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Research Article

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Compensatory Increase in α1-Globin Gene Expression in Individuals Heterozygous for the α-Thalassemia-2 Deletion

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Abstract

α-Globin is encoded by the two adjacent genes, α1 and α2. Although it is clearly established that both α-globin genes are expressed, their relative contributions to α-globin messenger RNA (mRNA) and protein synthesis are not fully defined. Furthermore, changes that may occur in α-globin gene activity secondarily to the loss of function of one or more of these genes (α-thalassemia [Thal]) have not been directly investigated. This study further defines the expression of the two human α-globin genes by determining the relative levels of α1 and α2 mRNA in the reticulocytes of normal individuals and in individuals heterozygous for the common 3.7-kilobase deletion within the α-globin gene cluster that removes the α2-globin gene (the rightward type α-Thal-2 deletion). To quantitate accurately the ratio of the two α-globin mRNAs, we have modified a previously reported S1 nuclease assay to include the use of ^{32}P end-labeled probes isolated from α1- and α2-globin complementary DNA recombinant plasmids. In individuals with a normal α-globin genotype (as determined by Southern blot analysis [αα/αα]), α2-globin mRNA is present at an average 2.8-fold excess to α1. In individuals heterozygous for the rightward type α-Thal-2 deletion (−α/αα) the α2/α1 mRNA ratio is 1:1. These results suggest that the loss of the α2-globin gene in the α-Thal-2 deletion is associated with a 1.8-fold compensatory increase α1-globin gene expression.

Introduction

The human globin genes are organized in two clusters: the α-globin gene cluster is located on chromosome 16 and the β-globin gene cluster is located on chromosome 11 (1, 2). The structural organization of the genes in both clusters is now defined in detail. The α-globin cluster consists of five genes and pseudogenes organized over a 30-kilobase (kb)[1] distance in the order: 5′ζ–ψζ–ψα—α2–α1 (3). The two adjacent α-globin genes, α1 and α2, are located 3.7 kb apart and each is situated within a segment of duplicated DNA (3–5). During primate evolution the sequence composition of these duplicated segments encompassing the two α-globin genes has changed in parallel (6, 7). The result is that the sequence of the two α-globin genes and their 5′ flanking regions is highly homologous (7–9).

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1. *Abbreviations used in this paper:* bp, base pair; Hb, hemoglobin; kb, kilobase; SSPE, sodium chloride, sodium phosphate, EDTA hybridization buffer; Thal, thalassemia.

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The relative levels of expression of the two α-globin genes are at present not firmly established. Genetic studies imply that the two α-globin genes produce equal amounts of α-globin protein (10, 11). These studies are consistent with the high degree of structural homology between the two genes. However, two recent studies have demonstrated that the concentration of α2 messenger RNA (mRNA) exceeds that of α1 by 1.5- (12) to 2.8-fold (13). It has been suggested that the disparity between the imbalance in mRNA expression and the balanced protein expression may be reconciled at the level of protein synthesis by a higher translational efficiency of the α1 mRNA (14).

The exact extent of and molecular basis for the relative excess of α2 mRNA in the normal human reticulocyte remain undefined. It is also not known whether or not a decrease in or a total loss of expression of one or more α-globin genes (α-thalassemia [Thal] mutations) is associated with compensatory changes in the expression of the remaining, intact α-globin genes. In the present report, we attempt to clarify these issues further by establishing the relative activity of the two α-globin genes in the normal α-globin gene cluster (αα/αα) and by comparing this value to the relative level of expression of the α1 and α2 genes subsequent to the loss of an α2-globin gene in individuals heterozygous for the α-Thal-2 deletion (−α/αα).

Methods

Sample collection and preparation. 15–50 ml of heparinized blood was obtained by venipuncture from each patient after obtaining informed consent. The blood was sedimented and washed three times in normal saline, and reticulocyte RNA was extracted from acid-precipitated polysomes as previously detailed (15). High molecular weight leukocyte DNA was isolated from the buffy coat by Proteinase K (Sigma Chemical Co., St. Louis, MO) digestion and phenol extraction (16).

α-Globin gene mapping. 15 μg of DNA from each patient was digested overnight with 30 U of the indicated restriction endonuclease (Bam H1 or Bgl II) under conditions suggested by the supplier (New England Biolabs, Beverly, MA) The DNA digest was then electrophoresed on a 0.8% agarose gel, denatured, transferred to nitrocellulose paper, and hybridized to a nick-translated α-globin-specific probe (pMC18 [17]). After washing, the filter was exposed to XAR5 film (Eastman Kodak Co., Rochester, NY) at −70°C in the presence of a Cronex Lightening Plus intensifying screen (DuPont Instruments, Wilmington, DE), all as detailed elsewhere (16).

S1 analysis of α-globin mRNA. The method of S1 mapping the human α-globin mRNAs is a modification of the technique of Orkin and Goff (12). This technique allows the separate identification of the α1 and the α2 mRNAs based upon the ability of the single strand-specific nuclease, nuclease S1, to recognize and hydrolyze DNA at the single-stranded regions that form between the nonhomologous 3′ nontranslated sequences of an α1–α2-globin mRNA–complementary DNA (cDNA) duplex. To prepare ^{32}P end-labeled probes, the α1 and α2 cDNA plasmids (pJW101 [18] and pRP9 [13, 19], respectively) were digested with Hind III, and end-labeled with DNA polymerase 1 (Klenow fragment, New England Biolabs). Hind III cuts both cDNA inserts at codon 90–91. In the pRP9 plasmid (α2-globin cDNA), the vector Hind III site is immediately 3′ to the insert (13, 19) whereas in the pJW101 plasmid (α1-globin cDNA)

the Hind III site lies ~250 base pairs (bp) 3′ to the cDNA insert (12, 18). The α2 cDNA-derived probe is therefore smaller than that derived from the α1 cDNA (298 bp vs. 550 bp; see Fig. 2 *B*, lanes *1* and *7*, respectively). The ^{32}P end-labeled digest was phenol-extracted, ethanol-precipitated, and run on a 5% acrylamide strand-separating gel (20). The slower migrating of the two separated strands was eluted from the gel, phenol-extracted, and co-precipitated with 25 μg/ml of yeast transfer RNA (tRNA) carrier. Recovery was calculated by comparing counts originally in the gel slice with counts in the final preparation. 200 ng of total reticulocyte RNA was hybridized to an estimated 25 ng of probe. This ratio was determined to be in greater than 10-fold molar probe excess both by calculation and by titration of the probe with increasing amounts of the RNA (data not shown). The RNA was hybridized to the probe in 30 μl of formamide buffer (80% formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA) at 40°C for 3 h. Initial control studies (see Fig. 2) conducted at lower ratios of probe to mRNA demonstrated that at low temperatures (35°C) the hybridization becomes inefficient whereas at higher temperatures selective hybridization to the homologous mRNA becomes increasingly favored. The conditions used in the subsequent assays (see details above), which included a higher probe to mRNA ratio, allowed use of a slightly higher temperature (40°C). These conditions resulted in efficient hybrid formation without preferential association of probe with homologous RNA. Hybridization was terminated by adding directly to the samples 300 μl of ice-cold S1 buffer (30 mM NaAc pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol) containing ~60–1,500 U of S1 nuclease (Miles Laboratories, Inc., Elkhart, IN). The exact concentration of S1 was determined separately for each new lot of S1. The S1 digestion was carried out at 37°C for 4 h, and was terminated by ethanol precipitation with 25 μg/ml of tRNA. The pellet was redissolved in 15 μl of formamide dye (80% formamide, 10 mM NaOH, 0.05% of both xylene cyanol and bromphenol blue) and electrophoresed on a 6% acrylamide/8 M urea gel to size and separate the end-labeled S1 nuclease-resistant fragments. The gels were exposed to Kodak XAR5 film at −70°C for varied durations to obtain bands of comparable intensity that were in the linear range of the densitometer. The intensity of each band was quantitated either by a Zeineh Soft Laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA), or by scintillation counting of the excised gel slices with appropriate background correction. Both methods of quantitation give equivalent results.

Dot-blot analysis of α/β mRNA ratios. Total reticulocyte RNA isolated as detailed above was applied to duplicate sets of nitrocellulose paper using the Schleicher & Schuell Minifold dot-blot apparatus (Schleicher & Schuell, Inc., Keene, NH). The dot-blot procedure is a modification of that peviously reported (21). Samples containing 0.1 and 0.01 μg of total RNA were dissolved in 300 μl of 15× SSPE (2.24 M NaCl, 0.13 M NaH_2PO_4, 16 mM/EDTA, pH 7.0). The samples were then denatured by adding to each 80 μl of 37% formaldehyde, heating to 60°C for 15 min, and ending the incubation by adding an additional 450 μl of ice-cold 15× SSPE. Half of each sample was applied under gentle vacuum in a parallel array to each of two nitrocellulose filters fitted on the dot-blot apparatus and prewetted with 15× SSPE. After application of all the samples, the wells were washed with 500 μl of 15× SSPE, and the filter was air-dried, baked in a vacuum oven at 80°C, for 2 h, and prehybridized with hybridization mix (16) overnight at 50°C. The duplicate

Table I. Evaluation of Patient Studies

Patient no.	Hemoglobin	Reticulocyte count	Mean corpuscular volume	α-Globin genotype	(α1/α1 + α2) mRNA Probe 1	(α1/α1 + α2) mRNA Probe 2	Mean % α1/α1 + α2
1	9.8	N/A	84	−α/αα	0.48	0.48	48.0
2	8.6	4.0	83	−α/αα	0.51	0.53	52.0
3	10.7	3.4	94	αα/αα	0.24	0.26	25.0
4	7.2	4.0	82	−α/αα	0.48	0.49	48.5
5	9.9	2.2	85	−α/−α	1.00	1.00	100.0
6	12.1	1.6	89	αα/αα	0.15	0.20	17.5
7	12.1	1.8	84	−α/αα	0.53	0.53	53.0
8	9.2	2.7	87	−α/αα	0.52	0.50	51.0
9	11.9	1.2	91	αα/αα	0.27	0.24	25.5
10	11.1	3.4	92	−α/αα	0.48	0.54	51.0
11	13.0	2.2	96	αα/αα	—	—	—
12	11.3	N/A	99	αα/αα	0.19	0.31	25.0
13	12.5	N/A	99	αα/αα	0.24	0.24	24.0
14	9.8	2.4	85	−α/αα	0.52	0.48	50.0
15	10.2	3.0	90	αα/αα	0.24	0.24	24.0
16	12.6	4.0	87	−α/αα	0.49	0.48	48.5
17	13.9	1.6	93	αα/αα	0.31	0.27	29.0
18	12.9	6.6	94	αα/αα	0.27	0.32	29.5
19	10.6	3.6	93	−α/αα	0.42	0.56	49.0
20	10.4	3.5	80	−α/αα	0.49	0.57	53.0
21	10.8	2.2	99	αα/αα	0.25	0.37	31.0
22	11.6	N/A	91	αα/αα	0.28	0.32	30.0
23	12.6	2.0	95	αα/αα	0.21	0.33	27.0
24	9.9	3.2	101	αα/αα	0.22	0.27	24.5
25	12.6	2.8	101	αα/αα	0.23	0.32	27.5
26	N/A	N/A	N/A	αα/αα	0.25	0.34	29.5
27	9.8	3.6	73	−α/−α	1.00	1.00	100.0
28	10.2	N/A	81	αα/αα	0.23	0.26	24.5
29	10.8	3.4	99	αα/αα	0.32	0.39	30.5

filters were hybridized to either a nick-translated full-length-globin cDNA -pMC18 [17]) or β-globin cDNA (pSAR6 [22]) probe of approximately equal specific activities (1×10^8 cpm/μg). An equal number of counts were used on each filter. After overnight hybridization at 50°C, the filters were washed in separate containers with 2× standard saline citrate (0.3 M NaCl, 0.03 M Na citrate, pH 7.0) at room temperature for 30 min followed by two 45-min washes in 0.1× standard saline citrate/0.1% sodium dodecyl sulfate at 50°C and air-dried, and corresponding lanes from the α- and β-probed nitrocellulose were cut out, juxtaposed, and autoradiographed as described above. The relative α/β mRNA ratio was determined by scanning the α- and β-probed dots on the autoradiographs in a pairwise fashion with a soft laser densitometer.

Results

Sample selection. Venous blood samples were collected from 29 black women attending a prenatal clinical for routine care. None of these individuals had any known hematologic abnormalities. The hemoglobin (Hb), reticulocyte count, and erythrocyte mean corpuscular volume of each individual were measured in the hospital clinical laboratory as part of routine patient evaluation and are recorded in Table I. All individuals tested negative for homozygous sickle cell anemia.

α-Globin gene mapping. Leucocyte DNA from each of the 29 individuals was mapped for α-globin gene organization by the Southern blotting technique (23). Representative results for individuals with each of the three observed genotypes is shown in Fig. 1 along with the map of the α-globin genes on a normal chromosome and on a chromosome with the rightward type α-Thal-2 deletion (5, 24). Table I lists the genotype determined for each patient. Of the 29 individuals studied, 17 (59%) had a normal pattern (αα/αα), 10 (34%) were heterozygotes for the rightward α-Thal-2 deletion (−α/αα), and 2 (7%) were homozygotes for this deletion (−α/−α). The distribution of the three genotypes fits the Hardy–Weinberg distribution. The gene frequency of the rightward deletion in this population is 0.24.

α2:α1 mRNA analysis. RNA was isolated from acid precipitated reticulocyte polysomes and used without further fractionation. All samples were analyzed by the S1 mapping technique as first described by Orkin and Goff (12) with several modifications. The procedure as originally described used a single probe isolated from an α1 cDNA clone. It is theoretically possible that an α1 probe used in this assay could bias the results towards the detection of α1 mRNA by preferentially hybridizing to the homologous (α1) versus the less perfectly matched (α2) mRNA. For this reason the relative levels of α1 and α2 mRNA were determined using probes isolated from both the α1 and α2 cDNA clones (pJW101 and pRP9, respectively). A schematic of this assay using reciprocal probes is shown in Fig. 2 *A*. A number of parameters of hybridization were tested with these two probes to establish conditions of hybridization and S1 digestion that would minimize any bias and result in internally consistent results. We found that this bias could only be overcome by hybridizing the RNA to a large (10-fold) excess of probe at a low stringency. The results of an experiment demonstrating the sensitivity of this result to the hybridization temperature is shown in Fig. 2 *B* and *C*. In this experiment the ratio of probe to RNA was only in a threefold excess (see Methods). By performing the hybridization at decreasing temperatures at this level of probe excess, the α2/α1 ratio with the reciprocal probes become identical at 35°C. The ratio of α2 to α1 in the reticulocyte RNA of this particular individual (αα/αα) measured by both probes under these conditions is 3.2:1. Because the efficiency of hybridization at 35°C is quite low, as seen in Fig. 2 *B*, it was not practical to use this temperature, and the subsequent hybridizations were therefore carried out with a higher probe to mRNA ratio at 40°C (see Methods). To insure that these conditions continued to result in an unbiased value, both probes were used for the α2:α1 mRNA determination on each of the individuals studied. These results, which are listed in Table I and demonstrated in Fig. 3, substantiate the accuracy of the results under these conditions.

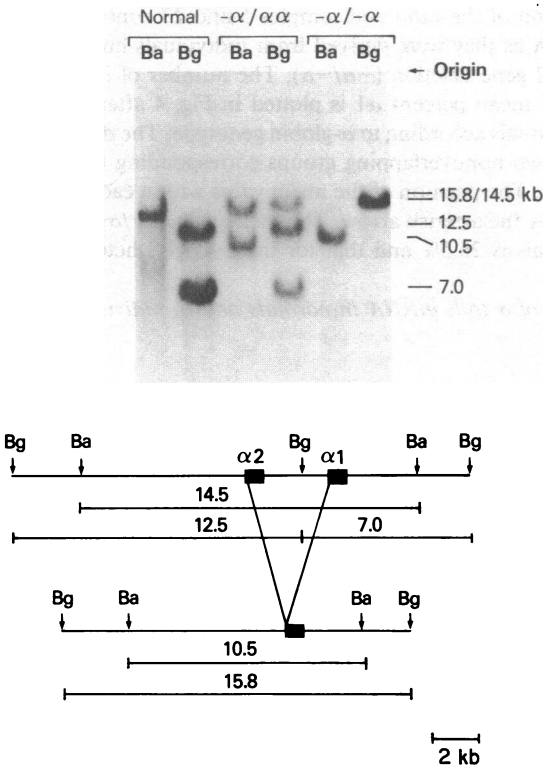


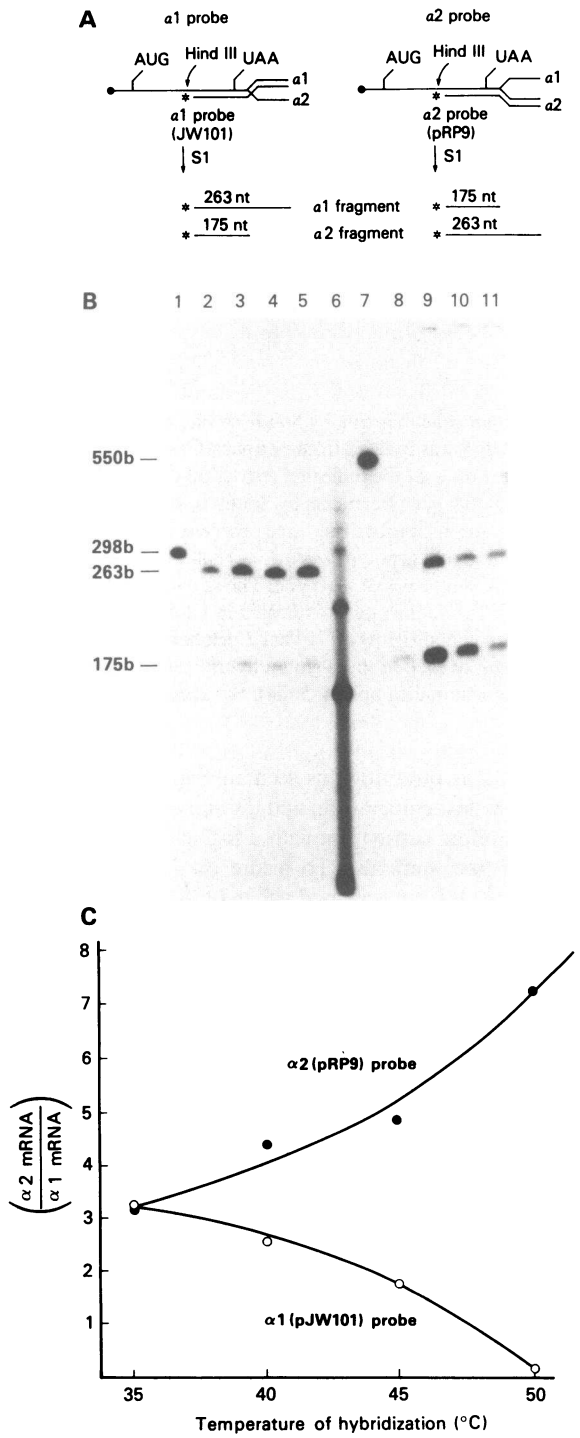
Figure 1. α-Globin gene mapping by Southern blot analysis. High molecular weight DNA was isolated from peripheral blood leucocytes, digested to completion with the indicated restriction endonuclease, and analyzed for α-globin gene fragments by Southern blotting using a ^{32}P-labeled α-globin-specific probe. The three observed patterns are shown: normal α-globin genotype αα/αα, α-Thal-2 heterozygote (−α/αα), and α-Thal-2 homozygote (−α/−α). The size of each hybridizing fragment on this autoradiograph is indicated in kilobases (kb). The maps of the normal and rightward α-Thal-2 deletion chromosomes indicating the origin of each of the α-globin hybridizing fragments are shown below the autoradiograph (4, 5, 24). Ba (BamH1), Bg (Bg1II).

Percent α1 mRNA of total α-globin mRNA in the reticulocytes of 29 black women. Reticulocyte RNA from each of the 29 black women described above was assayed for percent α1 mRNA using both the α1 and the α2 cDNA probes described. Fig. 3 shows a representative S1 mapping study of the mRNA isolated from one individual of each of the three genotypes. The reciprocal nature of the results as quantitated by densitometric scans suggests a lack of detection bias for either mRNA under the essay conditions described.

The results of the S1 analysis of each individual in the study is listed in Table I. Each experimental determination is listed under the type of probe used and the mean of the two values is listed in the last column as a mean percent α1/α1 + α2. The RNA in sample 11 was too degraded to allow an accurate de-

termination of the ratio and samples 5 and 27 contained only a1 mRNA as they were derived from individuals homozygous for the $\alpha 2$ gene deletion ($-\alpha/-\alpha$). The number of individuals with each mean percent $\alpha 1$ is plotted in Fig. 4 after grouping the individuals according to α-globin genotype. The data clearly fall into two nonoverlapping groups corresponding to the two genotypes. The position of the mean value within each group is denoted by the asterisk arrow. The mean value $\alpha 1/\alpha 1 + \alpha 2$ for the normals is 26.5% and that for the α-Thal-2 heterozygotes is 50.4%.

Ratio of α to β mRNA in normals and in individuals with the α-Thal-2 deletion. To determine the incremental drop in total α-globin mRNA with the loss of the $\alpha 2$ globin gene in the rightward α-Thal-2 deletion (Fig. 5), the α/β mRNA ratio was determined in each of the 29 described individuals by dot-blot analysis (21). If the level of β-globin mRNA is assumed to be equal in all cases (i.e., no β-thalassemia), then the α/β ratio should directly reflect the relative content of total α-globin ($\alpha 1 + \alpha 2$) mRNA. It should be noted however that these values do not necessarily represent the absolute α/β mRNA ratios due to the inherent difficulties in accurately determining the specific activities of two separate probes. A representative dot-blot analysis of reticulocyte RNA from an individual with each of the α-globin genotypes is displayed in Fig. 6 *A* along with the densitometer tracing used for quantitation of the relative signal strength of each dot. The α/β ratio for each individual averaged from at least two separate determinations is plotted in the graph in Fig. 6 *B* against the α-globin genotype. The mean of the values in each group is marked with a horizontal bar. In establishing the mean of the normal population, the highest value (noted in parentheses) was not included as this individual has β-thalassemia as indicated by an α/β synthetic ratio three times normal (data not shown). The arrows indicate the α/β mRNA ratios, which based upon the findings in this report are predicted to exist in the reticulocytes of heterozygotes and homozygotes for α-Thal-2.



Discussion

It has been known for some time that the two human α-globin genes are coexpressed in normal erythroid cells (10, 11). The relative contribution of these two genes to overall α-globin synthesis has, however, been difficult to estimate accurately by conventional techniques because both genes encode an identical α-globin protein product (25). The recent isolation and structural analysis of the $\alpha 1$- and $\alpha 2$-globin genes has revealed that the $\alpha 1$ and $\alpha 2$ mRNAs differ in primary structure in their 3′ nontranslated regions (7, 26, 27). Assays that can detect this structural

Figure 2. S1 nuclease mapping of α-globin mRNA using $\alpha 1$ and $\alpha 2$ probes. (*A*) ^{32}P end-labeled probes isolated from an $\alpha 1$ (pJW101) or an $\alpha 2$ (pRP9) cDNA clone are hybridized to total reticulocyte RNA and subsequently digested to completion with the single strand-specific nuclease S1 to generate fragments specific to the homologous mRNA or the nonhomologous mRNAs (263 nucleotides (nt) and 175 nucleotides, respectively). (*B*) The effect of different hybridization temperatures upon the relative detection of each α-globin mRNA species by the two S1 probes (lanes *2–5* with $\alpha 2$ cDNA-derived probe; lanes *8–11* with $\alpha 1$ cDNA-derived probe). Hybridization was carried out at 35°C (lanes *2* and *8*), 40°C (lanes *3* and *9*), 45°C (lanes *4* and *10*), and 50°C (lanes *5* and *11*); the ratio of $\alpha 2$ to $\alpha 1$ mRNA was subsequently quantitated by S1 digestion and analysis on a 5% acrylamide, 8 M urea gel. Equal aliquots (equal total counts per minute) of each reaction were loaded on the indicated gel lanes. The autoradiograph of the analytic gel is shown. Lanes *1* and *7* contain the ^{32}P end-labeled (undigested) $\alpha 2$ and $\alpha 1$ S1 probes, respectively, and lane *6* contains ^{32}P end-labeled pBR322/Hinf 1 restriction fragments as size markers. The size of each probe and the S1 digestion fragments are indicated in bases (b). (*C*) The bands in lanes *2–5* and *8–11* of the autoradiograph in *B* were quantitated by excision from the gel and scintillation counting. The ratio of counts in the $\alpha 2$- and $\alpha 1$-specific bands in each lane (*ordinate*) was calculated and plotted as a function of temperature of hybridization (*abscissa*). (●) Values generated using the $\alpha 2$ cDNA probe; (○) values generated using the $\alpha 1$ probe.

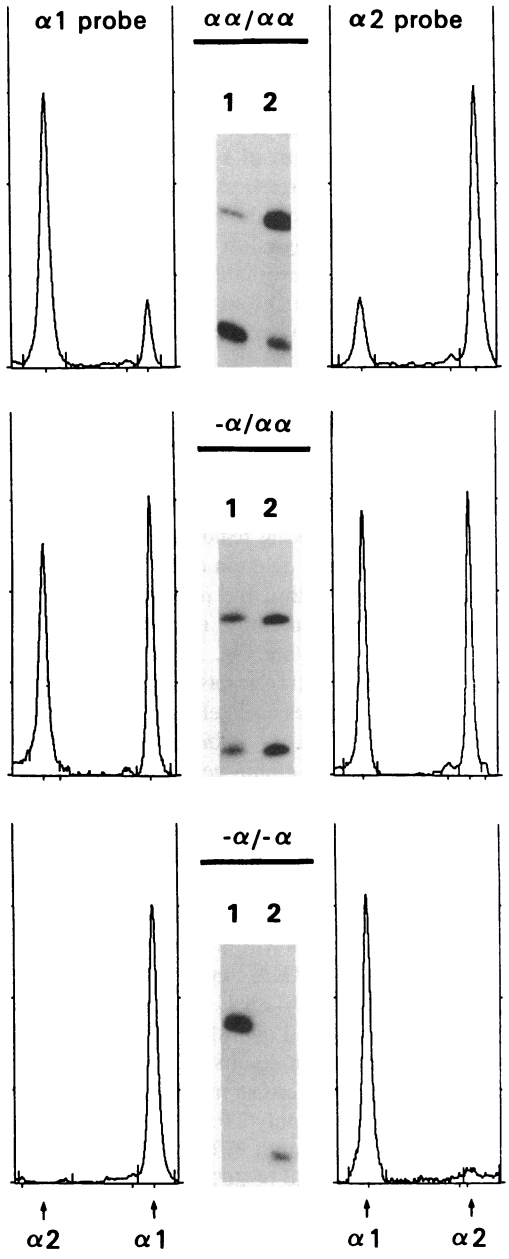


Figure 3. S1 analysis of the α1 and α2 mRNA content in the reticulocytes of individuals with a normal, α-Thal-2 heterozygote (−α/αα), and α-Thal-2 homozygote (−α/−α) genotype. Analysis was carried out as detailed in text. Lanes *1* and *2* contain S1-resistant fragments generated by hybridizing reticulocyte RNA with a ^{32}P end-labeled probe isolated from an α1 or α2 cDNA clone, respectively. Densitometric scans of lanes *1* and *2* are shown to the left and right of the gel, respectively. The position of the α1- and α2-specific signals on the densitometric scans is indicated at the bottom of the figure.

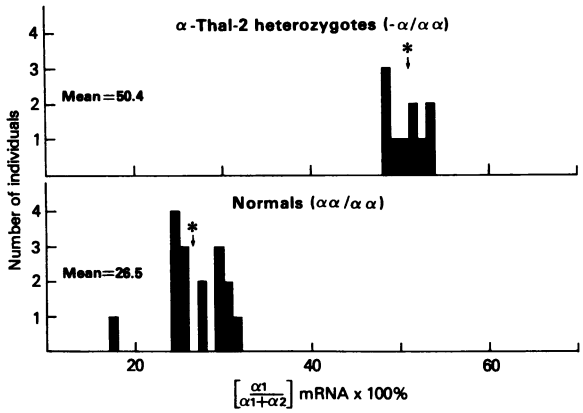


Figure 4. The fraction of α1 mRNA in reticulocyte α-globin mRNA of normals (αα/αα) and α-Thal-2 heterozygotes (−α/αα). The mean percent of α1/α1 + α2 mRNA in each of the individuals was calculated from the values obtained by S1 analysis using probes derived from both α1 and α2 cDNA clones. The mean percent α1 mRNA (*abscissa*) is plotted as a function of the number of individuals with that value (*ordinate*). The individuals are separated by genotype as determined by Southern blot analysis into α-Thal-2 heterozygotes (*top*) and normals (*bottom*). The *asterisk arrow* indicates the mean value in each group.

divergence have been used to compare directly the relative concentrations of the two α-globin mRNAs (12, 13). In the present report, S1-mapping analysis detects a mean α2:α1 mRNA ratio of 2.8 in normal reticulocytes. This result compares favorably with our previously reported ratio of 2.8, as measured by a technically independent primer-extension assay (13). To avoid preferential hybridization of each S1 probe to its homologous mRNA, we found it necessary to carefully adjust hybridization conditions as demonstrated in Fig. 2. The lower a2/a1 mRNA ratio of 1.5:1 reported by investigators using the S1-mapping approach with a single α1-derived probe (12) may be attributed to preferential hybridization to the α1 mRNA. The two coexpressed α-globin genes in individuals with normal α-globin genotypes appear to encode substantially different steady-state levels of α-globin mRNA.

A number of arguments suggest that the observed excess of α2 over α1-mRNA in normal reticulocytes is due to a greater transcriptional activity of α2-globin gene. On the basis of known structural data, it is unlikely that significant differences exist in the processing of the α1 and α2 transcripts. The two-base substitutions and seven-base deletion/insertion that occur in the

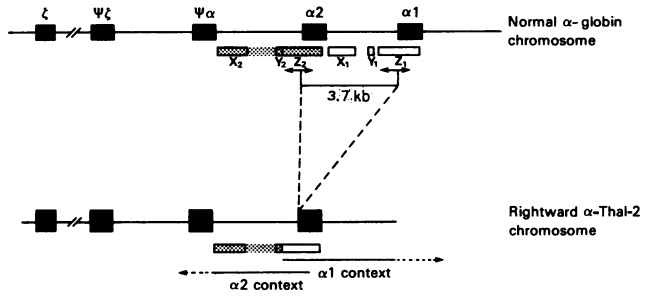


Figure 5. Diagram of the normal α-globin gene cluster and the rightward type-Thal-2 gene cluster. The genes in each cluster are indicated by the solid rectangles and arranged in order and spacing as previously described (3). The three major regions of homology within the α-globin gene duplication unit are designated by the empty rectangles and located as previously reported (3). The set of homology boxes in the α2 duplication unit are stippled to distinguish them from those in the α1 unit. The borders of the 3.7-kb region of the α-globin cluster, which is deleted in the rightward α-Thal-2 deletion, is indicated with the double-headed arrows describing the region in which the unequal crossover may occur.

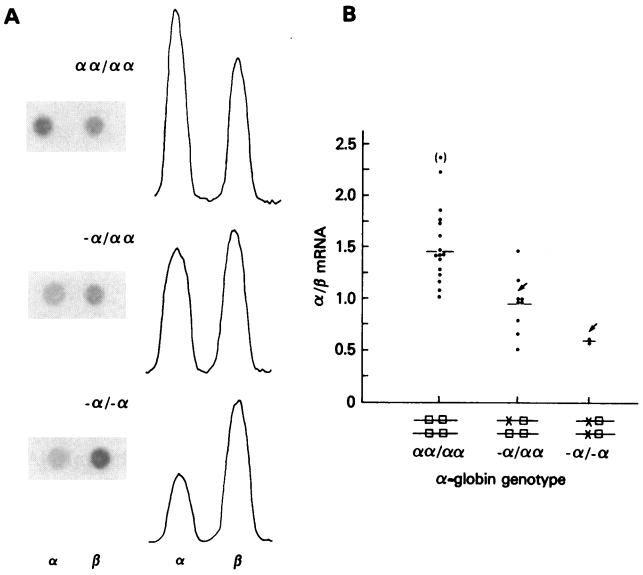


Figure 6. Dot-blot hybridization analysis of the α/β mRNA ratio in the reticulocytes of normals, α-Thal-2 heterozygotes and α-Thal-2 homozygotes. (*A*) Representative autoradiograph and densitometric scan of reticulocyte mRNA applied as a dot to nitrocellulose paper and hybridized either with an α-globin-specific ^{32}P-labeled cDNA probe (*left dot*) or a β-globin-specific ^{32}P-labeled cDNA probe (*right dot*). (*B*) Reticulocyte α/β mRNA ratio (quantitated by dot-blot hybridization) of each individual in the study is plotted as a function of the respective genotype. The experimentally derived mean of each group is marked by a horizontal line and the predicted mean values for the two thalassemic groups is indicated by the arrows.

second intervening sequence neither create a new splice site concensus sequence nor interrupt the concensus sequence *PyXPyTPuAPy* of possible importance in the formation of the lariate structure intermediate in the splicing reaction (28–30). Significant differences in the half-lives of the two α-globin mRNA species also appear unlikely as the $\alpha 2$:$\alpha 1$ mRNA ratio in transcriptionally active erythroid tissue is indistinguishable from that in the transcriptionally inactive reticulocyte (12, 13). The different levels of the two α-globin mRNAs are therefore more likely to result from a difference in transcriptional activity of their respective genes. Such a difference might be secondary to sequence divergence between the two genes in the remote 5′-flanking regions, in the 3′-flanking regions or to as yet undefined effects of the relative positioning of the genes within the α-globin cluster. The relative positioning of the globin genes becomes particularly important when one considers the position dependence of globin gene transcriptional activation during the 5′ to 3′ progression of gene switching during ontogeny (31), and the marked changes that can occur subsequent to positional changes of the β-globin gene within its gene cluster (32).

The possibility that deletion of an α-globin gene might result in a compensatory increase in the expression of the remaining α-globin genes has been suggested by the finding that the absolute level of the α-globin structural mutant HbG-Philadelphia is increased by the coexistence of α-thalassemia in the same genome (33). In the present report, the effect of the rightward α-Thal-2 deletion upon the expression of the remaining α-globin genes has been measured directly at the level of mRNA expression. This deletion removes a 3.7-kb fragment of DNA containing the $\alpha 2$ gene from the α-globin cluster (3) (Fig. 5). The single remaining α-globin gene encodes $\alpha 1$ mRNA (8, 12, 13). In 10 individuals heterozygous for this deletion ($-\alpha/\alpha\alpha$), the mean ratio of $\alpha 1/\alpha 2$ mRNA is 1.05. Comparison of these values to the 2.8 mean ratios in normals indicates that $\alpha 1$-globin gene expression has increased. The present data cannot distinguish a 1.4-fold increase in the expression of both $\alpha 1$-genes (a *trans* acting effect) from a 1.8-fold increase of expression limited to the repositioned $\alpha 1$-globin gene on the α-Thal-2 chromosome (a *cis* acting effect). A *trans* acting effect would suggest the existance of soluble factors that coordinate the expression of the α-globin gene clusters on the two chromosome homologs. The *cis* acting effect might result from local structural effects of the deletion upon the $\alpha 1$-globin gene. In the latter case the level of expression of the repositioned $\alpha 1$-globin gene would be midway between that of the native $\alpha 1$ and $\alpha 2$ genes. Because the first major difference in the 5′ direction between the native $\alpha 1$ gene structure and the α-Thal-2 gene begins 864 bp 5′ to the site of transcription initiation (8, 34) and the region 3′ to the transposed $\alpha 1$ gene derives from the 3′-flanking region of the $\alpha 1$ gene (8, 34), the net effect of the α-Thal-2 deletion is to shift the $\alpha 1$ gene to the more central position within the α-globin gene cluster normally occupied by $\alpha 2$ and juxtapose it with the remote 5′ flanking region of the $\alpha 2$ gene (see Fig. 5). Inasmuch as the identical structure of the mRNA transcripts from the native and repositioned $\alpha 1$ genes (8) makes unlikely any difference in mRNA processing, modification, or stability, the increase in expression of the repositioned α-globin gene (in the *cis* acting model) is most likely transcriptional in origin.

The present determination of α-globin gene function can be compared to previous attempts to assess total α-globin mRNA levels in α-thalassemia. The present report would pedict a linear relationship between α-globin gene number and total α-globin mRNA concentration. In normals with a 2.8:1 ratio of $\alpha 2$ and $\alpha 1$ mRNA, the total α-globin mRNA concentration can be assigned an arbitrary level of 7.6 U (2.8 U from each of the $\alpha 2$ genes and 1 U from each of the $\alpha 1$ genes). Deletion of a single $\alpha 2$ gene in the α-Thal-2 mutation results in the loss of 2.8 U. However, with the consequent increase in $\alpha 1$ gene expression, the net loss of α-globin mRNA is only 2.0. The result is that the predicted decrement in total α-globin mRNA in α-Thal-2 heterozygotes and homozygotes would be 26% and 53% of normal, respectively. To test this prediction directly, we determined the relative levels of α-globin mRNA in the defined population by hybridization analysis. The results that are summarized in Fig. 6 demonstrate that the mean values of α-globin mRNA in the heterozygotes and homozygotes (represented by the horizontal bars) are quite close to the predicted levels (indicated by the small arrows). Although a previous attempt to correlate α-thalassemia phenotypes with the relative levels of α-globin mRNA demonstrated a similar relationship (35), that data could not be directly compared to the present results as the number of α-globin genes in those individuals was assumed and not directly determined.

The actual phenotype of α-thalassemia, that is, the severity of the disease, must also be compared to predictions from the current data. The clinical diagnosis of α-thalassemia in individuals heterozygous for the single α-globin gene deletion has not been possible prior to the advent of gene mapping. These individuals previously referred to as silent carriers are entirely normal by hematologic parameters and could only be identified in kindred studies as obligate carriers of a mild thalassemia defect (36). Sensitive biochemical tests of globin-chain synthesis, such

as radiolabeling newly synthesized globin chains in reticulocytes, or detecting γ4 tetramers (HbBarts) in cord blood has been applied to this population (36, 37). Although a large number of problems (degredation of uncomplexed chains, differences in the pools of preexisting unlabeled chains, and the fact that none of these individuals have established α-globin genotypes) must be considered in these labeling studies, the data imply that the loss of one gene has a minimal, although detectable effect, suggesting the existence of a partial compensation in α-globin expression. In that the data in the present report would predict that there should be no net loss of α-globin synthesis subsequent to loss of the α2 gene in the rightward α-Thal-2 deletion, and the studies cited above do, in fact, document some loss, the measurements of α-globin gene function at the mRNA level and at the level of protein synthesis, although qualitatively in agreement, cannot be fully reconciled. Further studies that integrate newer techniques of gene mapping, mRNA quantitation, and in vitro protein synthesis may further clarify these issues.

The present study deals exclusively with a single subset of α-thalassemia: the rightward type α-Thal-2 deletion. Although the severity of the α-thalassemia in general correlates with the number of functional α-globin genes remaining in the genome (38, 39), certain unexplained variations in this relationship do exist. For example, the loss of function of the α2-globin gene due to the nondeletion defect Hb Constant Spring appears to result in a more severe loss of total α-globin gene expression than does deletion of the same gene (40). The increase in α1-globin gene expression associated with the rightward type α-Thal-2 mutation, which is demonstrated in the present report, suggests that the deletional loss of an α-globin gene may be associated with some degree of compensation by the remaining α-globin genes. One possible explanation for the greater severity of a nondeletion defect might be that such an associated compensatory effect does not occur or is less marked. Analysis of relative α1 to α2 mRNA levels in individuals heterozygous for a variety of different α-thalassemia mutations (deletional and nondeletional) may reveal such differences in the overall compensatory response of α-globin gene expression to these defects. In this way, a comprehensive study of α-globin gene expression in the various α-thalassemia syndromes may allow further correlation between the severity of the α-thalassemic phenotype and the specific molecular defect involved.

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