Posttranscriptional Defects in β-Globin Messenger RNA Metabolism in β-Thalassemia

ABNORMAL ACCUMULATION OF β-MESSENGER RNA PRECURSOR SEQUENCES

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ABSTRACT The production of β-globin messenger RNA (mRNA) in β-thalassemic erythroblasts was studied during pulse-chase incubations with [^{3}H]uridine. Globin [^{3}H]mRNA was quantitated by molecular hybridization to recombinant DNA probes complementary to globin mRNA and mRNA precursor sequences. Each of six patients with β^+-thalassemia produced normal amounts of globin α and β [^{3}H]mRNA during a 20-min pulse incubation, but the β/α [^{3}H]mRNA ratio declined to steady-state levels during a chase incubation, suggesting posttranscriptional defects in β-globin mRNA metabolism. β-globin mRNA precursor production was estimated by measurement of [^{3}H]RNA sequences hybridizing to a pure DNA probe containing only the large intervening sequence (intron) of the β-mRNA precursor. Four of the patients exhibited abnormal accumulation of ^{3}H-β-^{intron} sequences (2–10 times normal), indicating abnormal posttranscriptional processing. In the remaining two patients, one of whom is known to carry a mutation in the small intron of the β-globin gene, accumulation of large ^{3}H β-^{intron} RNA and β-globin [^{3}H]mRNA was normal in nuclei, but the ratio of β/α [^{3}H]mRNA in cytoplasm was reduced, suggesting a different posttranscriptional defect in β-mRNA processing. These findings imply the existence of heterogeneous posttranscriptional abnormalities in β-globin mRNA metabolism in different patients with β-thalassemia. The initial rates of γ- and δ-mRNA synthesis were low in all patients, suggesting that the low level of expression of these genes in adults is mediated at the transcriptional level.

INTRODUCTION

β-thalassemia is characterized by partial reduction (β^+-thalassemia) or total absence (β^0-thalassemia) of the synthesis of the β-globin subunit of Hb A ($\alpha_2\beta_2$) (1). Defective β-globin synthesis arises from reduced accumulation of the messenger RNA coding for β-globin chains (2–4). Some forms of β^0-thalassemia are due to partial gene deletion or terminator codon mutations. However, the β-globin gene is present and grossly intact in patients with β^+-thalassemia (3, 4), who produce small but detectable amounts of normally functioning β-globin mRNA (3, 4). These disorders must involve abnormal synthesis or metabolism of the mRNA product of the β-globin gene.

Inadequate accumulation of β-globin mRNA could arise from impaired synthesis (transcription), abnormal posttranscriptional processing of β-mRNA precursors to yield mature β-globin mRNA, defective transport of mature mRNA to cytoplasm, or instability of cytoplasmic mRNA sequences. The β-globin gene consists of mRNA coding sequences (exons) aligned in tandem with two intervening sequences (introns) (Fig. 1). The entire gene, including exons and introns, is transcribed into a β-globin RNA precursor ~1,800 nucleotides long (5–11), which is converted into mature mRNA (650–700 nucleotides long) by excision of the intron segments and ligation of the exons to form mRNA (Fig. 1). In mice, processing of newly synthesized transcripts is 95% complete within 30 min (7–9). Mature mRNA is rapidly transported to the cytoplasm where it is stable, with a half-life of at least 20 h (12, 13). Maquat et al. (14) have suggested that human β-globin mRNA

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synthesis and processing proceed within a similar time frame.

Several studies suggest that some patients with β-thalassemia accumulate insufficient β-globin mRNA because of posttranscriptional defects. Nienhuis et al. (15) observed higher levels of β-mRNA in erythroblast nuclei than in cytoplasm or reticulocytes from three patients. Maquat et al. (14) demonstrated that thalassemic erythroblasts pulse labeled with [^{3}H]-uridine-synthesized normal amounts of β-mRNA sequences in three cases. An abnormally high proportion of β-mRNA sequences remained at higher molecular weights than mature globin mRNA during a "chase" incubation, suggesting abnormal accumulation of β-mRNA precursors. Kantor et al. (16) observed normal initial rates of β-mRNA synthesis, but a posttranscriptional decline of β-mRNA in two patients with β-thalassemia who also exhibited abnormally high steady-state levels of $\beta^{\text{-intron}}$ sequences. Our preliminary studies of a single patient (17) suggested that abnormal accumulation of newly synthesized transcripts of the large β-intervening sequence occurred in pulse-labeled erythroblasts. These findings imply defective β-mRNA processing, rather than impaired transcription.

In this communication, we present studies of newly synthesized β-globin mRNA and β-mRNA precursor sequence metabolism in six patients with phenotypic β^+-thalassemia. By measurement of [^{3}H]β-mRNA sequences in pulse-labeled RNA, we encountered mRNA processing defects involving abnormal accumulation of large intron RNA in some individuals, but normal large intron RNA accumulation in others. A β-thalassemia gene in one of the latter individuals is known to contain only a single mutation, which is located in the small intron (18). We thus propose that mRNA processing defects are a common cause of β^+-thalassemia and that these defects are heterogeneous with respect to mRNA precursor accumulation.

METHODS

Patients. Six patients with "phenotypic" homozygous β^+-thalassemia were studied. Such individuals produce some β-globin (cf. Table III) and could be either homozygous for β^+-thalassemia or doubly heterozygous for β^+- and β^0-thalassemia. All were unrelated Caucasians of either Greek and Italian origin, 6–21-yr-old, with typical biochemical and clinical features of β-thalassemia. Patients (3–6) had transfusion-dependent β-thalassemia major, while patients 1 and 2 had β^+-thalassemia intermedia. Globin biosynthetic data are included in Results (Table III). Eight nonthalassemic controls (ages 6–75 yr) with erythroid hyperplasia were also studied. Their diagnoses included hereditary spherocytosis (two patients), Hb SC disease (one patient), polycythemia vera with hypersplenism and erythroid hyperplasia (two patients), cold agglutinin-mediated hemolytic anemia (one patient), thrombotic thrombocytopenic purpura (one patient), and idiopathic autoimmune hemolytic anemia (one patient). Informed consent was obtained for all bone marrow aspirations, which were performed during general anesthesia administered for required surgical procedures. Erythroid/myeloid ratios ranged from 1.5:1 to 10:1.

Preparation of bone marrow cells. Aspirates ($1–5 \times 10^8$ nucleated cells) collected in heparinized syringes were immediately transferred to 10 ml of sterile, prewarmed, heparinized medium A (RPMI 1640, containing 10% fetal calf serum), washed three times with medium A, and fractionated by Ficoll-Hypaque density gradient centrifugation, as described (19). The mononuclear layer was washed three times with medium A with heparin.

Pulse-chase labeling of erythroblasts with [^{3}H]uridine. Washed cells were suspended ($2–5 \times 10^7$ cells/ml) in medium A without heparin and preincubated at 37°C in a 5% CO_2 humidified atmosphere for 30 min. An equal volume of medium A containing 0.5 mCi/ml [^{3}H]uridine (New England Nuclear, Boston, Mass. 40 Ci/mmol) was then added. For pulse incubations, cells were exposed to isotope for 20 min. For pulse-chase incubations, approximately two-thirds of the cells were removed after 20 min, washed three times in medium B (medium A containing 20 mM nonradioactive uridine), resuspended at a concentration of 1×10^7 cells/ml in medium B, and incubated for an additional 20 h. Cells were harvested by rapid chilling in an ice bath, centrifuged at 700 *g* for 10 min at 0°C, and washed twice with medium B.

RNA extraction. Total cellular RNA was obtained by proteinase K digestion and phenol extraction of cell pellets (5). To obtain nuclear and cytoplasmic RNA, the procedure of Laevis and Penman (20) was used. Reagents and glassware were autoclaved or pretreated with 0.1% diethyl pyrocarbonate to eliminate nucleases. RNA preparations were stored in water (1 mg/ml) at −80°C. Reticulocyte RNA was prepared as described (21, 22).

Preparation of filter-immobilized molecular hybridization probes from recombinant DNA plasmids. Recombinant DNA probes for β-mRNA and β-mRNA precursor sequences were obtained as outlined in Fig. 1. To detect mRNA coding sequences (exons), recombinant plasmids containing inserted human globin complementary (c)DNA copies of α-, β- and γ-globin mRNA were used. Plasmids JW101, JW102, and JW151 (cf. 23) were used as probes for α, β, and γ-mRNA coding sequences, respectively. To obtain a probe for the large intervening sequence of β-mRNA precursors ($\beta^{\text{-intron}}$ probe), the large intervening sequence was excised from recombinant bacteriophage HβGl DNA with Eco RI and Bam HI restriction endonucleases (Fig. 1). HβGl contains the cloned cellular (genomic) β- and δ-globin genes, as well as intergenic DNA (24). The $\beta^{\text{-intron}}$ DNA fragment thus generated is ~1,000 base pairs long and contains only small regions of mRNA coding sequence (~60 base pairs, Fig. 1). This fragment was subcloned into the Eco RI and Bam HI sites of plasmid pBR322, using described methods (23, 24). Recombinant plasmid sequences were cultivated in *Escherichia coli* HB101 hosts, and plasmid DNA isolated as described (23).

To use each plasmid DNA as a hybridization probe, the entire plasmid was denatured to single-stranded form by boiling DNA (1 mg/ml) in 2 M sodium chloride, 0.2 M NaOH for 5 min (25), and bound to nitrocellulose filter discs by spotting 50 μg of each boiled DNA preparation onto separate discs (Schleicher & Schuell, Inc., Keene, N. H., BA85, 25-mm disc diameter, 0.45-micron pore diameter). The discs were air-dried, baked in vacuo at 80°C for 2 h, and washed in 300 mM sodium chloride, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS) for 30 min at 45°C (10 ml/ disc). Binding efficiency was 75–85%. Filters were used immediately for the hybridization incubations with [^{3}H]RNA.

Molecular hybridization assay for [^{3}H]mRNA. [^{3}H]RNA (usually $2 \times 10^5–8 \times 10^5$ cpm), in 10 ml of hybridization buffer

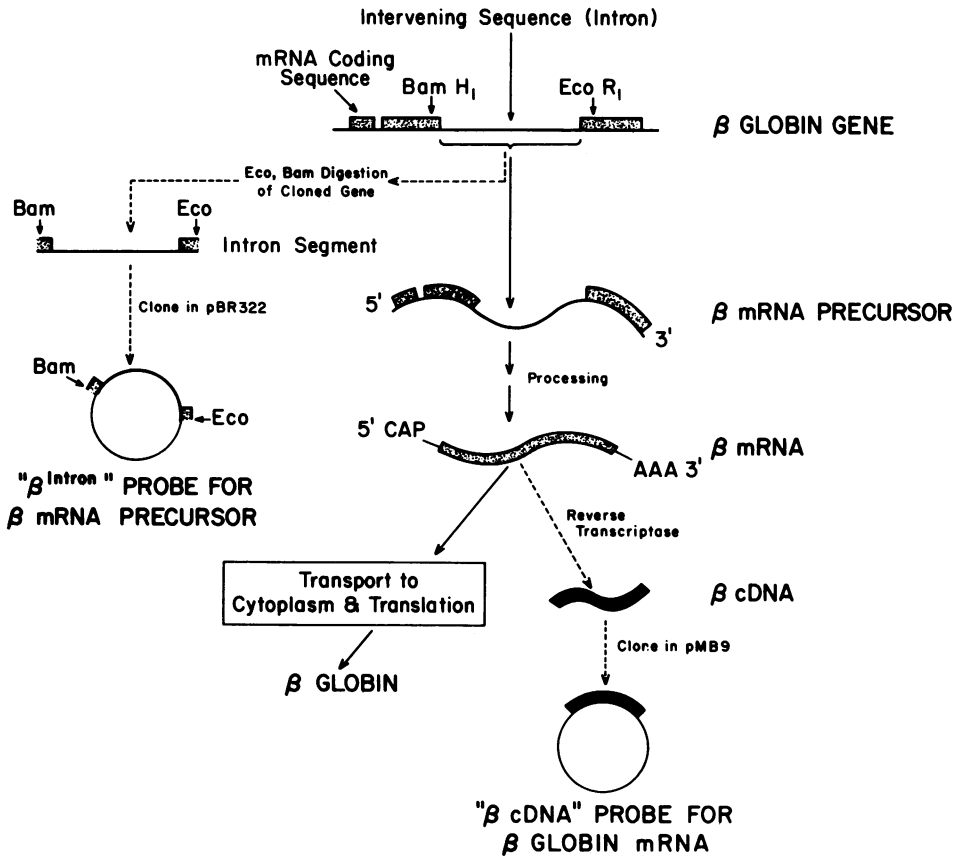

FIGURE 1 Pathway of β-globin gene expression and preparation of probes for analysis of β-globin mRNA and β-mRNA precursor sequences. The solid lines with arrows indicate the normal intracellular pathway of β-globin gene expression. Shaded blocks indicate regions of the gene containing the nucleotide sequences destined to be included in mature mRNA. The solid lines indicate sequences transcribed into β-mRNA precursor, and removed by mRNA processing, and, therefore, absent from mature β-mRNA. The dashed lines with arrows show the preparation of: (*a*) a recombinant DNA probe for the large intron transcript of the β-mRNA precursor from a cloned genomic β-globin gene (24) by subcloning of a restriction endonuclease fragment consisting largely of the large intervening sequence; and (*b*) preparation of a probe for mature β-mRNA from a synthetic DNA copy (cDNA) of mature β-mRNA (23). The cDNA is indicated by the solid black blocks. "5'-CAP"-5'-oligonucleotide cap sequence; "AAA-3"-poly-(A) tail. Both are added to the initial transcript of the β-globin gene during processing. Eco RI, Bam HI, restriction endonuclease sites used for purifying and cloning $\beta^{\text{-intron}}$ DNA probe.

(5 × SSC, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidine, 0.1% Ficoll 400 [Pharmacia Div. Pharmacia Fine Chemicals, Piscataway, N. J.], 50 mM sodium phosphate, pH 7.0, 0.1% SDS, 1% dextran sulfate, 100 μg/ml salmon sperm DNA; cf. 26) was placed in a sterile 30-cm³ plastic jar. The nitrocellulose filter discs bearing the individual DNA sequences complementary to the ^{3}H mRNA's to be measured were added. Each reaction mixture thus consisted of a "stack" of three to six filter discs immersed in the hybridization buffer containing [^{3}H]RNA. The sealed jars were incubated with gentle shaking for 48 h at 42°C. These high salt, low temperature conditions were chosen on the basis of preliminary experiments which established equal efficiencies of hybridization to α-, β-, γ-, and δ-DNA probes occurred (data not shown).

The amount of nonradioactive single-stranded DNA sequence complementary to the desired [^{3}H]mRNA was 2.5–10 μg on each filter disc used. These amounts constituted at least a fourfold excess of DNA probe over complementary hybridizing mRNA sequences. The excess of DNA sequences insured that all complementary mRNA sequences, [^{3}H]mRNA as well as preexistent nonradioactive mRNA present in the cells at the time of sampling, would be bound into mRNA-DNA hybrids.

At the end of the hybridization reactions, each filter disc was washed to remove nonspecifically adherent radioactive RNA (7). Each set of filters was placed in 20 ml buffer A (450 mM sodium chloride, 0.1% mM EDTA, 0.5% SDS, 10 mM Tris-HCl, pH 7.5) for 5 min at 24°C with gentle shaking, then transferred successively to fresh buffer A, 30 min, 68°C; buffer A, 5 min, 24°C; 1 × SSC, 0.5% SDS, 5 min, 24°C; 1 × SSC, 5 min, 24°C; low salt buffer 10 mM Tris-HCl, pH 7.5, 2 mM EDTA), 1 min, 24°C; low salt buffer, 10 min, 68°C; and low salt buffer, 1 min, 24°C. The discs were dried under a heat lamp, and bound ^{3}H counts per minute determined in a

liquid scintillation counter in 10 ml Liquifluor (New England Nuclear).

In all hybridization reactions, a filter disc to which only pMB9 plasmid DNA had been attached was included as a control for background levels of [^{3}H]RNA binding. Nonspecific adherence of ^{3}H counts per minute to these filters was between 15 and 30 cpm. The number of ^{3}H counts per minute of each globin [^{3}H]mRNA was determined by subtracting background values attributable to the plasmid control filters from the total counts per minute bound to the washed filters containing the individual DNA probes.

Measurements of globin synthesis and steady-state mRNA levels. Globin biosynthesis was measured by incubating intact bone marrow cells and/or reticulocytes with [^{3}H]leucine, as described (21, 22). Measurements of the steady-state amounts of globin mRNA present in the bone marrow aspirates at the time the sample was taken were accomplished by saturation hybridization techniques, using [^{32}P]deoxy cytidine triphosphate (dCTP) purified α- and β-cDNA (specific activity 8×10^7 cpm/μg cDNA) under stringent hybridization conditions (78°C, 0.2 M sodium phosphate buffer, pH 6.8, 0.5% SDS); the methods used were those described by us earlier (22, 27). The steady-state β/α-mRNA ratios in total erythroblast RNA were measured by comparing the slopes of the saturation hybridization curves. The values presented in Results were determined using the same pulse-labeled RNA preparations used to detect newly synthesized [^{3}H]mRNA.

RESULTS

Experimental approach. To investigate β-mRNA production in β-thalassemia, we have compared the synthesis and subsequent fate of β-mRNA and β-mRNA precursor sequences in β-thalassemic and nonthalassemic erythroblasts. To accomplish this, we labeled erythroblasts for 20 min with [^{3}H]uridine, and detected the production of newly synthesized globin mRNA by molecular hybridization of [^{3}H]RNA to filter-immobilized recombinant DNA probes complementary to mRNA or precursor (large intron) sequences. We also examined the subsequent fate of each [^{3}H]mRNA sequence after a 20-h chase in the absence of [^{3}H]-uridine.

In previously reported preliminary experiments using a less sensitive and specific hybridization system (17), we observed normal production of β-[^{3}H]mRNA during 20-min pulse incubations of β^+-thalassemic bone marrow, implying normal transcription with subsequent defects in processing, transport, or stability of β-mRNA transcripts. For the present study, we examined processing by comparing the relative amounts of pulse-labeled β-[^{3}H]mRNA precursor and β-mRNA produced by β^+-thalassemic erythroblasts, to those produced by nonthalassemic erythroblasts. The probe for β-mRNA precursor (Fig. 1) contains essentially only DNA sequences representing the large intron of the β-globin gene. The complementary RNA transcript that it detects can be present only in a β-mRNA precursor. The ratio of [^{3}H]β^{intron}/[^{3}H]β-mRNA is thus a rough but useful index for comparing the "precursor-to-product" radio in β^+-thalassemic erythroblast [^{3}H]RNA to that of nonthalassemic [^{3}H]RNA. In addition, we have examined a larger number of patients than in previous studies (14, 16, 17) in order to determine whether heterogeneity in β-mRNA metabolism existed in β-thalassemia.

Detection of [^{3}H]globin mRNA in pulse-labeled [^{3}H]RNA. The amounts of α/β-[^{3}H] and β^{intron} RNA sequences observed in pulse-labeled erythroblast RNA from eight nonthalassemic and six β^+-thalassemic patients are shown in Table I. The specific activity of total [^{3}H]RNA recovered ranged between 2×10^4 and 1×10^5 cpm/μg. The hybridizing radioactivity was destroyed by incubation with ribonuclease or alkaline treatment, but not by prior DNase treatment (data not shown). Globin [^{3}H]mRNA sequences ranged from 0.15 to 0.3% of total RNA input, except in nonthalassemic patients 7 and 8, in whom erythroid hyperplasia was only moderate (Table I), values in agreement with other studies (5–7, 12, 14, 16). Over a range of RNA inputs, including inputs two- to fourfold higher than those included in the figures and tables, the net counts per minute bound varied linearly with input and β/α-^{3}H ratios were essentially constant (data not shown).

To verify that all available [^{3}H]mRNA bound to the filters during the annealing reaction, we reincubated several [^{3}H]RNA hybridization mixtures with a second set of filters. Binding of [^{3}H]mRNA to the second set of filters was 0–10% of the [^{3}H]mRNA binding observed with the first set. Conversely, 80–85% of the [^{3}H]mRNA cpm that annealed to filter-bound DNA probes were shown to hybridize to a second set of filters after elution from the first filter (90% formamide, 65°C, 10 min) and reincubation with a second set of filters containing the same globin DNA probe (unpublished data).

Specificity of [^{3}H]mRNA-DNA filter disc hybridization. We established that no crosshybridization occurred among the mRNA and the DNA probes for β-, γ-, and δ-globin mRNA, which have closely related (homologous) nucleotide sequences. This was especially important for studies of β^+-thalassemic erythroblasts, because these patients synthesize elevated amounts of the γ-chain of Hb F and the δ-chain of Hb A_2 (1). Nonthalassemic erythroblasts produced almost no [^{3}H]mRNA sequences binding to γ- or δ-globin DNA; the amounts of γ- and δ-[^{3}H]mRNA binding to these probes in β^+-thalassemic erythroblast [^{3}H]RNA were small, and did not alter significantly the estimation of non α/α-[^{3}H]mRNA ratios (Table II). In separate experiments (unpublished data), we have shown that the amounts of γ- and δ-[^{3}H]mRNA measured in this manner corresponded well to the production of γ- and δ-mRNA and of γ- and δ-globin by erythroblasts. Moreover, bone marrow [^{3}H]RNA showed no binding to filters containing DNA complementary to embryonic

TABLE I

Measurement of Globin [^3H]mRNA in Erythroblasts Pulse-labeled with [^3H]Uridine for 20 min

Patient	Input [^{3}H] cpm	Net [^{3}H] cpm bound			Ratio		
		α	β	β^{intron}	β/α	β^{intron}/β	[^{3}H]globin mRNA[1]
							%
Nonthalassemic							
1	2×10^5	227	222	13	0.98	0.06	0.23
2	2×10^5	297	321	28	1.08	0.08	0.31
3	2×10^5	274	184	23	0.67	0.12	0.23
4	2×10^5	223	185	—	0.83	—	0.20
5	2×10^5	265	225	—	0.86	—	0.24
6	2×10^5	154	144	—	0.95	—	0.15
7	4×10^5	73	90	—	1.24	—	0.04
8	2×10^5	87	81	—	0.93	—	0.08
β^+-thalassemia							
1	2×10^5	278	253	274	0.91	1.08	0.31
2	2×10^5	255	202	22	0.79	0.11	0.24
3	2×10^5	111	121	121	1.09	1.00	0.14
4	4×10^5	284	246	100	0.87	0.41	0.15
5	4×10^5	578	543	130	0.93	0.21	0.24
6	2×10^5	271	223	18	0.81	0.08	0.24

[^{3}H]RNA was extracted and analyzed by hybridization to DNA filters as described in Methods. Each value shown represents cpm bound to each globin DNA filter after subtraction of ^{3}H counts per minute binding to a control filter (included in every incubation) which contained only plasmid pMB9 DNA (50 μg). The measured cpm bound to these control filters ranged from 15–30 cpm. Dashed lines indicate that the measurements were not performed.

[1] Sum of net cpm binding to α-, β-, γ-, and δ-filters/input cpm $\times$ 100. No binding to probes for embryonic globin mRNA (ϵ, ζ) observed.

(ϵ, ζ) globin mRNA. Specificity of hybridization was also verified by competition hybridization with excess amounts of nonradioactive globin mRNA. Binding of ^{3}H counts per minute to the β-mRNA probe was selectively inhibited by adult reticulocyte mRNA (α + β-mRNA), whereas binding of ^{3}H counts per minute to the γ-mRNA probe was preferentially inhibited by fetal reticulocyte mRNA (predominantly α + γ-mRNA). [^{3}H]RNA from lymphoblastoid cell lines exhibited no globin [^{3}H]mRNA sequences above background. [^{3}H]-RNA from the K562 human leukemia cell line, which produces only embryonic and fetal hemoglobin (27), yielded predominantly globin [^{3}H]mRNA binding to the cloned DNA probes for fetal and embryonic mRNA (γ, ϵ, ζ) (data not shown).

Initial production of globin mRNA in nonthalassemic erythroblasts. Pulse-labeled RNA from nonthalassemic erythroblasts of eight patients contained approximately equal amounts of α- and β-[^{3}H]mRNA sequences (range 0.67 to 1.24; Table I). These ratios correlated well with those reported by Kantor et al. (16), but are somewhat higher than those observed by Maquat et al. (14). As shown in Fig. 2, the β/α-[^{3}H]mRNA ratio did not decline during 20-h chase incubations in the absence of [^{3}H]uridine, indicating approximately equal stabilities of α/β-globin [^{3}H]-mRNA transcripts.

Initial production and posttranscriptional fate of globin mRNA sequences in β-thalassemic erythroblasts. The initial rate of β-mRNA production by six patients with phenotypic β^+-thalassemia approximated the normal range (β/α-[^{3}H]mRNA = 0.79–1.09, Table I and Fig. 2). The ratio of newly synthesized β- and α-globin mRNA was substantially higher than the steady-state β/α-mRNA ratios (Table III), which measured the relative amounts of α- and β-globin mRNA accumulating in the cells before the pulse-labeling period. By contrast, the initial production of α- and β-mRNA in nonthalassemic erythroblasts corresponded well to the relative steadystate accumulation of α- and β-mRNA (Fig. 2).

In two patients with β-thalassemia (patients 1 and 2), from whom sufficient erythroblasts were obtained for measurement of posttranscriptional stability of β-mRNA sequences during a 20-h chase incubation, a substantial decline in the β/α-[^{3}H]mRNA ratios oc-

TABLE II
Specificity of [³H]non-α-mRNA Binding to Filter Bound Plasmid DNAs

Patient	Net [³H] cpm bound			Ratio	
	β	γ	δ	γ/β	δ/β
Nonthalassemic					
1	222	6.4	8.7	0.029	0.039
2	321	9.5	0	0.029	0
3	184	2.3	0.8	0.013	0.004
β⁺-thalassemia					
1	253	61.4	30	0.22	0.12
2	202	26.8	2.3	0.13	0.01
3	121	24	16.4	0.20	0.14
4	246	64	17.2	0.23	0.07
5	543	54.8	7.2	0.13	0.02
6	223	8	1.0	0.03	0.005

Data were obtained as discussed in Methods and Table I. The probe for δ-mRNA was a plasmid subclone containing the 3′-end of the δ-mRNA coding sequence, as well as some extragenic DNA beyond the 3′-end of the δ-gene. The subclone was prepared by Eco RI digestion (Eco RI fragment F) of HβGl, a λ-phage recombinant containing the entire length of normal human δ- and β-genes (21). The Eco RI fragment was subcloned into the Eco RI site of plasmid pBR322. The probe for γ-mRNA was γ-cDNA plasmid JW151 (cf. 20). Patients and RNA inputs were the same as in Table I.

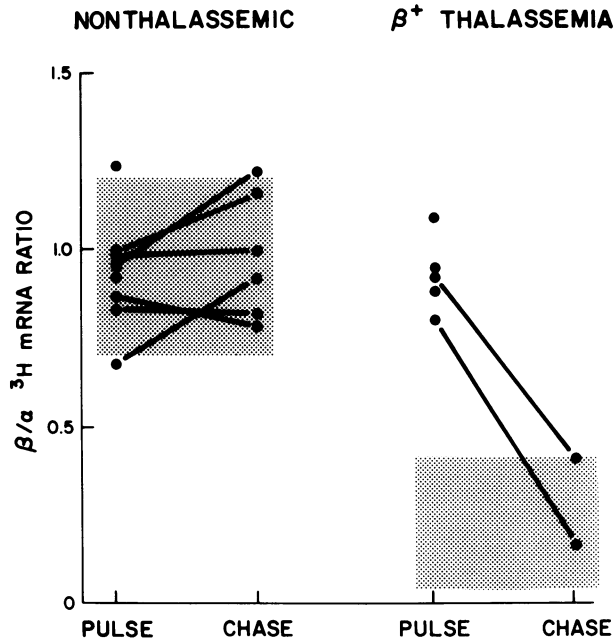

FIGURE 2 Production and stability of globin mRNA in nonthalassemic and β⁺-thalassemic erythroblasts after pulse and pulse-chase labeling with [³H]uridine. Data were obtained as described in the text and Tables I and II. The solid lines connect pulse and pulse-chase results obtained with the same bone marrow samples. Some patients could be studied only with pulse incubations. Shaded areas indicate range of "steady-state" β/α-mRNA ratios obtained with the bone marrow samples from which the [³H]mRNA was obtained. Effectiveness of the chase incubation is suggested by: (*a*) the alteration of β/α-ratios in the β⁺-thalassemic patients (1 and 2) studied with chase incubations; (*b*) by the decline in total [³H]RNA cpm (per 10^8 cells) of 66–78% in all patients studied; and (*c*) a shift of [³H]mRNA cpm from predominantly nuclear (50–70%) after the pulse to predominantly cytoplasmic (88–95%) after the chase incubation in separate experiments (Cf. Table V).

curred (Fig. 2). Moreover, the β/α-[³H]mRNA ratios measured after a chase incubation (Table III) correlated well with the steady-state levels of preexistent β- and α-mRNA. The steady-state levels of α- and β-mRNA shown in Table III were obtained with the RNA specimen containing ³H pulse-labeled RNA; the RNA samples obtained after the chase incubation also exhibited similar β/α-ratios.

Abnormal accumulation of large intron mRNA precursor sequences in β⁺-thalassemia. The initial production of RNA transcripts complementary to the large intron of the β-globin gene in nonthalassemic erythroblasts (patients 1–3) and β-thalassemic erythroblasts (patients 1–6) is shown in Table I. [³H]RNA binding to the β-^{intron} probe represented only 6–12% of β-mRNA coding sequences in nonthalassemic erythroblasts, indicating relatively rapid turnover (degradation) of the β-^{intron} RNA during normal processing. During a 20-h chase incubation, the level of intervening sequences declined to 1–3% of β-mRNA (Table IV), suggesting that essentially no radioactive intervening sequence remained after the chase incubation.

In contrast to normal erythroblasts, some β-thalassemia erythroblasts accumulated abnormally large amounts of [³H]β-^{intron} during the pulse incubation (Table IV). In patients 1, 3, and 4 substantial increases in both the total number of β-^{intron} counts per minute (Table I), and in the β-^{intron}/β-mRNA ratio (Table IV), ranging from 4 to 10 times normal, were observed. In patient 5, [³H]β-^{intron} RNA accumulation was also increased (twice normal), but less dramatically. These results suggest that the posttranscriptional defect in β-globin mRNA metabolism involves anomalous β-mRNA precursor processing in these patients. Apparently, [³H]β-^{intron} RNA was also not degraded normally, because large amounts of these sequences accumulated during the pulse incubation. [³H]β-^{intron} RNA accumulation was apparently normal in two cases, patients 2 and 6, as discussed later.

In patient 1, the quantity of [³H]β-mRNA precursor sequences was documented to be nearly elevenfold greater during the pulse incubation than in normal pulse-labeled [³H]mRNA (Tables I and II). Intervening sequence continued to comprise nearly 25% of the [³H]β-mRNA levels, even after a 20-h chase incubation during which intervening sequences essentially dis-

TABLE III

Comparison of Newly Synthesized and Steady-State Levels of Globin mRNA in β⁺-thalassemia Erythroblasts

Patient	β/α ratio		[³H]mRNA	
	Intact cell globin synthesis	"Steady-state" mRNA	Pulse	Chase
β-thalassemia				
1	0.14	0.42[1]	0.92	0.41
2	0.07	0.10	0.79	0.17
3	0.11	0.21	1.09	—
4	0.10	0.25	0.87	—
5	0.06	0.20	0.93	—
6	0.23	0.15	0.81	—

[³H]mRNA data are derived from Table I and Fig. 2. Chase incubations were for 20 h in the presence of 20 mM uridine after removal of [³H]uridine from the medium (cf. Methods). Globin synthesis and steady-state mRNA were measured as described in Methods. Dashed lines indicate insufficient RNA was available for analysis. As noted in the legend to Fig. 2, total [³H]RNA, after the chase, were one-fourth to one-third of the pulse values. [³H]α-mRNA (α-cpm/10^6) remained at 50–100% of pulse values, while [³H]β-mRNA cpm/10^6 cpm declined to 0.1–0.3 of pulse-labeled [³H]β-cpm/10^6 cpm (in patients 1 and 2). Data for pulse-labeled RNA are from Table I. Data for chase samples are from 400,000 cpm RNA inputs; net α-[³H] counts per minute were at least 200 cpm above background for both patients.

[1] mRNA ratio in total cellular erythroblast RNA; βα-mRNA = 0.18 in reticulocytes (cf. Fig. 3). Patient numbers refer to the same patients as Table I.

TABLE IV

Abnormal Accumulation of [³H]β mRNA Precursor Sequences in β⁺-thalassemia

Patient	[³H]β-intron/[³H]β-mRNA	
	Pulse	Chase
Nonthalassemic		
1	0.06	0.01
2	0.08	0.03
3	0.12	0.03
β-thalassemia		
1	1.08	0.23
2	0.11	0.05
3	1.00	—
4	0.41	—
5	0.21	—
6	0.08	—

Data were obtained as described in Methods and in Table I. Patients are numbered as in Table I. Ratios shown represent net cpm bound to β-intron probe ÷ net cpm bound to β-cDNA probe. Dashed lines indicate insufficient RNA was available for analysis. Absolute ratios of chase/pulse [³H]β-intron counts per minute per 10^6 counts per minute total RNA were 0.08 for β⁺-thalassemia patient 1, and 0.005–0.03 for the other patients. Pulse-labeled RNA values from Table I. Pulse-chase labeled RNA data were obtained from experiments in which [³H]β-mRNA net counts per minute were at least 100 cpm.

appeared from nonthalassemic erythroblast [³H]RNA (Table IV). Total cellular erythroblast RNA from this patient contained substantially higher levels of β-mRNA (Fig. 3; β/α-mRNA = 0.41) than expected on the basis of β-globin synthesis (β/α-globin synthesis = 0.14, Table III). In each of the remaining three patients with abnormally high β-intron mRNA production (patients 3, 4, 5), the levels of β-mRNA were also higher than β-globin synthesis although the differences were less striking (Table III). These results are consistent with earlier studies (15). The β/α-mRNA ratio in anucleate peripheral blood reticulocytes from patient 1 was 0.18 (Fig. 3), a value that did correlate well with β-globin synthesis (Table III). The correlation of anomalous persistence of β-mRNA precursor sequences with an unexpectedly high level of total erythroblast β-mRNA sequences in the steady state could reflect substantial accumulation of abnormally processed β-mRNA during erythropoiesis, possibly in the form of nuclear β-mRNA and/or RNA precursor, as reported earlier (15).

Posttranscriptional decline in [³H]β-mRNA in two patients with no apparent abnormalities of processing of the large intervening sequence. In patients 2 and 6 with β-thalassemia, the initial [³H]β/α-mRNA levels (Table III), the β^1/β-[³H]RNA levels in total cellular pulse-labeled RNA (Table IV), and the β^1/β-[³H]RNA levels after the chase incubation (patient 2, Table IV) were all within the normal range. No apparent abnormality in either β-globin gene transcription or processing of large intron RNA was observed to account for the posttranscriptional decline in β/α-[³H]mRNA, or the β-thalassemic levels of steady-state mRNA documented in Tables III and IV. In an effort to localize better the posttranscriptional defect in patients 2 and 6, we prepared pulse-labeled [³H]RNA from fractionated nuclei and cytoplasm (Table V). In addition, sufficient material from patient 2 was available for examination of ³H nuclear and cytoplasmic mRNA after a 20-h chase in the absence of [³H]uridine.

Nuclear [³H]RNA accounted for 70% of the radioactive globin mRNA sequences in the pulse-labeled samples, but only 5% of the remaining radioactivity after the chase incubation (data not shown). As shown in Table V, the β/α-[³H]mRNA ratios were close to normal in pulse and pulse-chase labeled nuclear RNA. In contrast, a substantial reduction in the β/α-[³H]mRNA ratios in the cytoplasm was evident in both patients even within the 20-min pulse incubation period (Table V). A further decline in the β/α-[³H]mRNA ratio of cytoplasmic RNA from patient 2 occurred during the subsequent 20-h chase incubation (Table V), but the most substantial alteration occurred during the pulse

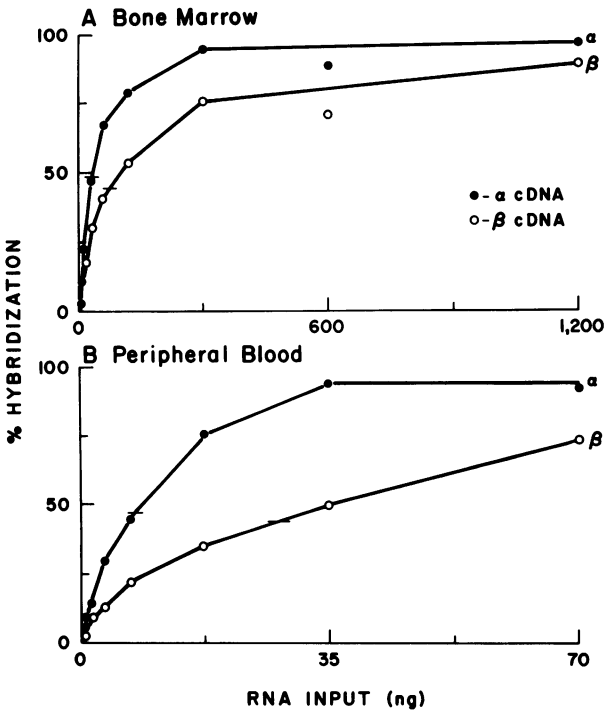

FIGURE 3 Relative "steady-state" levels of α- and β-mRNA from erythroblasts and reticulocytes of β^+-thalassemic patient 1. Data were obtained as discussed in Methods by incubation of increasing concentrations of each RNA sample with equal amounts of purified α-[^{32}P] (closed circles) or β (open circles) cDNA. mRNA ratios were obtained by comparing the RNA inputs required to achieve "half saturation" (50% of maximum hybridization) of the α- and β-cDNA probes. α/β-mRNA ratio for bone marrow RNA was 0.42 and for reticulocytes, 0.18. Dash marks on hybridization curves indicate the half saturation values.

incubation. In patient 6, cytoplasmic pulse-labeled RNA yielded a β/α-[^{3}H]mRNA ratio that was already in the range of steady-state cytoplasmic mRNA (Table V). In both patients, steady-state nuclear β/α-mRNA ratios were substantially higher in nucleus than in cytoplasm. The defects in patients 2 and 6 thus appear to be intranuclear, leading rapidly to deficiency of newly synthesized cytoplasmic β-mRNA. As discussed later, previously published nucleotide sequence data from patient 6 (18) have revealed only a single base change in the cloned β^+-thalassemia gene, occurring within the small intron. The large intron was normal (cf. Discussion).

DISCUSSION

Our studies suggest strongly that the initial rate of β-globin mRNA transcription is normal in β^+-thalassemia. A distinct posttranscriptional decline of β-mRNA occurred (Fig. 2, Table III). These findings extend earlier studies (14, 16, 17), which presented evidence for defective mRNA processing as a cause of β^+-thalassemia. The present studies argue strongly that posttranscriptional defects, most likely involving abnormal intranuclear processing, occur in the majority of patients with phenotypic β^+-thalassemia.

Table V
Nuclear and Cytoplasmic [^{3}H]globin mRNA in β-thalassemia

	β/α-[^{3}H]mRNA		β/α-mRNA
	Pulse	Chase	Steady state
Nonthalassemic patient 1			
nucleus	0.67	0.83	1.1
cytoplasm	0.91	0.92	0.8
β-thalassemia patient 2			
nucleus	1.34	0.90	0.7
cytoplasm	0.41	0.20	0.2
β^+-thalassemia patient 6			
nucleus	1.45	—	0.5
cytoplasm	0.26	—	0.2

Pulse and pulse-chase labeling of cells with [^{3}H]uridine and molecular hybridization analysis of [^{3}H]RNA and steady-state RNA were performed as described in Methods. Nuclear and cytoplasmic RNA were extracted by the methods of Laevis and Penman (20). 70% of pulse-labeled globin mRNA cpm was nuclear; only 5% of [^{3}H]globin mRNA cpm was in the nucleus after the chase. Of the total [^{3}H]RNA cpm recovered from the pulse, 50% remained after the chase, but the percent of total cpm recovered as globin mRNA was 80–100% of pulse values, results consistent with other laboratories (cf. 30). Net α-[^{3}H] cpm in all cases was at least 150 cpm. Cytoplasmic β/α-[^{3}H]RNA ratios were repeated at several RNA inputs and yielded essentially the same β/α-ratios as those shown in the table.

An additional finding of the present study was heterogeneity in the amount of large intron [^{3}H]RNA accumulating during pulse incubations among the six patients studied (Table IV). Three patients (1, 3, and 4) exhibited abnormally high large intron [^{3}H]RNA, confirming earlier suggestions (14, 16, 17) that abnormal processing intermediates containing transcripts of the large intron accumulated in β^+-thalassemia. In one case, patient 5, this accumulation was less dramatic, but still abnormally high (twice normal). However, in two patients (2 and 6), no abnormality of ^{3}H large intron RNA accumulation was detected. These results could arise from different lesions in mRNA processing in different patients, or from individual patient differences in handling of the abnormally processed precursors.

The results in Table V argue strongly that the defects in β-mRNA metabolism in patients 2 and 6 are intranuclear despite normal large intron [^{3}H]RNA accumulation. Indeed, it is interesting that results from patient 6 are compatible with nucleotide

sequence data (18) from a cloned cellular β-globin gene. The only nucleotide sequence abnormality is located in the small intron, 20 base pairs from the 3′-junction between the intron and mRNA coding sequence. The mutation creates a potentially anomalous splicing signal that should result in abnormal processing of the small intron (18). It is tempting to speculate that the small intron processing defect does not lead to accumulation of large intron transcripts (Table IV) seen in some of the other patients. However, our data do not justify this conclusion because the methods are not sensitive enough to detect slight increases in large intron RNA accumulation, evident in the steady state as the result of accumulation throughout erythropoiesis, but not appreciable within a short 20-min pulse incubation.

Neither the structure of the cloned β-globin gene of patient 6 (18) nor our data define the exact mechanisms by which this mutation leads to defective β-mRNA accumulation. However, our results (Tables III and V) establish some of the features of abnormal β-mRNA metabolism that must result from this mutation. Because the cytoplasmic β/α-mRNA ratio at the end of the pulse incubation is already close to that of steady-state mRNA, and because this β/α-mRNA ratio also correlates well with β-globin synthesis, the small intron mutation results in delivery to, or accumulation within, the cytoplasm of only the more stable, normally functioning β-mRNA molecules resulting either from normal processing events, or further processing of the anomalous mRNA product created by the alternative splice site. One must assume that the abnormal splice signal is preferentially utilized to generate the abnormal mRNA product. Otherwise, it is difficult to account for the fact that only a minority of β-globin mRNA in this patient (Table III) survives as normally functioning cytoplasmic β-mRNA sequences. We found no evidence for accumulation of substantial amounts of nonfunctioning β-mRNA. Finally, the abnormal processing product is either not delivered to cytoplasm, or is exceedingly labile, because the normal half-life of cytoplasmic globin mRNA is at least 20 h (12, 13, 30). The data in Table V demonstrate that any abnormal β-mRNA delivered to cytoplasm is already largely destroyed during the 20-min pulse incubation.

The patients studied by ourselves and others (14, 16, 17) had phenotypic β^+-thalassemia. Some of these patients could be doubly heterozygous for β^+- and β°-thalassemia. The heterogeneity of $\beta^{\text{-intron}}$ RNA accumulation that we have observed may not merely represent different types of β^+-thalassemia (e.g., large vs. small intron defects). For example, double heterozygosity for the mutation identified in patient 6 and a recently identified mutation for β°-thalassemia resulting in ablation of the processing signal at the 5′-end of the large intron (31, 32) would account for our results from patients 1, 3, and 4. Patients with higher levels of large intron RNA could well be β^+/β° double heterozygotes with exaggerated large β-intron RNA accumulation due to β°-thalassemia of the type described above.

A corollary of our studies of the specificity of our hybridization system (Table II) is that the low level of γ- and δ-globin gene expression in adult erythroid cells is determined primarily at the level of initial mRNA synthesis (transcription). Similarly, the low level of large intron [^{3}H]RNA detected in nonthalassemic cells implies rapid turnover of these sequences during normal mRNA processing. Conversely, in those patients with β^+-thalassemia who exhibited large amounts of large intron [^{3}H]RNA, defective processing must also impair degradation sufficiently to allow the abnormal accumulation documented in Table IV.

In conclusion posttranscriptional intranuclear defects in β-globin mRNA processing cause β^+-thalassemia. There is heterogeneity in the extent to which accumulation of precursors retaining the large intron occurs. This variability could be due to different types of mRNA processing mutations or variability in the metabolism of abnormal β-mRNA precursors. Further kinetic and structural analysis of cloned genes and mRNA precursors will be required to identify the precise defects in individual patients.

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