β^0 Thalassemia in Sardinia Is Caused by a Nonsense Mutation

RICHARD F. TRECARTIN, STEPHEN A. LIEBHABER, JUDY C. CHANG,
KATHLEEN Y. LEE, and YUET WAI KAN, *Howard Hughes Medical Institute
Laboratory and Department of Medicine, University of California,
San Francisco, California 94143*

MARIO FURBETTA, ANTONELLA ANGIUS, and ANTONIO CAO, *2nd Pediatric Clinic,
University of Cagliari, Sardinia, Italy*

ABSTRACT We report the characterization of a molecular lesion of β thalassemia in Sardinia. Beta thalassemia in this area is predominantly the β^0 type with low levels of β -globin mRNA. Translation assay of this messenger RNA in a cell-free system showed β -globin chain synthesis only with the addition of an amber (UAG) suppressor transfer RNA. Double-stranded complementary DNA prepared from reticulocyte mRNA from a Sardinian patient was cloned in a bacterial plasmid and a β -globin complementary DNA containing clone was isolated and sequenced. At the position corresponding to amino acid number 39, a single nucleotide mutation converted a glutamine codon (CAG) to an amber termination codon (UAG). We previously reported an amber nonsense mutation at amino acid 17 as a cause of Chinese β^0 thalassemia. Thus, β^0 thalassemia in Sardinia represents the second example of a nonsense mutation, and we predict that other β^0 thalassemias with mutations at various points along the β -globin chain will be found to form a discrete subgroup of β^0 thalassemia. These experiments further illustrate the heterogeneity of lesions that lead to defective globin chain synthesis in β thalassemia.

INTRODUCTION

The molecular mechanisms that lead to defective β -globin chain synthesis in the β thalassemia syndromes are extremely heterogeneous (1-3). Deletions of various segments of DNA on chromosome 11 in the region of the β -globin gene complex (4) produce the phenotypes of β^0 thalassemia (5, 6), $\delta^0\beta^0$ thalassemia,

hereditary persistence of fetal hemoglobin (7-12), and $\gamma\delta\beta$ thalassemia (13, 14). The more common types of β thalassemia, however, are not the result of gene deletions. The nondeletion thalassemias, which are usually accompanied by elevated hemoglobin A₂ levels, are divided into two broad categories: β^+ thalassemia, in which β -globin chain synthesis is reduced but detectable, and β^0 thalassemia, in which no β -globin chains are produced (15, 16). The molecular defects in these forms of β thalassemia are currently under investigation with molecular cloning, DNA sequence analysis, and functional assays. Recent studies suggest that some β^+ thalassemia may be due to defective processing of the primary β -globin messenger RNA (mRNA) transcript into mature mRNA (17, 18).

The molecular bases of β^0 thalassemia are also heterogeneous. In some individuals, β -globin mRNA synthesis is absent; in others, a variable quantity is demonstrable in the reticulocyte (19). Previously we investigated the lesion in the mRNA from a Chinese patient affected by β^0 thalassemia where β -globin mRNA was detectable (20, 21), and found the defect to be a nonsense mutation that caused premature termination of the β -globin chain (22). In this study we investigated thalassemia on the island of Sardinia (23), where β^0 thalassemia, the predominant molecular form, is characterized by low levels of β -globin mRNA. We cloned the complementary DNA (cDNA), isolated and sequenced a β -globin cDNA clone, and found that the lesion was also due to a nonsense mutation, although in a different position from that in the Chinese. This study further illustrates the heterogeneity of the lesions that produce β thalassemia.

METHODS

RNA and DNA preparation and analysis. Previous studies have identified 331 patients with β^0 thalassemia in Sardinia

Address correspondence to Dr. Kan, University of California.

Received for publication 30 March 1981 and in revised form 22 May 1981.

(23). We prepared poly (A+) RNA from 20 patients and cellular DNA from 10 patients, as previously described (21, 24). RNA from 20 patients and DNA from five patients was quantitated by solution hybridization with cDNA enriched in α - and β -globin sequences as previously described (20, 24). Genomic DNA from 10 patients was digested with the enzymes *Hpa* I, *Bam* HI, *Pst* I, and *Eco* RI (Bethesda Research Laboratories, Gaithersburg, Md.) and analyzed by Southern blotting. The globin-gene containing fragments were identified by 32 P-labeled cDNA or by nick-translated β -globin cDNA as described (25).

Cell-free synthesis. Poly(A+) RNA from five patients was pooled and the β -globin mRNA was enriched by selective adsorption to β -globin cDNA covalently bound to cellulose. The β -globin cDNA recombinant plasmid (pJW 102) (26) was covalently bound to diazotized cellulose by the method of Noyes and Stark (27), the only modification being the use of 200 mM borate buffer at pH 6.0. A small amount of radioactive β -globin cDNA was used to monitor the coupling efficiency, which was found to be 1.8 μ g of pJW 102/mg of cellulose. Poly(A+) RNA (200 ng) was hybridized to 5 mg β -globin cDNA cellulose in a 65- μ l hybridization buffer containing 60% formamide (vol/vol), 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 0.1 g/100 ml SDS, and 0.25 mg/ml wheat germ transfer RNA (tRNA), with constant inversion at 37°C for 44 h. The cellulose was then washed twice with the 100- μ l hybridization mixture and twice with the mixture without tRNA at 37°C. The bound mRNA was eluted at 65°C in two 50- μ l aliquots of hybridization buffer. The RNA was precipitated in 70% (vol/vol) ethanol at -20°C after adding 20 μ g/ml deacylated wheat germ tRNA and 200 mM potassium acetate (pH 7.5).

The ability of the enriched β -globin mRNA to direct protein synthesis was assayed in a micrococcal nuclease-treated rabbit reticulocyte lysate system (28) in the presence or absence of a yeast (*Saccharomyces cerevisiae*) serine-inserting amber suppressor tRNA (29). A 25- μ l reticulocyte lysate mixture containing 50 μ Ci of [35 S]methionine (Amersham Corp., Arlington Heights, Ill.; 1,000 Ci/mmol) with or without the addition of 120 μ g/ml yeast suppressor tRNA was incubated at 30°C for 90 min. 5 mg of hemoglobin containing equal amounts of lysate from adult and cord blood was added as carrier, globin was prepared by acid acetone precipitation, and the globin chains were separated on carboxymethyl cellulose in 8 M urea as described (30). The β -globin polypeptide recovered from the column was digested with trypsin and the [35 S]methionine-containing β -globin peptides were identified by high voltage electrophoresis and autoradiography, as previously described (29).

cDNA preparation. Total RNA extracted from the reticulocytes of one of the Sardinian subjects with β^0 thalassemia was used as a template for cDNA synthesis. Single-stranded cDNA was synthesized for 3 h in a 200- μ l reaction mixture containing 390 μ g reticulocyte RNA, 5,000 pmol dT₆GC primer, 400 μ M each of deoxyATP (dATP), deoxyguanosine triphosphate (dGTP), and thymidine 5'-triphosphate (TTP), and 100 mM α -[32 P]deoxycytidine triphosphate (dCTP) diluted to sp act 2 Ci/mmol, 228 U avian myeloblastosis virus (AMV) reverse transcriptase, 50 mM Tris-HCl (pH 8.3), 10 mM MgCl₂, 2 mM dithiothreitol, and 25 mM KCl. The reaction mixture was extracted with phenol, and the large molecular weight products were separated from unincorporated nucleotide triphosphates by chromatography on a Sephadex G-50 column in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA, and precipitated in 70% ethanol. RNA was hydrolyzed by dissolving the pellet in 25 μ l of 0.6 N NaOH and by incubating the mixture at 37°C for 3 h. The base was neutralized to pH 7.0 with an equivalent amount of acetic

acid followed by the addition of 2 M Tris-HCl (pH 7.5) to a final concentration of 150 mM Tris-HCl. The sample was precipitated in 70% ethanol. About 1 μ g of single-stranded cDNA was obtained.

Oligo deoxycytidine monophosphate (dCMP) was added to the 3' end of the cDNA (dC tailing) to provide a template for priming the synthesis of the second DNA strand (31). This was done by incubating the single-stranded cDNA with 275 μ M α -[32 P]dCTP (sp act 4 Ci/mmol) and 35 U terminal deoxynucleotidyl transferase (P-L Biochemicals, Inc., Milwaukee, Wis.) in a 35- μ l mixture containing 140 mM potassium cacodylate, 30 mM Tris-HCl (pH 7.5), 1 mM cobalt chloride, and 100 mM dithiothreitol for 18 min at room temperature. An average of 33 dCMP residues was added to the 3' end of the cDNA. The reaction was extracted with phenol and precipitated in ethanol.

To synthesize the complementary strand, the oligo dC-tailed single-stranded cDNA was incubated for 2.5 h at room temperature with 2 nmol dG₁₂₋₁₈, 500 μ M each of dATP, dGTP, and TTP, 100 μ M α -[32 P]dCTP (sp act 10 Ci/mmol), and 54 U AMV reverse transcriptase in 40 μ l of the same buffer used to synthesize the first strand. The mixture was extracted with phenol, fractionated on a Sephadex G-75 column, and the resulting double-stranded cDNA was precipitated in ethanol. A dC tail was added to the 3' end of the double-stranded cDNA by incubation with terminal deoxynucleotidyl transferase and dCTP for 15 min under the tailing conditions given above. An average of 130 dCMP residues was added to each end.

Preparation of the plasmid vector. 5 μ g of the plasmid pBR 322 was linearized by digestion with *Pst* I and dG tails were added by incubating the cleaved plasmid for 3 h at room temperature in the described tailing buffer containing 100 μ M α -[32 P]dGTP (New England Nuclear, Boston, Mass.) diluted to sp act 24 Ci/mmol, 0.1 μ g/ μ l gelatin, and 35 U terminal deoxynucleotidyl transferase, with a second addition of 15 U TDT at 37°C for 15 h. An average of six guanosine 5'-monophosphate residues was added to each 3' end.

Equimolar amounts (0.074 pmol each) of the double-stranded cDNA (dC-tailed) and the pBR 322 (dG-tailed) were annealed to each other by incubating 200 μ g cDNA with 28 μ g pBR 322 in a 200- μ l mixture containing 100 mM NaCl, 1 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA. The reaction mixture was heated to 70°C and allowed to cool slowly to 42°C over ~30 h, and then to room temperature overnight and stored at 4°C.

Transformation and isolation of the β -cDNA clone. *Escherichia coli* of the strain χ 1776 was prepared for transformation according to previously described methods (32, 33). 200 μ l of the above-mentioned annealing mixture was incubated with 400 μ l of calcium-treated χ 1776 at 4°C for 1 h. 600 μ l L broth was added, and the entire mixture was heated for 3 min at 42°C and incubated at 37°C for 30 min. The mixture was grown at 37°C on L broth agar plates supplemented with diaminopimelic acid (100 μ g/ml), thymidine (840 μ g/ml), and tetracycline (15 μ g/ml). Colonies appeared over the next 2 d.

Colonies were screened by *in situ* filter hybridization with a nick-translated β -globin cDNA probe excised from the β cDNA-containing recombinant plasmid pNC 1-7¹. Filters were hybridized and washed under conditions similar to those previously reported (34), with the final wash at 55°C for 2 h to decrease cross hybridization of the β -globin probe with γ - and δ -globin sequences.

Colonies that hybridized strongly with the β -cDNA probe

¹ Liebhauer, S. A. Unpublished observation.

were grown in 25 ml culture and plasmid was prepared from a clarified lysate by banding on a CsCl gradient. 1 μ g of each plasmid was digested with *Pst* I and the recombinant (pSAR 6) with the largest insert (~600 base pairs) was used to transform the host Hb101, grown in M-1 medium to OD₆₃₀ 0.77, and amplified with 150 μ g/ml chloramphenicol. The plasmid supercoil was isolated from a clarified lysate.

DNA sequencing. The complete sequence of the β -globin cDNA insert was determined by the Maxam and Gilbert method (35), with a modification in the A reaction (31). DNA fragments generated by restriction endonuclease digestion were either labeled at the free 5' ends with γ -[³²P]ATP and polynucleotide kinase after dephosphorylation with bacterial alkaline phosphatase, or at the 3' ends with *E. coli* DNA polymerase I (Klenow Fragment) and the appropriate α -[³²P]deoxynucleotide triphosphate (2–3000 Ci/mm, Amersham Corp.).

RESULTS

β -globin mRNA and β -globin gene quantitation. The proportion of α - to β -globin mRNA in the reticulocytes of 20 Sardinian patients with β^0 thalassemia was measured by cDNA-RNA hybridization using cDNA probes enriched in α - and β -globin sequences. A representative hybridization analysis is shown in Fig. 1. Analysis of the 1/2 Rot value (the concentration of RNA where 50% of the radioactive probe is annealed) demonstrated an average β/α ratio of 0.054 (range 0.043–0.062), which indicates that the concentration of β -globin mRNA is ~5% that of the α -globin mRNA. Solution hybridization of the labeled cDNA to cellular DNA showed that the DNA from these Sardinian patients contained the full complement of β -globin genes. The structure of these β -globin genes was normal by Southern blot analysis; the sizes of β -globin gene-containing fragments generated by cleavage with *Eco* RI, *Bam* HI, *Pst* I, and *Hpa* I were identical to normal controls. (The DNA data are not shown.)

Analysis of the function of the β -globin mRNA. We previously showed that the nonsense mutation respon-

FIGURE 1 Rate of annealing of α - and β -globin cDNA to reticulocyte RNA from a Sardinian β^0 thalassemia patient. $\circ$, ³²P-labeled α -globin cDNA; $\bullet$, ³H-labeled β -globin cDNA.

sible for the nonfunctional β -globin mRNA in a Chinese patient could be suppressed in in vitro translation systems by adding a yeast suppressor tRNA. To analyze the function of the β -globin mRNA in Sardinian β^0 thalassemia, we pooled the mRNA from five subjects and enriched the β -globin mRNA sequences by selective adsorption of reticulocyte poly(A+) mRNA to β -globin cDNA cellulose. The β -globin-enriched mRNA directed both γ - and α -globin chain synthesis. When the yeast serine inserting amber suppressor tRNA was added, a small but definite radioactivity peak in the β -globin chain region was clearly discernible (Fig. 2). We pooled the [³⁵S]methionine-labeled globin in this fraction, digested it with trypsin, and separated the peptides by high voltage electrophoresis. We identified the methionine-containing peptide with β -T5 as previously described (29) (result of peptide analysis is not shown).

In our previously reported Chinese β^0 thalassemia patient, the normal codon for the 17th amino acid AAG (lysine) is changed to the amber termination codon UAG (22). Placing a serine in this position with a serine inserting amber suppressor tRNA produces a more acidic globin chain, which elutes earlier than the β^A chain on chromatography (29). In the Sardinian patient, inserting serine in a UAG codon produced a β -globin chain with the same electrophoretic charge as the normal β^A . This indicates that the β^0 Sardinia mutation affects a codon for a neutral amino acid and is therefore different from the β^0 Chinese mutation.

A single nucleotide mutation affecting codons for the neutral amino acids leucine, serine, glutamine, tyrosine, or tryptophan can produce a UAG codon.

FIGURE 2 Carboxymethyl cellulose chromatography of globin chains synthesized in a cell-free system with mRNA enriched in β -globin sequences. The two experiments performed with (solid lines) and without (interrupted lines) suppressor tRNA are superimposed on each other. The OD (A280) markers are shown above the radioactive peaks. In the inset, the radioactivity scale was expanded 10-fold to show that an authentic β -globin peak appeared over the background radioactivity in response to the addition of the suppressor tRNA. The α -globin peaks, which were unaffected by addition of suppressor tRNA, are not shown.

However, the leucine codon (UUG) and the serine codon (UCG) are not used in the human β -globin mRNA (36). Therefore the β^0 Sardinia mutation probably affects one of the three other amino acids found in eight positions in the coding region (Trp in positions 15 and 17; Tyr in positions 35, 130, and 145; and Gln in positions 39, 127 and 131). The very low level of β mRNA made direct determination of β -globin sequences from the RNA impractical. We therefore cloned cDNA prepared from the reticulocyte mRNA to obtain enough material for sequencing.

Cloning of the cDNA. We primed synthesis of the first strand with dT₆GC and added oligo dC to the 3' end of the cDNA. By using oligo dG to prime the synthesis of the second strand, we avoided creating a hairpin connection between the two strands. The hairpin connection would necessitate S₁ cleavage that could overdigest and shorten the cDNA. The double-stranded cDNA was again dC-tailed and annealed to dG-tailed pBR 322. The recombinant plasmids were used to transform χ 1776.

238 recombinants were screened with nick-translated β -globin cDNA. Three recombinants which hybridized strongly to the β cDNA probe (Fig. 3) were analyzed by restriction endonuclease digestion with the enzyme *Mbo* II, which cleaves γ - and δ -, but not β -globin cDNA sequences. One clone containing an insert ~600 base pairs in length and resistant to digestion by *Mbo* II was grown up in large quantity for further analysis.

This cDNA insert contains the entire 5' noncoding region excluding the cap nucleotide and 69 base pairs of the 3' noncoding region. The entire cDNA insert was sequenced according to the strategy outlined in Fig. 3. Two changes from the published β -globin mRNA sequences (36) were observed and confirmed by sequencing both cDNA strands. First, at the position corresponding to amino acid 53, the alanine was encoded by GCU. This sequence differs from the codon (GCA) reported by Marotta et al. (36), but agrees with

FIGURE 3 Map of the β -globin cDNA clone that was characterized and the sequence strategy used. P, *Pst* I; Hf, *Hinf* I; Ha, *Hha* I; B, *Bam* HI. The solid circles indicate the ends of the fragments that were labeled; the arrows show the direction of sequencing.

FIGURE 4 Autoradiogram of the sequencing gel of the β^0 Sardinia cDNA clone showing the amber mutation at amino acid position 39. The G and A reactions of the opposite strand (G' and A') confirm the unambiguous nature of this mutation.

the result published by Lawn et al. (37), and represents a silent third-base substitution. The second change occurred at amino acid 39 where the codon CAG (glutamine) was changed to UAG, an amber termination codon (Fig. 4). This change accounts for the lack of complete β -globin chain synthesis in Sardinian β^0 thalassemia. The nucleotide substitution agrees with cell-free translation analysis, in that replacement of the neutral amino acid glutamine with serine by the suppressor tRNA results in a globin unchanged in charge from β^A .

DISCUSSION

Thalassemias constitute the major group of hereditary disorders on the island of Sardinia; the gene frequency of α thalassemia is 0.18², and that of β thalassemia is 0.07 (23). Homozygous β thalassemia poses a major health problem in this area; in Cagliari, for example, over 400 patients with homozygous β thalassemia are currently being followed. The molecular types of β thalassemia in Sardinia appear to be quite homogeneous. Hemoglobin electrophoresis and globin chain synthesis studies in southern Sardinia show that

² Kan, Y. W., and A. Cao. Unpublished observation.

virtually all cases are the β^0 type (23). The few patients with β^+ thalassemia either have Sicilian parentage or are from northern Sardinia, which once had Greek genetic influence. Genetic and biochemical studies indicate that β^0 thalassemia in Sardinia most likely arose as a single mutational event (38). Our study shows that this mutation is produced by a single nucleotide change at codon number 39, where CAG is changed to the termination codon UAG. In accordance with previously proposed terminology, this mutant is designated either $\beta^{39\text{CAG}\rightarrow\text{UAG}}$ or $\beta^{39\text{UAG}}$.

This is the second example of a nonsense mutation found in humans. A review of the nucleotide sequence of β -globin mRNA showed a single nucleotide mutation in the coding region could result in termination codons in 29 positions. Hence, one would expect to find a subgroup of β^0 thalassemia that is due to different nonsense mutations.

In both Sardinians and Chinese with nonsense mutations, there is a decreased amount of β -globin mRNA in the reticulocyte. It has been postulated that the lack of ribosomal binding to a large section of the mRNA may expose it to nuclease degradation (22, 29). But we do not know why the β/α globin mRNA ratio is lower in the Sardinian with a termination codon at amino acid position number 39 than in the Chinese with the termination codon at position 19. Other factors, such as the effect of the mutation on secondary mRNA structure, could influence the mRNA level in the reticulocyte. Studies of termination codon mutations at other positions on the β -globin chain may explain the mechanism of mRNA degradation.

Low level mRNA hybridization of the β -globin cDNA probe in β thalassemia has often been ascribed to a cross-reaction with δ -globin mRNA (19). This study further emphasizes that a low mRNA level does not rule out the presence of a β -globin mRNA.

β^0 thalassemia comprises a heterogeneous group of disorders. In addition to nonsense mutations, other possibilities include defective mRNA transcription resulting in the absence of β -globin mRNA synthesis, and abnormal processing causing incomplete degradation of β -globin mRNA in the nucleus. Our study illustrates the efficacy of the suppressor tRNA assay as a means of distinguishing the nonsense mutation from other causes.

ACKNOWLEDGMENTS

We thank Julie Bailey for performing some of the liquid hybridization experiments, Dr. Ray Gesteland and Norma Wells for providing the suppressor tRNA, 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, and Jennifer Campell for editorial comments.

This investigation was supported in part by grants from the National Institutes of Health (AM 16666, HL 24137), the

March of Dimes/Birth Defects Foundation, Assessorato Ingiene e Sanita Regione Sardegna, and Centro Nazionale Ricerche subprogetto finalizzato "Malattie Ereditarie dell'Eritrocita".

REFERENCES

1. Weatherall, D. J., and J. B. Clegg. 1979. Recent developments in the molecular genetics of human hemoglobin. *Cell* 16: 467-479.
2. Forget, B. G. 1979. Molecular genetics of human hemoglobin synthesis. *Ann. Intern. Med.* 91: 605-616.
3. Bank, A., J. G. Mears, and F. Ramirez. 1980. Disorders of human hemoglobin. *Science (Wash. D. C.)* 207: 486-493.
4. Maniatis T., E. F. Fritsch, J. Lauer, and R. M. Lawn. 1980. The molecular genetics of human hemoglobins. *Annu. Rev. Genet.* 14: 145-178.
5. Orkin, S. H., R. Kolodner, A. Michaelson, and R. Husson. 1980. Cloning and direct examination of a structurally abnormal human β^0 thalassemia globin gene. *Proc. Natl. Acad. Sci. U. S. A.* 77: 3558-3562.
6. Flavell, R. A., R. Bernards, J. M. Kooter, E. de Boer, P. F. Little, G. Annison, and R. Williamson. 1979. The structure of the human β -globin gene in β thalassemia. *Nucleic Acids Res.* 6: 2749-2760.
7. Kan, Y. W., J. P. Holland, A. M. Dozy, S. Charache, and H. H. Kazanian. 1975. Deletion of the β -globin structure gene in hereditary persistence of fetal hemoglobin. *Nature (Lond.)* 258: 162-163.
8. Forget, B. G., D. G. Hillman, H. Lazarus, E. F. Barell, E. J. Benz, Jr., C. T. Caskey, T. H. J. Huisman, W. A. Schroeder, and D. Housman. 1976. Absence of messenger RNA and gene DNA for beta globin chains in hereditary persistence of fetal hemoglobin. *Cell* 7: 323-329.
9. Mears, J. G., F. Ramirez, D. Leibowitz, F. Nakamura, A. Bloom, F. Konotey-Ahula, and A. Bank. 1978. Changes in restricted human cellular DNA fragments containing globin gene sequences in thalassemias and related disorders. *Proc. Natl. Acad. Sci. U. S. A.* 75: 1222-1226.
10. Ottolenghi, S., B. Gligioni, P. Comi, A. M. Gianni, E. Polli, C. T. Acquaye, J. H. Oldham, and G. Masera. 1979. Globin gene deletion in HPFH, $\delta^0\beta^0$ thalassemia and Hb Lepore disease. *Nature (Lond.)* 278: 654-657.
11. Fritsch, E. F., R. M. Lawn, and T. Maniatis. 1979. Characterization of deletions which affect the expression of fetal globin genes in man. *Nature (Lond.)* 279: 598-603.
12. Orkin, S. H., B. P. Alter, and C. Altay. 1979. Deletion of the γ -globin gene in $\gamma\delta\beta$ thalassemia. *J. Clin. Invest.* 64: 866-869.
13. van der Ploeg, L. H. T., A. Konings, M. Oort, D. Roos, L. Bernini, and R. A. Flavell. 1980. $\gamma\beta$ thalassemia studies showing that deletion of the γ and δ genes influences β -globin gene expression in man. *Nature (Lond.)* 283: 637-642.
14. Orkin, S. H., S. C. Goff, and D. G. Nathan. 1981. Heterogeneity of DNA deletion in $\gamma\delta\beta$ -thalassemia. *J. Clin. Invest.* 67: 878-884.
15. Weatherall, D. J. and J. B. Clegg. 1972. *The Thalassemia Syndromes*. Blackwell Scientific Publications, Oxford. 2nd ed.
16. Bunn, H. F., B. G. Forget, and H. M. Ranney. 1977. *Human Hemoglobins*. W. B. Saunders Co., Philadelphia.
17. Kantor J. A., P. H. Turner, and A. W. Nienhuis. 1980. Beta⁺ thalassemia: mutations which affect processing of the β -globin mRNA precursor. *Cell* 21: 149-157.

18. Maquat, L. E., A. J. Kinniburgh, L. R. Beach, G. R. Honig, J. Lazerson, W. B. Ershler, and J. Ross. 1980. Processing of human β -globin mRNA precursor to mRNA is defective in three patients with β^+ -thalassemia. *Proc. Natl. Acad. Sci. U. S. A.* **77**: 4287-4291.
19. Benz, E. J., Jr., B. J. Forget, D. G. Hillman, M. Cohen-Solal, J. Pritchard, and C. Cavalleco. 1978. Variability in the amount of β -globin mRNA in β^0 thalassemia. *Cell* **14**: 299-312.
20. Kan, Y. W., J. P. Holland, A. M. Dozy, and H. E. Varmus. 1975. Demonstration of non-functional β -globin mRNA in homozygous β^0 thalassemia. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 5140-5144.
21. Temple, G. F., J. C. Chang, and Y. W. Kan. 1977. Authentic β -globin mRNA sequences in homozygous β^0 thalassemia. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 3047-3051.
22. Chang, J. C., and Y. W. Kan. 1979. β^0 thalassemia: a nonsense mutation in man. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 2886-2889.
23. Cao, A., M. Furbetta, A. Ximenes, A. Angius, C. Rosatelli, T. Tuveri, M. T. Scalas, A. M. Falchi, L. Maccioni, M. A. Melis, and R. Gallanello. 1981. β thalassemia types in southern Sardinia. *J. Med. Genet.* **18**: 196-198.
24. Kan, Y. W., A. M. Dozy, H. E. Varmus, J. M. Taylor, J. P. Holland, L. E. Lie-Injo, J. Ganesan, and D. Todd. 1975. Deletion of α -globin genes in haemoglobin H disease demonstrates multiple α -globin structural loci. *Nature (Lond.)* **255**: 255-256.
25. Goossens, M. and Y. W. Kan. DNA analysis in the diagnosis of hemoglobin disorders. 1981. *Methods Enzymol.* In press.
26. 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. *Nucleic Acids Res.* **5**: 563-581.
27. Noyes, B. E., and G. R. Stark. 1975. Nucleic acid hybridization using DNA covalently coupled to cellulose. *Cell* **5**: 301-310.
28. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* **67**: 247-256.
29. Chang, J. C., G. F. Temple, R. F. Trecartin, and Y. W. Kan. 1979. Suppression of the nonsense mutation in homozygous β^0 thalassemia. *Nature (Lond.)* **281**: 602-603.
30. Kan, Y. W., E. Schwartz, and D. G. Nathan. 1968. Globin chain synthesis in the α thalassemia syndromes. *J. Clin. Invest.* **47**: 2515-2522.
31. Cooke, N. W., D. Coit, R. I. Weiner, J. D. Baxter, and J. Martial. 1980. Structure of cloned DNA complementary to rat prolactin messenger RNA. *J. Biol. Chem.* **255**: 6502-6510.
32. Dagert, M., and S. D. Ehrlick. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**: 23-28.
33. Norgard, M. V., K. Keem, and J. J. Monahan. 1978. Factors affecting the transformation of *Escherichia coli* strain χ 1776 by pBR 332 plasmid DNA. *Gene (Amst.)* **3**: 279-292.
34. Kan, Y. W., and A. M. Dozy. 1978. Polymorphism of DNA sequence adjacent to the human β -globin structural gene: relationship to sickle mutation. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 5631-5635.
35. Maxam, A. M., and W. Gilbert. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 560-564.
36. Marotta, C. A., J. T. Wilson, B. G. Forget, and S. M. Weissman. 1977. Human β -globin messenger RNA. III. Nucleotide sequences derived from complementary DNA. *J. Biol. Chem.* **252**: 5040-5043.
37. Lawn, R. M., A. Efstratiadis, C. O'Connell, and T. Maniatis. 1980. The nucleotide sequence of the human β -globin gene. *Cell* **21**: 647-651.
38. Kan, Y. W., K. Y. Lee, M. Furbetta, A. Angius, and A. Cao. 1980. Polymorphism in DNA sequences in the β -globin region. Application to prenatal diagnosis of β^0 thalassemia in Sardinia. *N. Engl. J. Med.* **302**: 185-188.