Differentiation of the mRNA Transcripts Originating from the α1- and α2-Globin Loci in Normals and α-Thalassemics

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ABSTRACT The α-globin polypeptide is encoded by two adjacent genes, α1 and α2. In the normal diploid state (αα/αα) all four α-globin genes are expressed. Loss or dysfunction of one or more of these genes leads to deficient α-globin production and results in α-thalassemia. We present a technique to differentially assess the steady-state levels of the α1- and α2-globin messenger RNA (mRNA) transcripts and thus delineate the relative level of expression of the two α-globin loci in a variety of α-thalassemia states. Only α1 mRNA was produced in the α-thalassemia-2 haplotype (−α) (one of the two α-globin genes deleted from chromosome 16). This confirms previous gene mapping data which demonstrate deletion of the α2 gene. The triple α-globin gene haplotype (ααα) is the reciprocal of the α-thalassemia-2 haplotype and thus contains an extra α2-globin gene. RNA from this haplotype contained a greater than normal level of α2- relative to α1-globin mRNA. This data implies that the extra α2 gene in the triple α-globin haplotype is functional. We detected a relative instability of the α2-globin mRNA encoding the α-globin structural mutant Constant Spring. This instability may contribute to the low level of expression of the α-Constant Spring protein. In a Chinese patient with nondeletion hemoglobin-H disease (− −/ααᵀ) (both α-globin genes are present but not fully functional) a normal ratio was maintained between the levels of α1- and α2-globin mRNA, implying that mRNA production from both α-globin genes is suppressed in a balanced manner. These observations extend previous findings concerning the structural rearrangements in the deletion types of α-thalassemia and the pathophysiology of two nondeletion variants.

Received for publication 6 February 1981 and in revised form 8 April 1981.

INTRODUCTION

The two α-globin genes, α1 and α2, encode for an identical α-globin product (1). These two genes, located ~3.0 kilobases (kb) apart (2,3) on chromosome 16 (4), are oriented in the same transcriptional direction (5). They comprise part of a cluster of α- and α-like globin genes including the embryonic ζ genes and the pseudo-alpha (Ψα1) gene which are oriented 5′-ζ2- ζ1-Ψα1-α2-α1 3′ with respect to each other (5). Since both the α1- and the α2-globin genes encode for an identical protein product, identifying the extent to which each of the two α-globin loci is expressed in normals has not been possible. We and others have reported the complete nucleotide sequence of both human α-globin genes (6–8) and have identified that the 3′ noncoding region of these genes varies at 18 positions and a single base insertion/deletion. We now utilize these sequence differences to devise an assay for α-globin messenger RNA (mRNA) which clearly discriminates between α1 and α2 mRNA and study the relative contribution of these two α-globin mRNA species to total α-globin production in α-thalassemia syndromes. The data confirms the conclusions of previous gene mapping studies that identify the possible boundaries of α-globin gene deletion in two separate α-thalassemia-2 haplotypes (leftward and rightward deletion) (5, 9, 10). We quantitated the relative levels of expression from the α1- and α2-globin loci in reticulocyte RNA from two cases of α-thalassemia associated with physically intact but dysfunctional α-globin genes (nondeletion α-thalassemia), and found two distinct patterns of α-globin mRNA expression. Finally, this assay measured a threefold higher level of α2 than α1 cDNA synthesis from normal reticulocyte RNA, it suggests a difference in the metabolism of the two α-globin mRNA species.

METHODS

Subjects. RNA was isolated from the peripheral blood reticulocytes and/or bone marrow of six nonthalassemic adults, four adults with different forms of α-thalassemia, and a subject with an extra α-gene locus (11). All samples were obtained after informed consent. The non α-thalassemic subjects (Table I) were chosen on the basis of elevated reticulocyte counts in order to maximize the yield of erythroid mRNA from peripheral blood samples. Their hematologic diagnoses were made by conventional clinical testing, and all had normal or increased mean corpuscular volume and negative family history for thalassemia. The nonthalassemic status of the three sickle cell anemia subjects in the control group was further substantiated with a normal α-globin gene map as determined by Southern blotting analysis (12) of Eco RI and Hpa I digested DNA.[1] Each of the four subjects with α-thalassemia (Table II) has a different genotype. Subject 7 is a Chinese with the leftward deletion type of hemoglobin-H disease (− −/−α) (3, 10), subject 8 is a Black, homozygous for the rightward deletion α-thalassemia-2 haplotype (−α/−α) (10), subject 10 has hemoglobin-H Constant Spring (− −/$\alpha^{C.S.}$α) (7, 13), and subject 11 has nondeletion hemoglobin-H disease (− −/αα^{T}) (14). Bone marrow samples from two additional subjects were obtained during diagnostic aspirations (acute leukemia in remission and pancytopenia secondary to hypersplenism). Both samples were morphologically normal.

Plasmid DNA preparation and primer construction. Plasmid pRP9 (15) is a recombinant of the plasmid pBR322 which contains a double-stranded DNA copy (cDNA) of the 3′ portion of the α-globin mRNA extending from the Hind III endonuclease recognition site at codon 90–91 to 16 adenines in the polyadenosine (polyA) tail. This recombinant plasmid was constructed by using synthetic Hind III linkers. The sequence of the inserted cDNA corresponds to the α2-locus.[2] The plasmid in the EK2 host bacteria HB101 was grown in M9 media in 1.5 liter batches, amplified at OD_{640} 0.7 with chloramphenicol (150 μg/ml), harvested 18 h later, and purified from a clarified lysate (16) by CsCl isopyknic centrifugation (17). All preparative work was done in a P2 facility according to National Institutes of Health Guidelines. To prepare a 5′ end-labeled primer specific for the synthesis of α-globin cDNA, this plasmid DNA was first digested with the restriction enzyme Hind III according to conditions specified by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.) (Fig. 1). The 5′ ends generated by Hind III digestion were labeled with [γ-^{32}P]ATP (4,000 Ci/mM) (New England Nuclear, Boston, Mass.) using T_4 polynucleotide kinase according to manufacturer's specification (New England Biolabs, Beverley, Mass.) after dephosphorylation with bacterial alkaline phosphatase (Bethesda Research Laboratories). The labeled fragments were digested with Dde I and separated on 5% polyacrylamide gels (18). The 31 nucleotide fragment corresponding to the 3′ terminal 9 bases of the α-globin mRNA and 16 nucleotides in the polyA tail, as well as 6 nucleotides derived from the Hind III linker, was isolated by electrolution from the appropriate gel slice. Specific activity of end-labeled fragments ranged from 1.1–1.5 × 10^6 dpm/μmol. Since the α1- and α2-globin transcripts are completely homologous over the terminal 40 bases (7, 8), this DNA fragment should be equally effective in priming the synthesis of α1- or α2-globin cDNA but not β-, γ- or δ-globin cDNA, where the sequences differ extensively in this region (19).

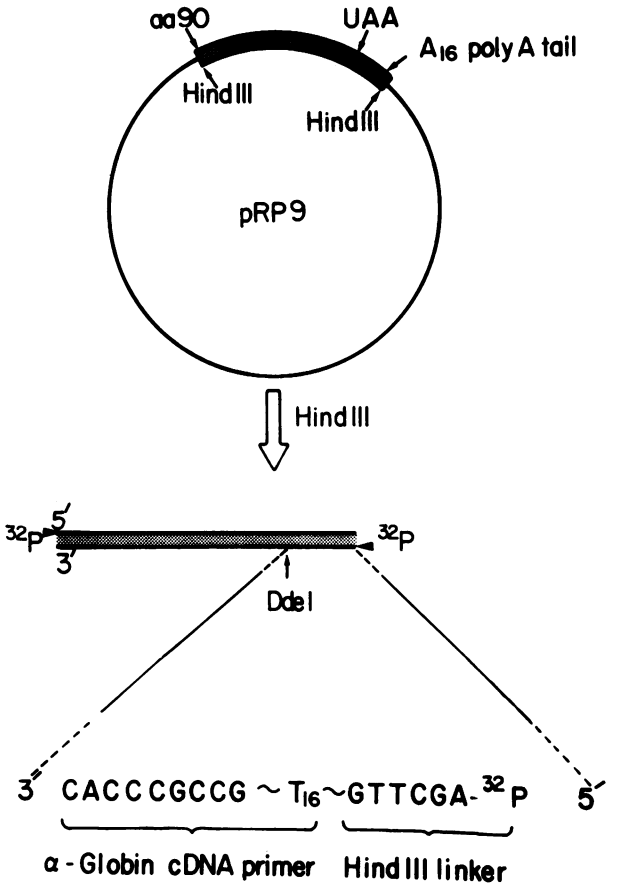

FIGURE 1 Construction of an α-globin specific primer. The insert (shaded) containing the 3′ portion of the α-globin cDNA was excised from plasmid pRP9 with Hind III and 5′ end-labeled with ^{32}P. Secondary digestion of the products with Dde I generated a 31 nucleotide end-labeled fragment that specifically primed α-globin cDNA synthesis under conditions described in Methods.

RNA preparation. The RNA used as a template for reverse transcription was prepared by one of three methods. Reticulocyte RNA was isolated from fresh blood by phenol extraction of acid-precipitated polysomes (20) after selective lysis of the erythrocytes (14). RNA from frozen blood samples and all bone marrow samples was isolated either by CsCl centrifugation of a guanidine thiocyanate cell lysate (21) (Fluka AG, Switzerland), or by phenol extraction from a sodium dodecyl sulfate (SDS) lysis buffer with subsequent precipitation of RNA with 0.25 M LiCl (22).

Synthesis and Cleavage of cDNA. Single-stranded α-globin cDNA was synthesized from peripheral reticulocyte or bone marrow RNA in buffer conditions designed to yield full-length transcripts (23). 360 pmol of end-labeled primer denatured by heating at 100°C for 2 min was added to 2–10 μg of total RNA and incubated with 600 U/ml avian myeloblastosis virus reverse transcriptase at 45° or 55°C for 1 h. The reaction was terminated by extraction with phenol/chloroform/isoamyl alcohol (50:49:1), and the cDNA precipitated in 0.2 M sodium acetate with 2.5 vol of ethanol and resuspended in 40 μl H_2O. cDNA synthesized from 1 μg of total RNA was digested with 24 U of Hae III (New England Biolabs) in the suggested buffer

[1] Kan, Y. W., and A. M. Dozy. Unpublished data.
[2] Liebhaber, S. Unpublished data.

at 37°C for 2 h. Hae III digestion products were separated on an 8% acrylamide denaturing gel (24) and autoradiographed for 1–7 d using Kodak XR5 film (Eastman Kodak Rochester, N. Y.) at −70°C with an intensifying screen. The radioactivity in the fragments was quantitated by either tracing the autoradiograph on a Joyce Loebl densitometer or by direct Cerenkov counting of excised gel slices. Preliminary results showed that the cleavage patterns and relative amounts of α1- and α2-globin cDNA synthesis were not affected by the method of reticulocyte RNA isolation. The α1/(α1 + α2) ratios obtained by densiometric scanning of autoradiographs or by direct gel slice counting agreed within 0.02 of each other.

DNA sequencing. cDNA sequencing was performed as described by Maxam and Gilbert (24) with one modification in the adenosine reaction (as noted in reference 6). The thin gel system (25) was used with 8 M urea and 8% acrylamide. DNA fragments to be sequenced were purified on a preparative 8 M urea, 8% acrylamide gel, and isolated by electroelution from excised gel slices.

RESULTS

Separate identification of α1- and α2-globin cDNA. In all samples studied, full-length cDNA (~580 nucleotides) comprised the predominant product of the reverse transcription reaction as assayed on an 8% acrylamide, 8 M urea gel. Complete cleavage of the end-labeled α1-globin cDNA by Hae III yielded a labeled 95 nucleotide fragment on the autoradiograph, and complete cleavage of the α2-globin cDNA yielded a 60 nucleotide band. The Hae III digest was often incomplete due to the relative resistance of single-stranded DNA to digestion (26, 27). However, the next Hae III sites on the α1- and α2-globin cDNA also differed from each other so that the first partial digestion products of 115 and 83 nucleotides, respectively, could be identified separately (Fig. 2). The origin of the 95 and 115 nucleotide fragments from α1- and the 60 and 83 nucleotide fragments from α2-globin mRNA was confirmed by two methods. (*a*) We isolated and sequenced the end-labeled Hae III-generated cDNA fragments. The sequences of both the complete and partial digest bands for the α1- and α2-globin cDNA Fig. 3) were identical to those of the 3′ termini of α1- and α2-globin mRNA respectively, as predicted by genomic sequence analysis (6–8). (*b*) We analyzed Hae III cleavage products of end-labeled α-globin cDNA synthesized from RNA preparations that contained synthesized from two RNA preparations that contained either the α1- or the α2-globin mRNA. The two mRNA species were obtained separately by transforming monkey kidney cells (CV1) with recombinant SV40 virus DNA containing cloned α1- or α2-globin genes (pSV3-α1 and pSV3-α2) (28). RNA isolated from these cells contained, together with CV1 and SV40 mRNA, fully processed mRNA from the α1- and α2-globin genes.[3] These RNA preparations were reverse transcribed using the end-labeled α-globin mRNA-specific primer,

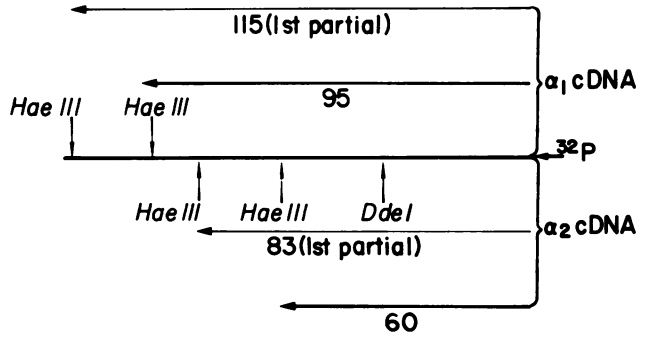

FIGURE 2 Hae III cleavage pattern of the α-globin cDNA 3′ terminus. Distance is shown in nucleotides from the terminal ^{32}P of the cDNA primer to the sites of Hae III cleavage in the α1- and α2-globin cDNA primer extension products. Dde I site marks the 3′ terminus of the primer (31 nucleotides). The heavy arrows describe the 3′ terminal Hae III fragment of α1 cDNA (95) and α2 cDNA (60); the light arrows describe products generated by the next Hae III site in each case.

the resultant cDNA cleaved with Hae III, and the expected 95 nucleotide α1 and 60 nucleotide α2 fragments were obtained from the RNA of the pSV3-α1 and the pSV3-α2 clones respectively (Fig. 4, lanes c and d).

Relative α1- and α2-globin cDNA synthesis in nonthalassemics. We investigated the relative contributions of α1- and α2-globin genes to the total α-globin cDNA synthesis in six nonthalassemic subjects with elevated reticulocyte counts. The α1/(α1 + α2) ratio was calculated in 14 independent assays of the adult nonthalassemic reticulocyte RNA samples, yielding a mean α1/(α1 + α2) ratio of 0.26±0.06 (mean ±2 SD) (Table I). Similar ratios of α1- and α2-globin transcripts were found in two nonthalassemic bone marrow samples (0.22 and 0.26), suggesting that the relative age of circulating reticulocytes does not significantly affect the ratio of the two α-globin mRNA species.

Relative α1- and α2-globin cDNA synthesis in α-thalassemia. We applied the cDNA assay to reticulocyte RNA from subjects with a variety of α-globin gene deletions or rearrangements (Table II). Two subjects with α-thalassemia-2 haplotypes were studied; one with hemoglobin-H disease ([– –/–α] leftward deletion) and one homozygous for the α-thalassemia-2 haplotype ([–α/–α] rightward deletion). As determined by cDNA synthesis, the α-globin mRNA present in the reticulocytes of both subjects corresponds to the sequence of the α1 locus (Table II, Fig. 4, lane e). We analyzed the relative amount of α1- and α2-globin transcription in subject 9 with three α-loci on one chromosome (11) and thus five α genes in total (αα*α*/αα). Gene mapping shows that this genotype is the reciprocal product of the unequal crossover which

[3] Kan Y. W., A. Dozy, and P. Berg. Manuscript in preparation.

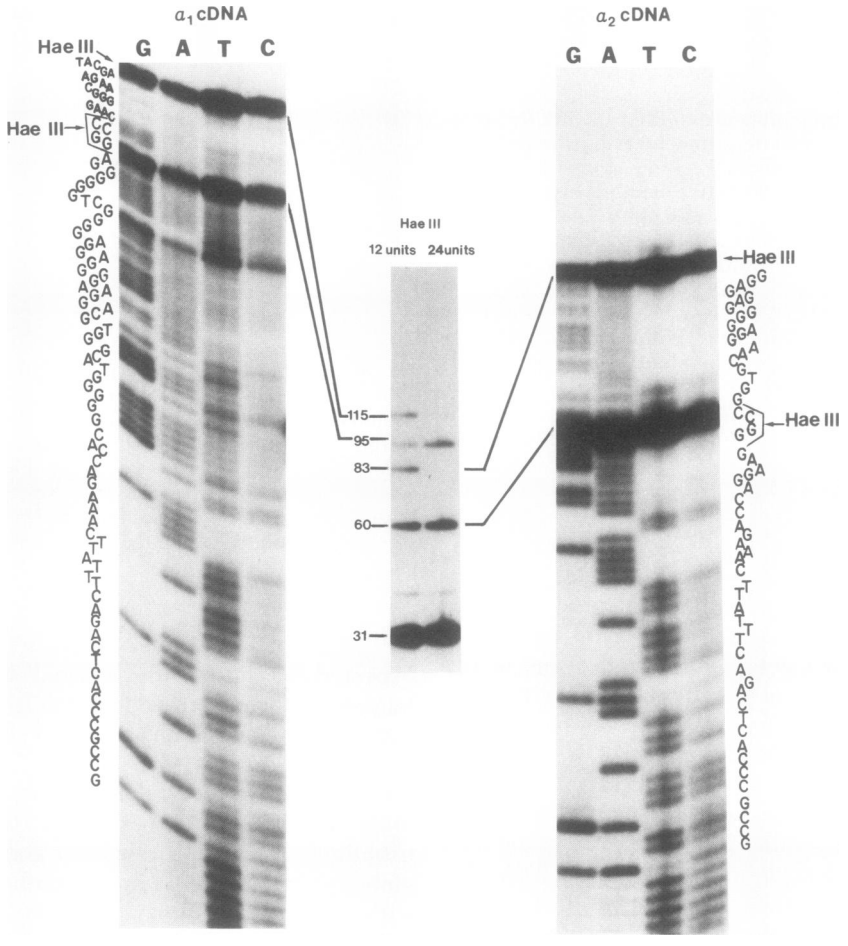

FIGURE 3 Identification of the Hae III cleavage products of α-globin cDNA by sequence analysis. End-labeled α-globin cDNA synthesized by extension of the 31 nucleotide primer was cut with two concentrations of Hae III to identify partial and complete cleavage patterns. The digestion products were separated on an 8% acrylamide denaturing gel and autoradiographed (center panel). The end-labeled α1-globin cDNA bands (95 and 115 nucleotides) and α2-globin cDNA bands (60 and 83 nucleotides) were isolated from gel slices and sequenced. The sequence of DNA pooled from the 95 and 115 nucleotide bands was that of the α1-globin locus (left panel) and that of the pooled 60 and 83 nucleotide bands that of the α2 locus (right panel). The GGCC Hae III sites are noted.

creates the α-thalassemia-2 rightward type deletion[4] and therefore the extra α locus should have the 3′ terminal sequence of the α2 gene (Fig. 5). The α1/(α1 + α2) cDNA synthetic ratio in this individual was 0.18 (Table II, Fig. 4, lane f).

The relative proportion of α1 and α2 mRNA was assayed in two cases where α-globin production is less than expected on the basis of the number of α-globin genes present. When reticulocyte RNA from subject 10 with hemoglobin-H Constant Spring ($--/\alpha^{C.S.}\alpha$) was reverse transcribed, only α1 cDNA was synthesized, while reverse transcription of bone marrow RNA from the same subject produced both α1- and α2-globin transcripts (Fig. 4, lanes h and i). Relative amounts of α1 and α2 cDNA from this bone marrow sample could not be accurately determined due to partial degradation of the RNA. In subject 11 with nondeletion hemoglobin-H disease ($--/\alpha\alpha^{T}$) (14) the α1 and α2 cDNA was found in the same proportions (0.24) as in normals (Fig. 4, lane g).

DISCUSSION

The approach described in this report allows clear discrimination between mRNA transcripts from the α1- and α2-globin gene loci on the basis of their se-

[4] Goossens, M., K. Lee, and Y. W. Kan. Manuscript in preparation.

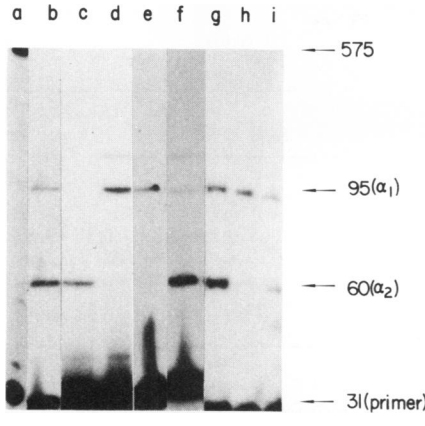

FIGURE 4 Hae III cleavage patterns of end-labeled α-globin cDNA. α-Globin cDNA synthesized by extension of the end-labeled 31 nucleotide primer was run on an 8% acrylamide denaturing gel and autoradiographed. Lane (a) contains the uncleaved α-globin cDNA transcribed from reticulocyte RNA of subject 1. In lanes (b) through (i), the end-labeled cDNA was cleaved with Hae III. Sources for the RNA were: (b) normal control, subject 1 (c) CV1 cells infected with pSV3-α2, (d) CV1 cells infected with pSV3-α1, (e) reticulocytes of subject 7 with Hb-H disease, (f) reticulocytes of subject 9 with a triple α locus, (g) reticulocytes from subject 11 with non-deletion Hb-H disease, (h) reticulocytes from subject 10 Hb-H Constant Spring, (i) bone marrow from subject 10. The numbers to the right of the panel are the sizes in nucleotides of the end-labeled fragments.

quence divergence in the 3′ noncoding region. It can be utilized to study the relative expression of the two α-globin loci in the normal state and to define functional abnormalities in several α-thalassemia syndromes at the mRNA level. The observed imbalance in α1- vs. α2-globin cDNA synthesis from control reticulocyte and bone marrow RNA deserves comment. Since mutant α-globin chains comprise 25% of total hemoglobin in most patients heterozygous for such mutations (29), expression of the α1- and α2-globin genes appears to be equal at the protein product level. The imbalanced α1:α2 cDNA ratio that we measured could be due to preferential synthesis of the α2 cDNA under the conditions of our assay. However, we have evidence that the assay accurately reflects the predominance of the steady-state α2-globin mRNA in the reticulocyte. When we reverse transcribed equivalent amounts of α-globin mRNA (as determined by liquid hybridization) isolated from CV1 cells separately infected with pSV3-α1 and pSV3-α2 recombinants, we obtained equivalent amounts of α-globin cDNA product,[5] indicating that our cDNA synthesis was not preferential for α2 transcripts. The predominance of steady-state α2 mRNA in the reticulocyte could be due to preferential transcription of the α2 locus or to the more rapid turnover of the α1-globin mRNA. We are presently investigating these two possibilities. Irrespective of the mechanism involved in the imbalance of the two α-globin mRNA species in the reticulocyte, analysis for the presence of the α1 and α2 mRNA products and their deviation from the normal ratio can be used to assess the function of the two genes in various types of α-thalassemia.

The results of the present experiments confirm at the mRNA level certain deductions regarding gene structure in α-thalassemia that have been made previously by restriction endonuclease mapping of genomic DNA and analysis of cloned α-globin genes. At least two different types of deletion events can produce the α-thalassemia-2 haplotype; a leftward deletion of 4.2 kb occurring more 5′ within the α-globin complex, and a

[5] Liebhaber, S., and K. Begley. Unpublished data.

TABLE I
α1/(α1 + α2)-Globin cDNA Ratio in Nonthalassemic Reticulocytes

Subject	Diagnosis	α-Globin genotype	Number of independent determinations	Mean ratio α1/(α1 + α2)
1	Sickle cell anemia	(αα/αα)	7	0.28*
2	Sickle cell anemia	(αα/αα)	1	0.24
3	Sickle cell anemia	(αα/αα)	2	0.26
4	Pyruvate kinase deficiency	N.D.‡	2	0.24
5	Autoimmune hemolytic anemia	N.D.	1	0.25
6	Treated nutritional folic acid deficiency	N.D.	1	0.33
Total	—	—	14	0.26±0.06 (mean±2 SD)

End-labeled α-globin cDNA transcripts from the reticulocyte RNA from the indicated subjects was cleaved with Hae III, separated on 8% acrylamide denaturing gels and autoradiographed. α1- and α2-globin mRNA specific bands were quantitated and compared as a ratio (α1/(α + α2).
* The range of 7 determinations is 0.26–0.31.
‡ Not done.

TABLE II

(α1/α1 + α2)-Globin cDNA Ratio in Human Reticulocyte RNA from Subjects with Abnormal α-Globin Genotypes

Subject	Diagnosis	α-Globin genotype	Number of determinations	Mean α1/(α1 + α2)	Range of values
7	Hemoglobin H disease	--/-α	3	1.00	—
8	α Thal trait sickle cell anemia	-α/-α	2	1.00	—
9	Triple α chromosome sickle cell anemia	ααα/αα	3	0.18	0.17–0.18
10	Hemoglobin H Constant Spring	$--/\alpha^{C-S}\alpha$	2	1.00	—
11	Nondeletion hemoglobin H disease	$--/(\alpha\alpha)^{T}$	2	0.24	0.23–0.25
	Controls (from Table I)			0.26±0.06	0.24–0.33

Results were obtained as described in Table I.

rightward deletion of 3.7 kb occurring more 3′ within the α-globin complex (5, 10). Both deletions occur by unequal crossing over of DNA between malaligned chromosomes at defined regions of repeated homology within the α-globin complex (Fig. 5) (5). Although it is clear that the more leftward deletion eliminates the α2-globin locus (3, 9, 10), the boundaries of the rightward deletion have not been delineated by gene mapping (5, 10). The results of the cDNA assay presented here confirm deletion of the α2 gene in the leftward crossover since only α1 cDNA is synthesized from the α-globin mRNA present. The synthesis of only α1 cDNA from a subject homozygous for the rightward crossover indicates that the 3′ nontranslated region of the α1 gene is present in the remaining gene and therefore the righthand border of this crossover event must occur 5′ to this region (Fig. 5). It will be difficult to be more precise about the point of crossover since the α1 and α2 genes are highly homologous for ~1.8 kb 5′ to this region (5, 8, 30).

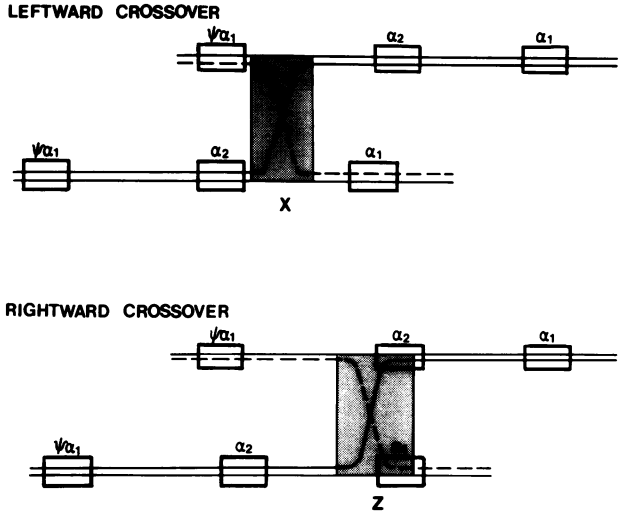

FIGURE 5 Probable sites of unequal crossover creating the leftward and rightward α-thalassemia-2 haplotyes (dotted line) and their reciprocal triple α-globin locus haplotype (solid line). The shaded regions, x and z, denote the regions of homology (as defined in references 5 and 30) within which the crossovers (leftward and rightward, respectively) can occur.

The data from subject 9 indicates that the additional α-globin gene present in a triple α-chromosome is active in transcription. The individual we studied has the α2.α1/α2.α2.α1 genotype which is created as the reciprocal product of the rightward crossover (Fig. 5). If the extra α2 locus is active, one would expect a decrease in the α1/(α1 + α2) cDNA ratio. The 0.18 result in the triple-α-subject (Table II, subject 9) is thus consistent with the expression of the extra α2-globin gene and implies that the crossover event did not remove any flanking DNA necessary for the function of this gene.

Although the α-thalassemias are usually caused by gene deletion, the α-thalassemia phenotype can also be associated with α-globin genes that are present but dysfunctional. In such situations, identifying which of the α-globin genes is expressed abnormally may help define the cause of the depressed α-globin production. We examined two situations where α-globin genes that were physically intact by Southern blotting analysis produced diminished quantities of α-globin protein. In the subject with hemoglobin-H Constant Spring ($--/\alpha^{C.S.}\alpha$) two species of α globin were produced. Normal α globin was synthesized by the α1-globin gene in amounts commensurate with those seen in hemoglobin-H disease (--/-α), whereas the α2 gene produced only trace amounts of the elongated α-globin chain termination mutant Constant Spring (31). The reason for the diminished production of a final protein product from the α2 Constant Spring gene has not been determined. This defect could be the result of decreased gene transcription, mRNA instability, abnormal translation, or instability of the Constant Spring protein. The present cDNA assay detected only α1 mRNA in reticulocytes of a subject with hemoglobin-H Constant Spring, whereas both α1 and α2 mRNA was synthesized from the bone marrow RNA (Fig. 4). This data implies that the low expression of the Constant Spring gene is due, at least in part, to a relative instability of the Constant Spring mRNA (i.e. α2-globin

mRNA). The cause of the decreased stability of this mRNA is now under investigation.

In the nondeletion hemoglobin-H syndromes, (14, 32, 33), physically intact α-globin genes produce a decreased amount of a normal α-globin protein. We have previously described a Chinese subject with nondeletion hemoglobin-H disease with an intact α1 and α2 gene on one chromosome ($--/\alpha\alpha^T$) (14). Genetic and biochemical data indicate that the two genes together produce α-globin protein at a level equivalent to or less than one gene (14). This could be due to both genes functioning at suboptimal levels, or one gene functioning relatively normally while the other is severely defective. The normal $\alpha 1/(\alpha 1 + \alpha 2)$ cDNA ratio (0.24) from this subjects' reticulocyte RNA (Table II; subject 11) indicates that both genes function at levels that may be suppressed but that are normal relative to each other and suggests that the inherited defect in this individual acts equally on α1- and α2-globin loci, possibly at a site remote from the structural gene itself. Since nondeletion hemoglobin-H disease occurs in a number of populations (32–34), and the thalassemias are a heterogenous group of disorders, other forms of this disease may display various patterns of dysfunction at the two α-globin loci. Our sequence comparisons of several genes at each of the two α-globin loci (8) failed to reveal any allelic polymorphisms, so we expect that the approach we present here will be generally applicable in the study of α-globin gene expression in diverse racial groups.

Note added in proof: A similar study has recently been published. (Orkin, S. H., and S. C. Goff. 1981. The duplicated α-globin genes: their relative expression as measured by RNA analyses. *Cell.* **24:** 345–352.)

ACKNOWLEDGMENTS

We thank Stephen H. Embury for kindly supplying several of the blood samples used in these studies, Andree M. Dozy for the generous gift of the pSV3-α1 and pSV3-α2 RNA, the Office of Program Resources and Logistics (Viral Cancer Program, Viral Oncology, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md.) for the reverse transcriptase, Kathi Begley for expert technical assistance, and Jennifer Gampell for editorial comments. This work was supported in part by grants from the National Institutes of Health (AM 16666) and the March of Dimes/Birth Defects Foundation.

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