RNA (moles of nucleotide/liter) × time (sec)].

RESULTS

The β-cDNA annealed to this patient's leukocytic DNA at the same rate and to the same extent as it did to the DNA of a nonthalassemic control (Fig. 1*A*). This confirms the previous findings that the β-globin structural genes are intact in β^0-thalassemia (11, 12). The rate of annealing of the β-cDNA to this patient's mRNA was about seven times slower than that of the α-cDNA (Fig. 1*B*), indicating that the relative concentration of α-mRNA to β-mRNA was about 7:1. Previous thermodenaturation studies have shown that γ-globin mRNA annealed to β-cDNA 20 to 50 times more slowly under these conditions. The proportion of β sequences observed in the mRNA from this patient, however, is lower than the levels described in two other patients (12).

To show that the RNA that hybridized to the β-cDNA was β-mRNA, we compared fingerprints of the ^{32}P-labeled T_1 ribonuclease digests of mRNAs from several sources. Fig. 2*A* shows the fingerprint of gel-purified β-globin mRNA with a β:α ratio of about 3:1 as determined by molecular hybridization. These patterns were matched to those previously published by Forget *et al.* (20, 27). Consistent with the higher concentration of β- than α-globin mRNA in this preparation, the darker spots

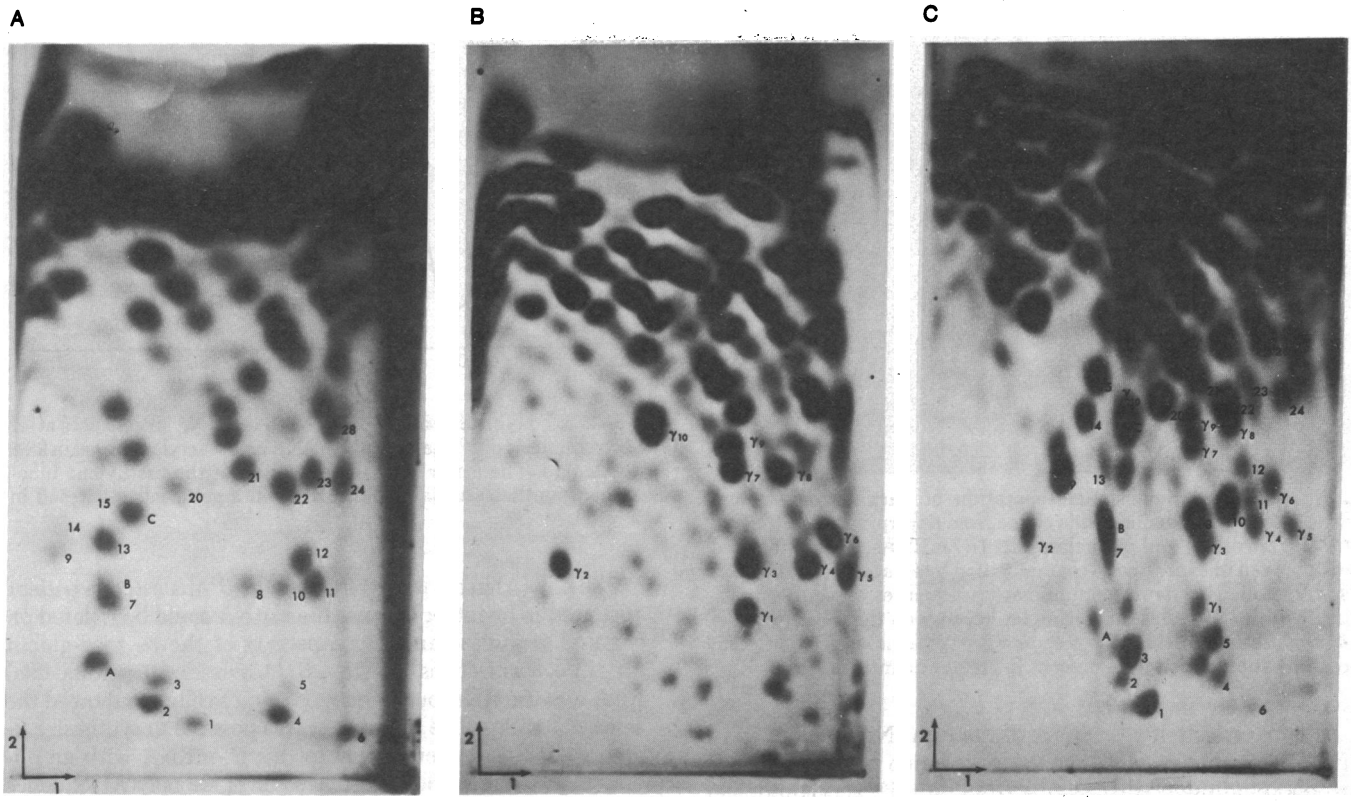

FIG. 2. Fingerprints of complete T_1 ribonuclease digests. (*A*) Gel-purified control β-mRNA (β:α ratio about 3:1). (*B*) Hydrops mRNA (γ:β about 8:1 with ribosomal RNA present as a contaminant). (*C*) β^0-thalassemia mRNA. Autoradiography was performed for 6–18 hr.

in this fingerprint were derived from the β-mRNA and the lighter spots from α-mRNA. Thus, the β-mRNA chromatogram included spot numbers 7, 11, 12, 13, 21, and 28, all of which have been shown by Forget *et al.* to be derived from β-globin mRNA (27). Two additional heavy spots, A and C, and a light spot B (overlapping spot 7) were seen. These were absent from the published fingerprints of Forget *et al.* (27) probably because in those experiments labeled cRNA transcripts of cDNA were sequenced. With this technique, the sequences near the 5′ end of the mRNA are underrepresented (27). The fingerprint of homozygous α-thalassemia mRNA is shown in Fig. 2*B*. This mRNA contained γ and β sequences in the proportion of about 8:1, with no α sequences. Thus, the dark spots were derived from the γ sequences, and the light spots were derived from the β sequences as well as from contaminating ribosomal RNA. [This oligo(dT)-cellulose-prepared RNA was not further purified by gel or sucrose density gradient centrifugation.] By comparing Fig. 2 *A* and *B*, we found that the γ sequences were readily distinguished from β sequences. The fingerprint of the β^0-thalassemia mRNA is shown in Fig. 2*C*. It consisted of dark spots derived from α-globin mRNA, medium spots derived from the γ-globin mRNA, and light spots that matched the pattern derived from the β-globin mRNA.

To show that the β-like pattern of the light spots of the β^0-thalassemia fingerprint was not derived from γ or δ sequences, we eluted and sequenced two putative β-globin oligonucleotides from both the nonthalassemia and the β-thalassemia fingerprints. The oligonucleotides of spot 7 and 11 have been matched to the amino acid sequence of positions 79–82 and 115–119, respectively, of the β-globin chain by Forget *et al.* (27), and the nucleotide sequences are shown in Table 1. The oligonucleotide of spot 7 cannot be present in a fingerprint of γ-mRNA as the change of asparagine (codon AAC) to aspartic acid (GAC) at amino acid position 80 introduces an additional site of T_1 ribonuclease cleavage in the RNA. Fig. 3*A* shows that when the oligonucleotide of spot 7 from the β^0-mRNA was analyzed, it indeed had the same sequence as that from the nonthalassemic control.

Because spot 7 could be derived either from β or δ sequences, the oligonucleotide of spot 11 was analyzed. This oligonucleotide is by necessity β-globin mRNA in origin and cannot be contributed by δ-mRNA because δ-globin contains arginine (CGU) instead of histidine (CAU) at amino acid position 116, again resulting in a new T_1 cleavage site in this oligonucleotide. Furthermore, the homologous oligonucleotide derived from γ-globin mRNA has a different sequence because the substitution of isoleucine for histidine in amino acid position 116 will result in the sequence AUX instead of CAU. Partial snake-venom digest analysis of this oligonucleotide from the β^0-thalassemia patient showed that it has the authentic sequence of β-globin mRNA (Fig. 4).

Table 1. Amino acid and nucleotide sequences* of homologous regions in β,γ-, and δ-globin chains

	T_1 Spot 7				T_1 Spot 11				
Chain	79	80	81	82	115	116	117	118	119
β	Asp	Asn	Leu	Lys	Ala	His	His	Phe	Gly
	(G)AC	AAC	CUC	AAG	(G)CC	CAU	CAC	UUU	G
γ	Asp	*Asp*	Leu	Lys	Ala	*Ile*	His	Phe	Gly
	(G)AC	*GAC*	CUC	AAG	(G)CC	*AUX*	CAC	UUU	G
δ	Asp	Asn	Leu	Lys	Ala	*Arg*	*Asn*	Phe	Gly
	(G)AC	AAC	CUC	AAG	(G)CC	*CGU*	*AAC*	UUU	G

The numbers refer to the amino acid position in the chain.
* The simplest nucleotide sequence alterations in the β-globin mRNA were used to account for the amino acid changes in δ and γ sequences.

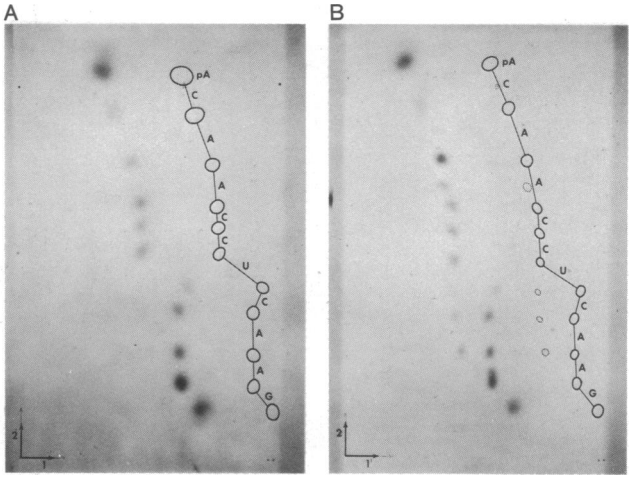

FIG. 3. Two-dimensional separation of partial snake-venom digests of spot 7 oligonucleotide eluted from fingerprints (Fig. 2 *A* and *C*) of control (*A*) and β^0-thalassemia (*B*) mRNAs. Spots indicated with broken lines are due to contamination by adjacent spot B. In this and the following figure, contaminating sequences were more prominent in the β^0-thalassemia samples because on the fingerprints of β^0-thalassemia mRNA, the β spots were contaminated by adjacent α and γ spots which were present in greater quantities.

To investigate if the failure of the β-mRNA to function in protein synthesis was due to its degradation *in vivo*, we electrophoresed the β^0-thalassemia RNA on a 5% acrylamide gel in 98% formamide, sliced the gel, eluted the samples, and hybridized them with β-[^{3}H]cDNA. The peak of hybridization activity coincided with an absorbance marker of nonthalassemic globin mRNA electrophoresed in a parallel gel (Fig. 5). Furthermore, in another experiment (results not shown), when α- and β-cDNAs were used together to hybridize to the mRNA from the gel slices, both cDNAs hybridized to the same slices, indicating that the β-mRNA is about the same length as the α-mRNA. The gel slices analyzed were too large to resolve small differences in the sizes of the α- and β-mRNAs.

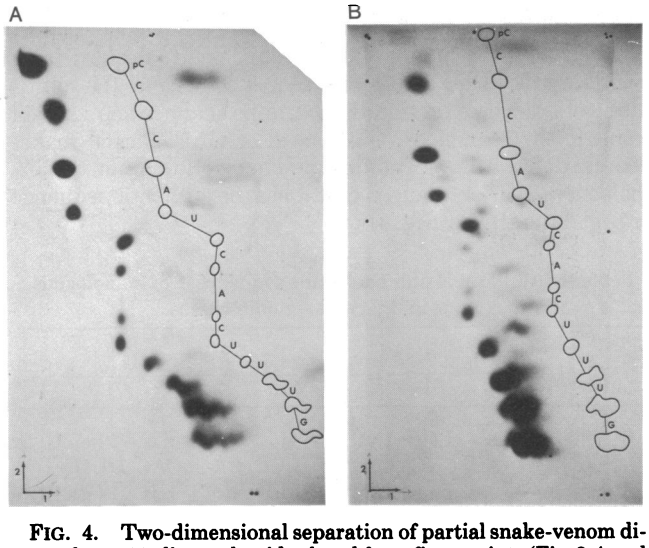

FIG. 4. Two-dimensional separation of partial snake-venom digests of spot 11 oligonucleotide eluted from fingerprints (Fig. 2 *A* and *C*) of control (*A*) and β^0-thalassemia (*B*) mRNAs. The 5′-terminal sequence of β^0-thalassemia spot 11 was unambiguously determined to be ^{32}pCpCpC by its coelectrophoresis (DE-81 paper, 5% pyridine acetate, pH 3.5) with a partial snake-venom phosphodiesterase digest of authentic ^{32}pC(pC)$_2$ marker prepared by ^{32}P labeling of C(pC)$_2$ (Collaborative Research).

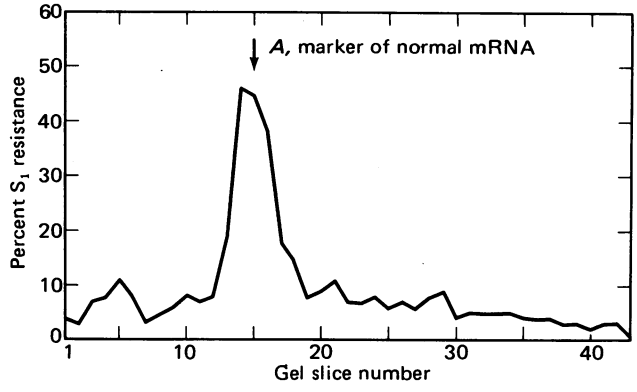

FIG. 5. Hybridization of β-cDNA to the RNA in gel slices after electrophoresis of β^0-thalassemia mRNA on 5% acrylamide gel in 98% formamide. The arrow shows the location of the methylene blue-stained nonthalassemia globin mRNA in a gel electrophoresed in parallel.

The poly(A) in the β-mRNA from the β^0-thalassemia patient appeared to be intact because the mRNA could be isolated on oligo(dT)-cellulose and a comparison of the α- to β-globin mRNA concentrations by cDNA·RNA hybridization of the total RNA and the RNA purified on oligo(dT)-cellulose showed the same β:α ratios. The fact that it was possible to synthesize β-globin DNA complementary to this β^0-mRNA with an oligo(dT) primer also indicates that some of the poly(A) sequence was present.

DISCUSSION

These results show that in this patient with β^0-thalassemia, the mRNA that hybridized to β-globin cDNA was β-globin mRNA. Analysis of the oligonucleotide sequence of spot 11 (coding for amino acids in positions 115–119) showed that the sequence could not have been derived from δ- or γ-globin mRNA. Furthermore, in agreement with previous hybridization studies (12), analysis of the oligonucleotide of spot 7 showed that authentic β sequences distinguishable from γ sequence were also present. A computer-based search for other possible sites for these β sequences in the coded region of the δ- and γ-mRNAs did not reveal any such sequences. Whereas it is possible that such sequences may be present in the noncoded region of the mRNA (about 230 nucleotides), the likelihood of finding an identical sequence of this size is less than 1 in 1000.

The reason for the failure of this mRNA to direct protein synthesis is not obvious from these experiments. The β-mRNA most likely contains poly(A) sequences because it was adsorbed on oligo(dT)-cellulose and could be primed with oligo(dT) in the synthesis of cDNA. The fact that the β^0-mRNA was approximately the same length as normal α- and β-globin mRNAs indicates that it probably retained most of the poly(A) sequences and was not extensively degraded to smaller sizes. However, these results do not rule out the possibility that a few nucleotides could have been deleted in a critical region of this mRNA.

Recently, it has been shown that certain sequences at the 5′ end of the mRNA are important for initiation of protein synthesis (28). For example, the presence of 7-methylguanine (added in 5′ to 5′ linkage with the first encoded base of the mRNA, "capping") is required for the initiation of translation of some (29–31) but not all (28, 32) mRNAs. Studies of the capping of this β^0-thalassemia mRNA may be revealing. However, the limited amount of material available has prevented us from studying this so far. Because cell-free translation experiments have localized the lesion to the mRNA (12), any

defect in capping must be secondary to some structural abnormality of the mRNA. It has been shown that certain parts of the sequence preceding the initiation codon AUG in prokaryotic mRNAs may serve as a binding site for the 3′ end of 16S ribosomal RNA (33, 34). If this mechanism also applies to eukaryotic mRNA, alterations in this region of the mRNA could result in the failure of ribosomal binding for the initiation of protein synthesis (35). Another possibility is that sequence alterations in the polypeptide coding regions of the mRNA may cause the premature termination of the globin chain, resulting in the failure of β-globin synthesis. Thus, complete sequencing of this mRNA should reveal the cause for its defect.

Forget *et al.* (9, 15), Housman *et al.* (10), Benz *et al.* (14), and Tolstoshev *et al.* (11) have reported appreciably lower levels (2–5%) of β-like mRNA in some patients. Whether or not in these cases authentic β-globin mRNA is present awaits similar studies. It may also be possible that β^0-thalassemia is the result of multiple molecular defects.

The type of β^0-thalassemia described herein probably represents the first example of a nonfunctional mRNA in a disease state. A definition of the molecular defect may further our understanding of normal and abnormal protein synthesis.

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