

Locus assignment of alpha-globin structural mutations by hybrid-selected translation.

S A Liebhaber, F E Cash

J Clin Invest. 1985;75(1):64-70. <https://doi.org/10.1172/JCI111698>.

Research Article

The two human alpha-globin genes, alpha 1 and alpha 2 located 3.4 kilobases apart on chromosome 16, encode identical alpha-globin proteins. A mutation in either gene could result in a structural hemoglobinopathy. It has only recently become possible to assign an alpha-chain mutant to one of these two loci by using recombinant DNA technology. While definitive, this approach has necessitated the cloning and sequencing of the specific gene in question. We present an alternative approach which results in rapid and definitive assignment of an alpha-globin mutation to its encoding genetic locus. This approach uses the technique of hybrid-selected translation. Reticulocyte RNA from individuals with alpha-globin mutations can be fractionated into beta-, alpha 9 (total)-, alpha 1-, and alpha 2-globin mRNA by selective hybridization of each mRNA species to its respective complementary DNA (cDNA) immobilized on nitrocellulose paper. Each mRNA purified in this way can be translated in vitro, and the mRNA species (and hence gene locus) encoding the globin mutant can then be directly identified by gel analysis of the radiolabeled translation products. This procedure can be used to identify globin mutants as alpha or beta and to localize alpha-globin mutants to the alpha 1 or alpha 2 gene. We have used this technique to localize the two alpha-globin mutants, alpha 125Pro (Hb Quong Sze) and alpha 47His (Hb Hasharon), [...]

Find the latest version:

<https://jci.me/111698/pdf>



Locus Assignment of α -Globin Structural Mutations by Hybrid-selected Translation

Stephen A. Liebhaber and Faith E. Cash

Departments of Human Genetics and Medicine, University of Pennsylvania School of Medicine/G3, Philadelphia, Pennsylvania 19104

Abstract

The two human α -globin genes, $\alpha 1$ and $\alpha 2$ located 3.4 kilobases apart on chromosome 16, encode identical α -globin proteins. A mutation in either gene could result in a structural hemoglobinopathy. It has only recently become possible to assign an α -chain mutant to one of these two loci by using recombinant DNA technology. While definitive, this approach has necessitated the cloning and sequencing of the specific gene in question. We present an alternative approach which results in rapid and definitive assignment of an α -globin mutation to its encoding genetic locus. This approach uses the technique of hybrid-selected translation. Reticulocyte RNA from individuals with α -globin mutations can be fractionated into β -, α (total)-, $\alpha 1$ -, and $\alpha 2$ -globin mRNA by selective hybridization of each mRNA species to its respective complementary DNA (cDNA) immobilized on nitrocellulose paper. Each mRNA purified in this way can be translated in vitro, and the mRNA species (and hence gene locus) encoding the globin mutant can then be directly identified by gel analysis of the radiolabeled translation products. This procedure can be used to identify globin mutants as α or β and to localize α -globin mutants to the $\alpha 1$ or $\alpha 2$ gene. We have used this technique to localize the two α -globin mutants, $\alpha^{125\text{Pro}}$ (Hb Quong Sze) and $\alpha^{47\text{His}}$ (Hb Hasharon), to the $\alpha 2$ locus. This approach could potentially be expanded to serve as an alternative to peptide analysis for the initial characterization of all globin structural mutants.

Introduction

Globin chain mutations are the best defined of all human protein defects. Due to the easy accessibility of red cells for analysis and the clinical presentation of many hemoglobinopathies, >110 α - and 190 β -globin structural mutants have now been identified (1). A variety of techniques have been employed to assign a globin structural mutation to either the α or the β gene. These techniques are based upon alterations in the physical or chemical properties of the mutant globin protein. Presently, the definitive assignment of a structural mutant as α or β (or less commonly γ or δ) depends on the direct analysis of peptide fragments (2, 3). Since the protein products of the two α -globin genes are normally indistinguishable (4), it is not possible to similarly assign an α -globin structural

mutant to its encoding gene on the sole basis of protein analysis. For this reason, a full genetic description of most α -globin structural mutations remains incomplete.

Despite the incomplete genetic description of most α -globin structural mutants, the level of α -globin protein present in individuals with α -globin structural mutations has been used to infer certain features of α -globin gene regulation. In most of these individuals, the α -globin mutation is present in one of the four α -globin genes, and is expressed at 25% of total α -globin (5). This finding suggests that the four α -globin genes are equally expressed at the protein level. Levels of α -globin mutant significantly <25% can often be attributed to a relative instability of the mutant globin chain, while in some cases high levels may result from coexisting α -thalassemia in the genome, which lowers production of the normal α -globin chains (6–8). In a number of situations, however, the factors which contribute to the final level of α -globin mutant are not understood (7). In order to further investigate such cases, it may be necessary to determine the number and organization of the α -globin genes in the affected individual, as well as the identity and location of the mutant locus. The organization of the α -globin gene cluster can be determined by Southern blot analysis (9–11), and the identity of the mutant globin chain can be determined by peptide analysis (2, 3). However, the location of the mutant locus within the α -globin cluster cannot be determined by either of these methods. The assignment of an α mutation to one of the two α -globin loci would be necessary to establish the exact genotype in each case. We present, in the present report, a technique based upon the divergent structure of the two α -globin mRNAs (12–14) which directly assigns α -globin structural mutations to either the $\alpha 1$ or the $\alpha 2$ locus. This approach, when combined with presently available protein analytic and gene mapping techniques, should allow a full genetic description of most α -globin structural mutations.

Methods

RNA preparation. Total RNA was isolated from the acid-precipitated polysomes of peripheral reticulocytes as previously described (15).

Recombinant DNA constructions. All restriction enzymes were obtained from Bethesda Research Labs (BRL),¹ Bethesda, MD, or New England Biolabs and were used at concentrations of 1 U/ μ g DNA in the recommended buffers. For blunt-end ligations, staggered ends generated by HindIII and BglI were filled in with DNA polymerase (Klenow fragment-BRL) and the appropriate nucleoside triphosphates under suggested conditions. To decrease vector self-ligation, 5'-terminal phosphates were removed from vector DNA with bacterial alkaline phosphatase (BRL) using 10 U/ μ g vector DNA in 20 μ l of the recommended buffer at 37°C for 30 min. The phosphatase was then removed by three successive phenol extractions. All ligations were

Dr. Liebhaber is a fellow of the John A. and George L. Hartford Foundation.

Received for publication 3 July 1984 and in revised form 17 September 1984.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/85/01/0064/07 \$1.00

Volume 75, January 1985, 64–70

1. Abbreviations used in this paper: bp, base pair; BRL, Bethesda Research Labs.

carried out at 4°C for 18 h in 20 µl reaction volume containing 5:1 molar ratio of insert to vector and 400 U of T4 DNA ligase (New England Biolabs) in 50 mM Tris HCl, pH 7.4, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM spermidine, and 1 mM ATP. The entire ligation reaction was used to transform *Escherichia coli* HB101 rendered competent by cold CaCl₂ treatment (16). Transformations were spread on L-broth plates containing ampicillin (35 µg/ml).

Preparation of plasmids and plasmid paper. All recombinant plasmids were maintained in the EK-2 host bacteria HB101. They were grown in 1 L batches to an OD₆₆₀ of 0.8, amplified with chloramphenicol (170 µg/ml), and harvested 18 h later. Plasmid supercoil was purified from a clarified lysate by cesium chloride isopycnic centrifugation in the presence of 0.75 mg/ml ethidium bromide. To bind plasmids to nitrocellulose paper, 100 µg of plasmid DNA was linearized by restriction endonuclease digestion (pMC18 [17] by EcoRI, pSAR6 [18] by HindIII, pH3α1B and pH3α2A by PstI—see Fig. 1), phenol extracted, ethanol precipitated, washed twice with 70% ethanol, dried, and dissolved in 50 µl of water. The DNA was denatured by heating to 90°C for 3 min, then quick-chilled in ice water, made 10× SSC by adding an equal volume of ice-cold 20× SSC (3 M NaCl, 0.3 M NaCitrate, pH 7.0), and added to a 2 cm by 1 cm piece of nitrocellulose paper resting on parafilm. The wetted nitrocellulose was then sealed with tape in a paraffin envelope, incubated 18 h at 4°C, removed from the parafilm, baked for 2 h at 80°C in a vacuum oven, and stored in a sterile dry container.

Hybrid selection of mRNA. Approximately 50 mm² of plasmid paper was placed in a sterile polypropylene tube with 500 µl of water and heated to 100°C for 1 min. The water was then removed and replaced by 100 µl of hybridization mix (50% formamide, 0.7 M NaCl, 10 mM Pipes, pH 6.4, 4 mM EDTA) and prehybridized at 47°C for 1 h. The paper was then transferred to a fresh tube containing 50–75 µg of total reticulocyte RNA which had been dissolved in 100 µl of hybridization mix and heated to 70°C for 10 min. After an 18-h incubation at 47°C, the hybridization mix was removed and the filter was successively washed three times at room temperature with 1× SSC/0.5% SDS, five times at 60°C with 0.1× SSC/0.1% SDS, two times at room temperature with 0.1× SSC, once with ice-cold 2 mM EDTA (pH 7.2), and two times with ice-cold water. The specifically hybridized mRNA was eluted from the paper by heating to 100°C for 1 min in 300 µl of water containing 10 µg calf liver tRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN) as carrier. Immediately after heating, the tube was snap frozen in an ethanol-dry ice bath and thawed slowly on wet ice. The filters were then removed, and the mRNA precipitated with 2 vol of ethanol in the presence of 0.2 M ammonium acetate.

S1 nuclease mapping of α-globin mRNA (19). To generate ³²P end-labeled, single-strand DNA probes for S1 nuclease mapping, the pRP9 (20) (α2) or pJW101 (21) (α1) cDNA plasmids were digested with HindIII, and the 3' ends were filled in with α³²P-dATP using DNA polymerase I (Klenow fragment). The labeled DNA was ethanol precipitated, dissolved in 30% dimethylsulfoxide, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue, heated to 90°C for 3 min, chilled on ice, and loaded on a 5% strand-separating gel (22). The labeled insert strand separates and migrates as two bands. The slower one in the case of both probes was determined to be complementary to the mRNA. This band was excised, electroeluted from the gel, phenol extracted, and precipitated. For S1 mapping, 25 ng of single-stranded probe was hybridized to 10% of the hybrid-selected mRNA in 30 µl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA) at 42°C for 3 h. This ratio of probe to mRNA was determined to be in substantial probe excess by demonstrating a commensurate increase in protected probe with increase in added RNA (data not shown). The hybridization reaction was terminated and S1 digested by adding 300 µl of S1 buffer containing 500 U of S1 (Boehringer Mannheim Biochemicals) nuclease per milliliter and incubated for 4 h at 37°C. The S1 reaction was terminated by ethanol precipitation, and the pellet was washed twice in 70% ethanol, dissolved in formamide loading dye (80% formamide,

10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue), heated to 90°C for two min, and run on a 6% acrylamide/8 M urea gel at 800 V for 1 h. The gel was exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70°C with an intensifying screen for 2 h–3 d, depending upon the signal strength. Fragment sizes on gels were established by comparison to an adjacent Maxam-Gilbert sequencing ladder of the end-labeled probe (data not shown).

In vitro translation and gel analysis. 1 µg of total reticulocyte RNA (total RNA) or 25% of a hybrid-selected mRNA sample was added to a 15 µl micrococcal nuclease-treated rabbit reticulocyte lysate translation system (23) as previously detailed (24). Translation was carried out at 27°C for 30 min in the presence of ³⁵S-methionine (1,500 Ci/mM, Amersham Corp., Arlington Heights, IL). 5 µl of each reaction was added to 12 µl of loading dye (8 M urea, 8% acetic acid, 8% β-mercaptoethanol, pyronin Y) and applied to a 12.5% acrylamide-Triton urea gel (25, 26). The gel was run, processed, and exposed as previously detailed (15).

Results

Subcloning the 3'-nontranslated region of the human α1- and α2-globin genes. The α1- and α2-globin mRNAs are identical in primary structure throughout their 5'-nontranslated regions and their entire coding regions. Structural divergence is limited to the 110 nucleotide 3'-nontranslated region, which contains 18 base differences and a single base insertion/deletion (12–14). To isolate a fragment of DNA that could hybridize specifically to the α1 or α2 mRNA, we subcloned the 3'-nontranslated region from both the α1 and α2 genes. The details of this subcloning are shown in Fig. 1. The α1-globin 3'-nontranslated region was isolated from a human α1-globin genomic clone (pRBα1) (27) on a 143 base pair (bp) BglI-PvuII fragment. After filling in its ends, this fragment was gel purified, ligated to the plasmid vector pBR322 between the HindIII and PvuII sites, and transfected into *E. coli* HB101. Recombinant plasmids were identified by in situ hybridization to a ³²P-labeled α-globin cDNA probe and further screened by restriction analysis to identify the expected 143-bp insert. The α2 3'-nontranslated region was subcloned in a similar fashion. A 132 bp AluI/HindIII fragment from the human α2-globin cDNA plasmid pRP9 (20) was gel purified and ligated to the plasmid vector pBR322 between the HindIII and PvuII sites. Colonies were screened and analyzed as described above. The subclone isolated from the 3'-nontranslated region of α1, pH3α1B, and the subclone isolated from the 3'-nontranslated region of α2, pH3α2A, were purified, linearized, and used to construct α1 and α2 plasmid paper.

Hybrid selection of β, α (total), α1, and α2 mRNA. The steps in the hybrid selection are schematicized in Fig. 2 and detailed in Methods. 25–50 µg of reticulocyte RNA was separately hybridized to each of the four plasmid papers. β, α, α1, and α2 plasmid papers contain, respectively, the full length β cDNA plasmid pSAR6 (18), the full length α-globin (chimpanzee) cDNA plasmid, pMC18 (17), and the two cDNA subclones, pH3α1B and pH3α2A (described above). The described washing and elution conditions routinely yield pure α and β mRNA and α1 and α2 mRNAs with little or no cross-contamination.

S1 nuclease mapping of hybrid-selected α-globin mRNAs. The relative content of α1- and α2-globin mRNA in each of the hybrid-selected samples, α, α1, and α2, was determined using the previously described S1 nuclease mapping procedure (19). To ensure accuracy of the results, we have modified this

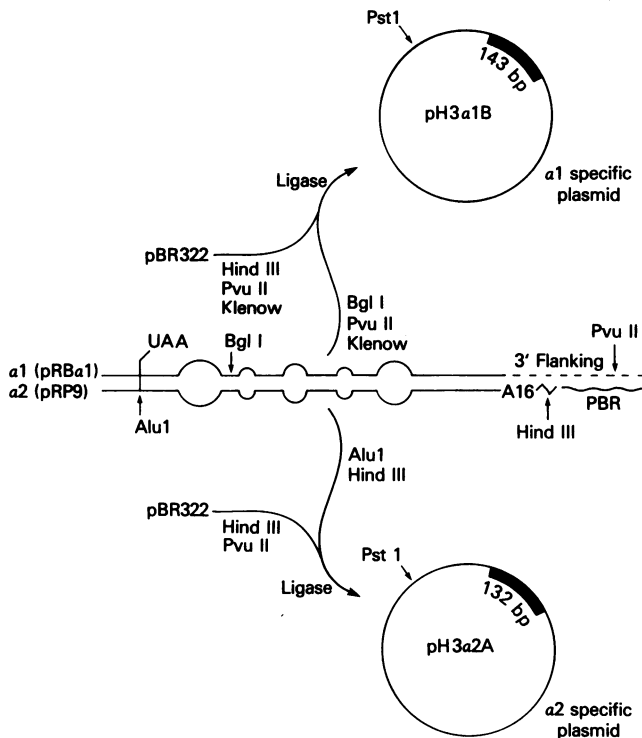


Figure 1. Construction of subclones containing the 3'-nontranslated regions of the human $\alpha 1$ - and $\alpha 2$ -globin genes. The 3'-nontranslated region of the $\alpha 1$ - and $\alpha 2$ -globin genes is shown in the center of the diagram as a solid line extending 3' from the termination codon (UAA). The regions of nonhomology between the two mRNAs are schematized as loops. The 3'-nontranslated region of $\alpha 1$ was excised from the $\alpha 1$ genomic clone pRBa1 (27) between the BglI and PvuII sites; the corresponding region of $\alpha 2$ was excised from the $\alpha 2$ cDNA clone pRP9 (20) between the AluI and HindIII sites. Each fragment was then separately inserted into pBR322 between the HindIII and PvuII sites as detailed in Methods. The $\alpha 1$ and $\alpha 2$ 3'-nontranslated inserts in the resultant recombinant plasmids pH3a1B and pH3a2A, respectively, are shown as solid bars.

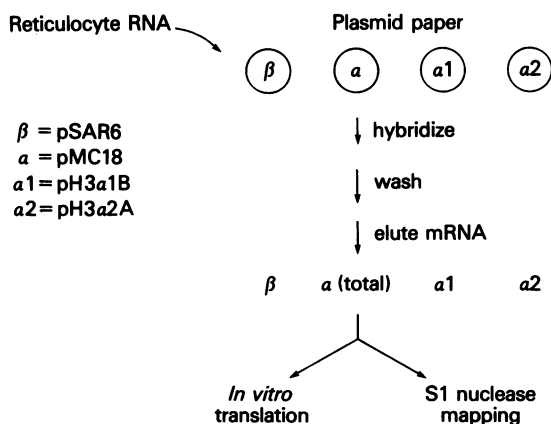


Figure 2. Experimental approach for hybrid-selected translation of globin mRNAs. Total reticulocyte RNA was added to nitrocellulose papers containing β -, α - (total), $\alpha 1$ -, or $\alpha 2$ -globin specific plasmids. After hybridization and extensive washing, the specifically bound mRNA was eluted from the paper. An aliquot was assayed by S1 nuclease mapping for relative content of the $\alpha 1$ and $\alpha 2$ mRNAs while a second aliquot was translated in vitro.

approach to include the use of two different S1 probes, one isolated from an $\alpha 1$ cDNA, and one from an $\alpha 2$ cDNA (Fig. 3). The results using both these probes should be reciprocal. Single-stranded DNA probes isolated from $\alpha 1$ and $\alpha 2$ cDNA plasmids were ^{32}P -end labeled at the HindIII site (codon 90-91) and hybridized in DNA excess to globin mRNA. When the probe hybridizes to its homologous RNA (i.e., $\alpha 1$ probe to the $\alpha 1$ mRNA), a labeled 263 nucleotide fragment is protected from S1 digestion. When the probe hybridizes to the nonhomologous (i.e., $\alpha 1$ to $\alpha 2$) mRNA, a smaller fragment of 175 nucleotides is protected, since the region of divergence between the two α -globin mRNAs begins 175 nucleotides 3' to the HindIII site.

Hybrid selection of normal $\alpha 1$ - and $\alpha 2$ -globin mRNA. The hybrid selection procedure was first used on reticulocyte RNA isolated from an individual who has normal α -globin chains. β , α , $\alpha 1$, and $\alpha 2$ mRNA were hybrid selected. The relative content of $\alpha 1$ and $\alpha 2$ in each α -globin mRNA sample was then determined by S1 mapping. The results in lanes 1-4 (Fig. 4 A) demonstrate the clean separation of $\alpha 1$ from $\alpha 2$ mRNA. In the total α -globin mRNA sample (Fig. 4 A, lanes 5 and 6), the S1 mapping demonstrated the previously reported excess of $\alpha 2$ mRNA in normal reticulocytes (19, 28). Each hybrid-selected sample was translated in vitro (Fig. 4 B). The $\alpha 1$ - and $\alpha 2$ -globin mRNAs encoded an identical α -globin protein. This was expected, as both genes produce identical proteins in the absence of a structural α -hemoglobinopathy. This translation also demonstrated the clean separation of α from β mRNA. Translation of the β mRNA sample was devoid of α -globin, and the α , $\alpha 1$, and $\alpha 2$ mRNA samples encoded no detectable β -globin. (Although the β -globin mRNA in this individual did in fact encode a structural mutation, β^S , this gel system does not resolve this mutant from β^A .)

Locus identification of $\alpha^{125\text{Pro}}$ (Hb Quong Sze) and $\alpha^{47\text{His}}$ (Hb Hasharon). Reticulocyte RNA was isolated from the blood of two individuals with known α -globin mutations. The individual with $\alpha^{125\text{Pro}}$ has been previously described in detail (29). He has HbH disease due to the deletion of both α -globin genes

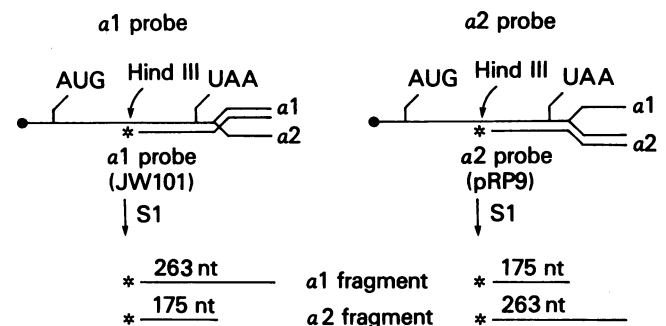


Figure 3. S1 nuclease mapping of α -globin mRNA using $\alpha 1$ and $\alpha 2$ probes (19). 5' ^{32}P -end labeled single-stranded DNA probes isolated from $\alpha 1$ or $\alpha 2$ cDNA was hybridized to globin mRNA samples and subsequently digested with S1 nuclease. The ^{32}P -label is represented by the asterisk. If the probe hybridized to its homologous mRNA (i.e., $\alpha 1$ to $\alpha 1$ or $\alpha 2$ to $\alpha 2$), a 263 nt labeled fragment would be protected. If the probe hybridized to the nonhomologous α -globin mRNA, a 175-nt fragment, which extended from the labeled end through to the first region of nonhomology between the two α -globin mRNAs (19), would be protected. The products of the S1 mapping were sized and quantitated on a 5% acrylamide, 8 M urea gel. nt, nucleotides.

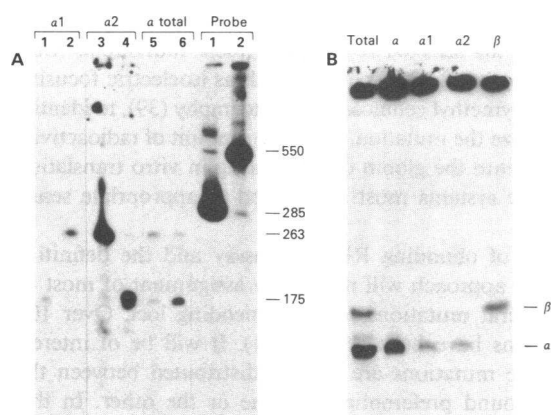


Figure 4. Hybrid-selected translation of globin mRNAs from an individual with normal α -globin genes. Total α -, $\alpha 1$ -, $\alpha 2$ -, and β -globin mRNAs were isolated by hybrid selection. (A) Aliquots from the indicated samples ($\alpha 1$ -, $\alpha 2$ -, α -total) were S1 mapped to determine relative content of $\alpha 1$ - and $\alpha 2$ -globin mRNA. Lanes 1, 3, and 5 display the fragments generated by S1 digestion of the $\alpha 2$ probe hybridized to the mRNA. Lanes 2, 4, and 6 display the fragments generated by S1 digests of the $\alpha 1$ probe hybridized to the mRNA. The starting $\alpha 2$ and $\alpha 1$ probes are shown in the two right-hand lanes 1 and 2, respectively. The size of each fragment is noted on the right in nucleotides. (B) In vitro translation of hybrid-selected globin mRNAs. Total reticulocyte RNA (total) or mRNA from the α -, $\alpha 1$ -, $\alpha 2$ -, or β -samples was translated in a rabbit reticulocyte lysate system in the presence of ^{35}S -methionine. The products were analyzed by autoradiography of a Triton-urea gel as detailed in Methods. The relative positions of β - and α -globin are marked. The faint band migrating between α - and β -globin in the Total lane is an α -globin oxidation product (15).

from one chromosome and a nondeletion α -thalassemia defect that destroyed the function of one of the two α -globin genes on the homologous chromosome. This nondeletion defect was defined by functional studies to be the result of an extremely unstable α -globin structural mutant encoded by the nondeletion α -thalassemia gene (15). This gene was localized to the $\alpha 2$ locus by DNA sequence analysis (30). To test the ability of the hybrid-selection approach to identify an α -globin mutant locus, we isolated $\alpha 1$ - and $\alpha 2$ -globin mRNA from this individual's reticulocytes and translated them in vitro. S1 analysis showed that $\alpha 2$ is in its normal threefold excess in the unfractionated α -globin mRNA as previously reported (28) (Fig. 5 A, lanes 5 and 6). The mRNAs in the $\alpha 1$ and $\alpha 2$ samples were highly purified and showed no evidence of cross-contamination. mRNA from the β -, α -, $\alpha 1$ -, and $\alpha 2$ -samples were translated in vitro (Fig. 5 B). Translation of the $\alpha 1$ yielded the normal α -globin, while translation of $\alpha 2$ produced the mutant α -globin $\alpha^{125\text{Pro}}$. Therefore, localization of the mutant to the $\alpha 2$ locus by the method of hybrid-selected translation agreed with the previous assignment by gene isolation and sequence analysis.

We next studied an individual with an α -globin structural variant that was not previously localized within the α -globin cluster. The identification of this α -globin as $\alpha^{47\text{His}}$ (Hb Has-haron) (31) was made by conventional peptide analysis (Lubin, B., personal communication). The individual we studied was a Caucasian female with normal hematologic parameters. A Southern blot analysis of her DNA demonstrated a normal α -

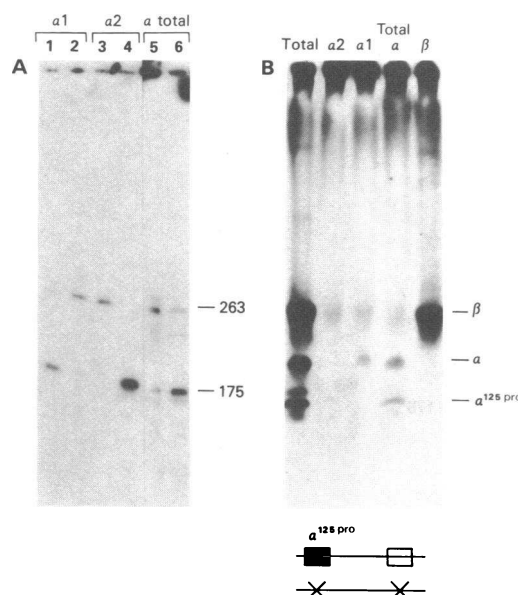


Figure 5. Hybrid-selected translation of globin mRNAs from an individual with HbH disease and an $\alpha^{125\text{Pro}}$ mutation. (A) Reticulocyte RNA was fractionated into α -, $\alpha 1$ -, and $\alpha 2$ -mRNA by hybrid selection. The relative concentration of $\alpha 1$ and $\alpha 2$ -mRNA in each sample ($\alpha 1$ in lanes 1 and 2; $\alpha 2$ in lanes 3 and 4; α -total in lanes 5 and 6) was determined by S1 mapping using an $\alpha 1$ -S1 probe (lanes 2, 4, and 6) or an $\alpha 2$ -S1 probe (lanes 1, 3 and 5). (B) In vitro translation of hybrid-selected mRNA. Total reticulocyte RNA and aliquots of $\alpha 2$ -, $\alpha 1$ -, α -, and β -hybrid-selected mRNA samples were translated and analyzed as in Fig. 4. The position of normal β -, α -, and the $\alpha^{125\text{Pro}}$ are noted. The band above $\alpha^{125\text{Pro}}$ is an α -globin oxidation product. The full α -globin genotype of this individual is noted below the gel.

globin gene organization (data not shown). The α -globin mRNA was separated into $\alpha 1$ and $\alpha 2$ samples by hybrid selection, and S1 analysis of these samples demonstrated no appreciable cross-contamination (Fig. 6 A). In vitro translation of the $\alpha 1$ sample produced normal α -globin, while the $\alpha 2$ mRNA encoded approximately equal amounts of α and $\alpha^{47\text{His}}$ (Fig. 6 B). This data directly localized the $\alpha^{47\text{His}}$ to the $\alpha 2$ locus on one chromosome while demonstrating that the $\alpha 2$ locus on the homologous chromosome encodes a structurally normal protein. This information, combined with the Southern mapping data, can be summarized in a fully defined genotype as shown at the bottom of Fig. 6 B.

Discussion

The present report describes a rapid and definitive method for the localization of α -globin structural mutants to one of the two α -globin loci. This method uses the technique of hybrid-selected translation (32–34), which has previously been used to map structural loci within viral genomes (32) and to confirm the identity of a variety of cDNA clones (33, 34). A number of factors make hybrid-selected translation particularly useful in the globin systems. mRNA can be isolated from easily obtainable peripheral blood samples. Even in the absence of reticulocytosis, sufficient mRNA for the assay as we describe can be isolated from as little as 50 ml of blood. This is in part due to the fact that reticulocyte mRNA is >90% enriched for

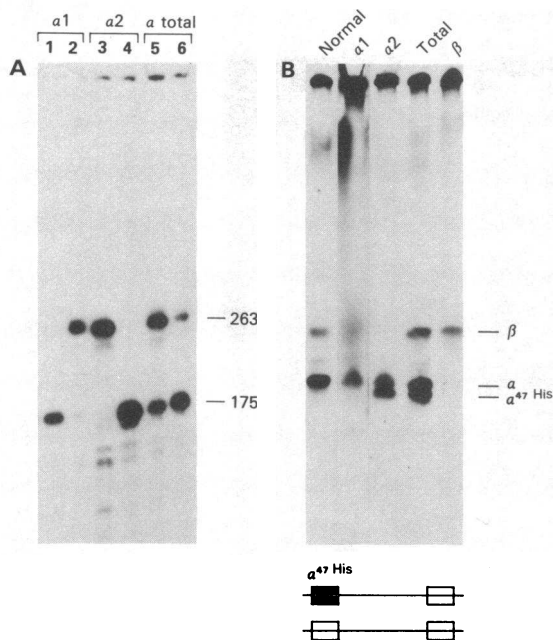


Figure 6. Hybrid-selected translation of globin mRNA from an individual with Hb Hasharon ($\alpha^{47\text{His}}$). (A) S1 mapping as described in Fig. 5 A. (B) In vitro translation of the hybrid-selected samples as detailed. The position of the $\alpha^{47\text{His}}$ is indicated and the individual's full α -globin genotype is noted below the gel.

globin mRNA (35). Genomic and cDNA clones encoding each of the globin chains are now available. Specific hybridization to any of the major globin mRNA species can be achieved by isolating regions that will achieve maximal specificity of hybridization to each mRNA species. Finally, based upon several decades of work analyzing the human globin proteins and their variants, numerous techniques are available both to identify specific hemoglobin variants and to separate and characterize the in vitro translation products of the selected globin mRNAs.

In adopting this approach to identify the genetic locus of $\alpha 1$ - and $\alpha 2$ -globin mutations, the primary problem was to isolate their respective $\alpha 1$ and $\alpha 2$ mRNAs. Previous sequence analysis has shown that the only region of structural divergence between the two α -globin mRNAs is in the 3'-nontranslated region (12-14). In this 110 nucleotide region, the two α -globin mRNAs differ by 18 nonhomologous bases and a single base insertion/deletion, which results in a net divergence of 16%. To maximize the usefulness of this region in differential hybridization to the two α -globin mRNA species, the homologous 5'-noncoding and entire coding regions had to be eliminated. The 3'-noncoding regions of $\alpha 1$ and $\alpha 2$ were therefore separately subcloned, attached to nitrocellulose paper, and the hybridization stringency was adjusted to maximize specific hybridization to their homologous mRNAs.

A difficulty which we have encountered in the general usefulness of this approach occurs in separating mutant from normal globin chain. For instance, in Fig. 4, the β -globin gene which is in fact a mutant (β^S) co-migrates with normal β^A . Similarly, several α structural mutants that we have studied (G^{Phila} [36], J^{Oxford} [37]) do not separate adequately from their normal counterparts on the Triton gel system which we are

using. Thus, the labeled globin chain synthesized by in vitro translation of the selected RNAs from these individuals must be analyzed by a different method, such as isoelectric focusing (38) or carboxymethyl cellulose chromatography (39), to identify and characterize the mutation. Since the amount of radioactivity incorporated into the globin chains during in vitro translation is small, these systems must be adapted to appropriate sensitivity.

The ease of obtaining RNA for assay and the definitive nature of this approach will now allow assignment of most α -globin structural mutations to their encoding loci. Over 100 such mutations have been reported (1). It will be of interest to see if these mutations are equally distributed between the two loci or found preferentially at one or the other. In this regard, it is of interest to note that of the nine α -globin mutations (structural and nondeletion α -thalassemia) which have now been assigned to a particular locus, eight are $\alpha 2$ (Table I). While this marked asymmetry in α -globin gene mutations may reflect an aberrance of the relatively small sample size, it may alternatively offer a clue to understanding the evolution of the α -globin gene cluster (see below). Assignment of the α structural mutations to their encoding loci will expand the sample size and significantly clarify this issue.

Are the two α -globin genes equally expressed? The α -globin mutants are generally expressed at 20-25% of total α , although a wide spectrum of levels has been documented (1, 5). The average level of 25% implies that all four α -globin genes are expressed equally at the protein level. Studies which have quantitated the relative concentrations of $\alpha 1$ and $\alpha 2$ mRNA in normal individuals have, however, documented a 2-3-fold excess of $\alpha 2$ mRNA over $\alpha 1$ mRNA (19, 28). In vitro translational studies suggest that $\alpha 1$ and $\alpha 2$ gene expression may be balanced to produce equal amounts of protein by a higher translation efficiency of the $\alpha 1$ mRNA (40). By correlating the level of expression of α -globin mutants with their encoding loci, it may be possible to determine if the two loci are indeed expressed equally in vivo or if the observed expression levels differ according to encoding locus.

Finally, locus assignment of α -globin mutations may shed light on the evolution of the α -globin cluster. Extensive sequence

Table I. α -Globin Mutations with Defined Location

	Locus	Reference
Nondeletion α -thalassemias		
1 Constant spring	$\alpha 2$	50
2 Icara	$\alpha 2$	51
3 Wayne	$\alpha 2$	52
4 Quong Sze	$\alpha 2$	30 (and present study)
5 Mutation of initiation codon	$\alpha 2$	53
6 IVS-1 splice donor mutation	$\alpha 2$	54
7 Codon 17 frameshift	$\alpha 1$	55
8 Poly A signal mutation	$\alpha 2$	55
Structural mutations		
1 Hb Hasharon ($\alpha^{47\text{His}}$)	$\alpha 2$	Present study

analysis of this cluster in a number of species implies that these two adjacent genes exchange structural information over evolutionary time (14, 17, 41–43). These events are currently referred to as gene conversions (41, 44–46) and result in concerted evolution (41) of both linked (14, 27, 41, 42, 47), as well as unlinked, genes (48). Gene conversion events between the two adjacent α -globin genes appears to occur frequently in primates (17, 41). If such an event occurred in a genome containing a preexisting α -globin mutation, that mutation might subsequently be found at both α -globin loci on the same chromosome. In such a genotype, the level of α -globin mutant would be expected to approach 50%. Association of such a chromosome with a single or double α -globin deletion on the homologous chromosome (α -Thal-2 or α -Thal-1 chromosome, respectively) would result in α -globin mutant levels of 66 or 100%, respectively. Assuming that the conversion unit does not include the 3'-nontranslated region (43), the hybrid-assisted translation would detect the mutant protein encoded by both $\alpha 1$ and $\alpha 2$ mRNAs. Recent studies on an individual with an unusually high (65%) level of the α -globin mutant $\alpha^{16\text{Glu}}$ (Hb^I) have in fact documented the presence of such a chromosome in which both α -globin loci on a single chromosome encode the identical mutation (49). It is anticipated that in the future other examples of gene conversion may be identified by α -globin locus assignment in individuals with unusually high levels of mutant chains. By establishing the full α -globin genotype in individuals with higher than expected levels of α -globin structural mutations, it may be possible to identify additional evidence of concerted evolution in the human α -globin gene cluster and estimate its frequency and importance.

Acknowledgments

We wish to thank Dr. Nancy E. Cooke for helpful advice and discussions, Dr. Gail Wagner for close cooperation, and Susan H. Shakin for critical comments on the manuscript.

This investigation was partially supported by grant 1-RO1-AM-33975 from the National Institutes of Health. Dr. Liebhaver is a recipient of a Basil O'Connor Starter Grant from the National Foundation-March of Dimes.

References

- Bunn, H. F., and B. G. Forget. 1984. Hemoglobin: Molecular, Genetic and Clinical Aspects. W. B. Saunders, Philadelphia.
- Ingram, V. M. 1956. A specific chemical difference between the globins of normal human and sickle cell anemia hemoglobin. *Nature (Lond.)*. 178:792–794.
- Jones, R. T. 1964. Structural studies of aminoethylated hemoglobins by automated peptide chromatography. *Cold Spring Harbor Symp. Quant. Biol.* 29:297–308.
- Dayhoff, M. O. 1969. Atlas of Protein Sequence and Structure. National Biomedical Research Foundation, Washington, DC. 5:56 and Supplements 1 and 1, 1975.
- Nute, P. E. 1974. Multiple hemoglobin α -chain loci in monkeys, apes and man. *Ann. NY Acad. Sci.* 241:39–61.
- Surrey, S., K. Ohene-Frempong, E. Rappaport, J. Atwater, and E. Schwartz. 1980. Linkage of $\alpha^{\text{Philadelphia}}$ to α -thalassemia in African-Americans. *Proc. Natl. Acad. Sci. USA*. 77:4885–4889.
- Sancar, G. B., B. Tatis, M. Cedeno, and R. F. Reider. 1980. Proportion of hemoglobin G Philadelphia ($\alpha^{268\text{Arg-Lys}}\beta 2$) in heterozygotes is determined by α -globin gene deletions. *Proc. Natl. Acad. Sci. USA*. 77:6874–6878.
- Old, J. M., J. B. Clegg, D. J. Weatherall, and P. B. Booth. 1978. Hemoglobin J Tongariki is associated with α -thalassemia. *Nature (Lond.)*. 273:319–320.
- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.
- Orkin, S. H. 1978. The duplicated human α -globin genes lie close together in cellular DNA. *Proc. Natl. Acad. Sci. USA*. 75:5950–5954.
- Embury, S. H., R. V. Lebo, A. M. Dozy, and Y. W. Kan. 1979. Organization of the α -globin genes in the Chinese α -thalassemia syndromes. *J. Clin. Invest.* 63:1307–1310.
- Liebhaver, S. A., M. J. Goossens, and Y. W. Kan. 1980. Cloning and complete nucleotide sequence of the human 5' α -globin gene. *Proc. Natl. Acad. Sci. USA*. 77:7054–7059.
- Michelson, A. M., and S. H. Orkin. 1980. The 3' untranslated regions of the duplicated human α -globin genes are unexpectedly divergent. *Cell*. 22:371–377.
- Liebhaver, S. A., M. J. Goossens, and Y. W. Kan. 1981. Homology and concerted evolution at the $\alpha 1$ and $\alpha 2$ loci of human α -globin. *Nature (Lond.)*. 290:26–29.
- Liebhaver, S. A., and Y. W. Kan. 1983. α -Thalassemia caused by an unstable α -globin mutant. *J. Clin. Invest.* 71:461–466.
- Dagert, M., and S. D. Ehrlich. 1979. Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. *Gene (Amst.)*. 6:23–28.
- Liebhaver, S. A., and K. A. Begley. 1983. Structural and evolutionary analysis of the two chimpanzee α -globin mRNAs. *Nucl. Acids Res.* 11:8915–8929.
- Liebhaver, S. A., R. F. Trecartin, and Y. W. Kan. 1981. β -thalassemia in Sardinia is the result of a nonsense mutation. *Trans. Assoc. Am. Physicians*. 94:88–96.
- Orkin, S. H., and S. C. Goff. 1981. The duplicated human α -globin genes: their relative expression as measured by RNA analysis. *Cell*. 24:345–351.
- Poon, R., K. H. Neumann, H. W. Boyer, A. M. Dozy, G. F. Temple, J. C. Chang, and Y. W. Kan. 1977. Cloning human globin genes in bacterial plasmid. *Blood*. 50(Suppl.):116.
- Wilson, J. T., L. B. Wilson, J. K. deRiel, L. Villa-Komaroff, A. Efstratiadis, B. G. Forget, and S. M. Weissman. 1978. Insertion of synthetic copies of human globin genes into bacterial plasmids. *Nucl. Acids Res.* 5:563–581.
- Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific cleavage reactions. *Methods Enzymol.* 65:499–560.
- Pelham, H. R. B., and R. B. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–257.
- Liebhaver, S. A., S. A. Shakin, and F. E. Cash. 1984. Helix destabilizing activity in rabbit reticulocyte lysate. *J. Biol. Chem.* 259:15597–15602.
- Rovera, G., C. Magarian, and T. W. Borun. 1978. Resolution of hemoglobin subunits by electrophoresis in acid urea polyacrylamide gels containing Triton X-100. *Anal. Biochem.* 85:506–518.
- Alter, B. P. 1979. The $\alpha\gamma\gamma$ composition of fetal hemoglobin in fetuses and newborns. *Blood*. 54:1158–1163.
- Lauer, J., C.-K. J. Shen, and T. Maniatis. 1980. The chromosomal arrangement of human α -like globin genes: sequence homology and α -globin gene deletions. *Cell*. 20:119–130.
- Liebhaver, S. A., and Y. W. Kan. 1981. Differentiation of the mRNA transcripts originating from the $\alpha 1$ - and $\alpha 2$ -globin genes in normals and α -thalassemics. *J. Clin. Invest.* 68:439–448.
- Kan, Y. W., A. M. Dozy, R. Trecartin, and D. Todd. 1977. Identification of a nondeletion defect in α -thalassemia. *N. Engl. J. Med.* 297:1081–1084.
- Goossens, M., K. Y. Lee, S. A. Liebhaver, and Y. W. Kan. 1982. Globin structural mutant $\alpha^{125\text{Leu-Pro}}$ is a novel cause of α -thalassemia. *Nature (Lond.)*. 296:864–865.

31. Halbrecht, I., W. A. Isaacs, H. Lehmann, and F. Ben-Porat. 1967. Hemoglobin Hasharon (α^{47} aspartic acid \rightarrow histidine). *Isr. J. Med. Sci.* 3:827-831.
32. Patterson, D. M., B. E. Roberts, and E. C. Kuff. 1977. Structural identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. *Proc. Natl. Acad. Sci. USA.* 74:4370-4374.
33. Harpold, M. M., P. R. Dobner, R. M. Evans, and F. C. Bancroft. 1978. Construction and identification by positive hybridization translation of a bacterial plasmid containing a rat hormone structural gene sequence. *Nucl. Acids Res.* 5:2039.
34. Parnes, J. R., B. Velon, A. Felsenfeld, L. Ramanathan, U. Ferrini, E. Appella, and J. G. Seidman. 1981. Mouse $\beta 2$ -microglobulin cDNA clones: a screening procedure for cDNA clone corresponding to rare mRNAs. *Proc. Natl. Acad. Sci. USA.* 78:2253-2257.
35. Lodish, H., and O. Desalu. 1973. Regulation of synthesis of non-globin proteins in cell-free extracts of rabbit reticulocytes. *J. Biol. Chem.* 248:3520-3527.
36. Baine, R. M., D. L. Rucknagel, P. A. Dublin, Jr., and J. G. Adams III. 1976. Trimodality in the proportion of hemoglobin G Philadelphia in heterozygotes: evidence for heterogeneity in the number of human α chain loci. *Proc. Natl. Acad. Sci. USA.* 73:3633-3636.
37. Liddell, J., D. Brown, D. Beale, H. Lehmann, and R. G. Huntsman. 1964. A new hemoglobin-Ja Oxford found during a survey of an English population. *Nature (Lond.)*. 204:269-270.
38. Comi, P., B. Gigliani, S. Ottolenghi, A. Gianni, G. Rocco, U. Mazza, G. Saglio, C. Camaschella, P. Pich, E. Gianazza, and P. Righetti. 1979. $G\gamma$ and $A\gamma$ -globin chains separation and quantitation by isoelectric focusing. *Biochem. Biophys. Res. Commun.* 87:1-8.
39. Bagliani, C. 1961. An improved method for the fingerprinting of human hemoglobin. *Biochim. Biophys. Acta.* 48:392-396.
40. Liebhaber, S. A., and Y. W. Kan. 1982. Different rates of mRNA translation balance the expression of two human α -globin loci. *J. Biol. Chem.* 257:11852-11855.
41. Zimmer, E. A., S. L. Martin, S. M. Beverley, Y. W. Kan, and A. C. Wilson. 1980. Rapid duplication and loss of genes coding for the α chains of hemoglobin. *Proc. Natl. Acad. Sci. USA.* 77:2158-2162.
42. Schon, E. A., S. N. Wernke, and J. B. Lingrel. 1982. Gene conversion of two functional goat α -globin gene preserves only minimal flanking sequences. *J. Biol. Chem.* 257:6825-6835.
43. Michaelson, A. M., and S. H. Orkin. 1983. Boundaries of gene conversion within the duplicated human α -globin genes: concerted evolution by segmental recombination. *J. Biol. Chem.* 258:15245-15254.
44. Chovnick, A. 1973. Gene conversion and transfer of genetic information within inverted region of inversion heterozygotes. *Genetics.* 75:123-131.
45. Hood, L., J. H. Campbell, and S. C. R. Elgin. 1975. The organization, expression and evolution of antibody genes and other multigene families. *Annu. Rev. Genet.* 9:305-353.
46. Baltimore, D. 1981. Gene conversion: some implications for immunoglobulin genes. *Cell.* 24:592-594.
47. Slightom, J. L., A. B. Blechl, and O. Smithes. 1980. Human fetal $G\gamma$ - and $A\gamma$ -globin genes: complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. *Cell.* 21:627-638.
48. Brown, A. J. L., and D. Ish-Hurowicz. 1981. Evolution of the 87A and 87C heat-shock loci in *Drosophila*. *Nature (Lond.)*. 290:677-682.
49. Liebhaber, S. A., E. F. Rappaport, F. E. Cash, S. K. Ballas, E. Schwartz, and S. Surrey. 1984. Hemoglobin I mutation encoded at both α -globin loci on the same chromosome. *Science*. In press.
50. Clegg, J. B., D. J. Weatherall, and P. F. Milner. Hemoglobin Constant Spring—A chain termination mutant? *Nature (Lond.)*. 234:337-340.
51. Clegg, J. B., D. J. Weatherall, I. Contopolou-Griva, K. Caroutsos, P. Pongouras, and H. Tsevrenis. 1974. Hemoglobin Icara, a new chain termination mutant which causes α -thalassemia. *Nature (Lond.)*. 251:245-248.
52. Seid-Akhavan, M., W. P. Winter, R. K. Abramson, and D. L. Rucknagel. 1976. Hemoglobin Wayne: a frameshift mutation detected in human hemoglobin α chains. *Proc. Natl. Acad. Sci. USA.* 73:882-886.
53. Pirastu, M., G. Saglio, A. Cao, and Y. W. Kan. 1984. An initiation codon mutation in α -thalassemia. *Clin. Res.* 32:550A.
54. Felber, B. K., S. H. Orkin, and D. H. Hamer. 1982. Abnormal RNA splicing causes one form of α -thalassemia. *Cell.* 29:895-902.
55. Higgs, D. R., S. E. Y. Goodbourn, Y. Lamb, J. B. Clegg, and D. J. Weatherall. 1983. α -thalassemia caused by a polyadenylation signal mutation. *Nature (Lond.)*. 306:398-400.