Two cloned β thalassemia genes are associated with amber mutations at codon 39

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Received 1 September 1981

ABSTRACT

Two β globin genes from patients with the β^+ thalassemia phenotype have been cloned and sequenced. A single nucleotide change from CAG to TAG (an amber mutation) at codon 39 is the only difference from normal in both genes analyzed. The results are consistent with the assumption that both patients are doubly heterozygous for β^+ and β^0 thalassemia, and that we have isolated and analyzed the β^0 thalassemia gene.

INTRODUCTION

The thalassemias are a group of inherited disorders of man characterized by decreased or absent synthesis of α or β chains of adult human hemoglobin A ($\alpha_2\beta_2$) (1, 2). There are two general types of β thalassemia: β^0 thalassemia, in which no β globin is synthesized, and β^+ thalassemia, in which structurally normal β globin is produced, but in markedly reduced amounts. In the homozygous state, both β^+ and β^0 thalassemia produce severe anemia. Deletions of DNA in the β globin gene complex have been implicated in rare cases of β^0 thalassemia (3, 4). However, most cases of both β^+ and β^0 thalassemia are not associated with any detectable deletions or rearrangements of DNA, since extensive restriction enzyme analysis of the DNA of these subjects has revealed no changes from normal (4-6).

In β^0 thalassemia, the presence of nonsense codons involving codons 17 and 39 in β globin mRNA presumably leading to premature termination of globin chains has previously been determined by analysis of β globin cDNA (7-9). Another class of β^0 thalassemia mutants is associated with a defect in the large intervening

sequence (IVS 2) of the β globin gene. The β globin gene contains two IVS: one small (IVS 1, 130 bases long) between codons 30 and 31, and one large (IVS 2, 850 bases long) between codons 104 and 105 (10, 11). Some β^0 thalassemia alleles have been shown to be associated with a base change at the 5' end of IVS 2 identified by restriction endonuclease mapping (12), and by direct nucleotide sequencing (13). Presumably, the nucleotide change prevents normal removal of the IVS and splicing of the β^0 thalassemic nuclear mRNA precursor. In β^+ thalassemia DNA, the only nucleotide defect reported to date is a single base change in IVS 1 which results in a nucleotide sequence very similar to that of the normal 3' IVS 1 splice junction (14, 15). This change is associated with incorrect splicing, and results in decreased normal β globin mRNA processing and decreased β globin mRNA.

In this study, we report nucleotide sequence analysis of two β thalassemia genes isolated from chromosomal DNA of two patients with β^+ thalassemia phenotypes. One of these patients is of mixed Scottish-Italian extraction and the other is Moroccan. In both cases, a single base change resulting in the same nonsense mutation at codon 39 is the only nucleotide change identified. The results suggest that both patients are probably compound heterozygotes for β^0/β^+ thalassemia, and we have isolated and sequenced the β^0 thalassemia alleles.

MATERIALS AND METHODS

Molecular cloning:

Total DNA was obtained from a Moroccan abortus, and from the spleen of a 19 year old male of mixed Scottish and Italian ancestry who had been subjected to splenectomy for therapeutic reasons (16, 17). Both the abortus and the 19 year old were clinically homozygotes for β thalassemia, and synthesized some β globin chains (β^+ thalassemia phenotype). DNA from the Moroccan abortus was digested to completion with Eco RI, which cleaves the β globin structural gene at codon 122, and the 5.2 kilobase (kb) 5' β globin gene fragment was isolated by electrophoresis. This 5.2 kb fragment was initially cloned in λ gt wes, and was subsequently subcloned in pBR 322.

A β globin gene from the Scottish-Italian patient (β 12) was isolated from a genomic library in λ charon 4A as previously described (18). Various fragments of the β globin locus were subcloned in plasmid pBR 322, and filamentous bacteriophage M13mp7 (19). The various subclones of β^+ 5.2 and β 12 were used as sources of DNA for restriction endonuclease mapping and DNA sequence analyses. All cloning and recombinant DNA propagation procedures were in accordance with the NIH Guidelines for Recombinant DNA Research that were in effect at the time.

Restriction endonuclease cleavage and nucleotide sequence analyses:

DNA was cleaved and fragments were isolated by standard methods. Restriction fragments were labelled with P^{32} at their 5' ends with alkaline phosphatase followed by T4 polynucleotide kinase and γ P^{32} ATP or their 3' ends with the large fragment of DNA polymerase and the appropriate α P^{32} dNTP. These fragments were then strand-separated or secondarily cleaved with a restriction endonuclease and separated on 4-8% polyacrylamide gels. Uniquely end-labelled fragments were eluted from the gels and sequenced by modifications of the method of Maxam and Gilbert (20). Portions of β 12 were sequenced by the dideoxy-terminator technique of Sanger and Coulson (21), using single-stranded DNA templates obtained by cloning into M13mp7 (19, 22). Sequencing reaction products were fractionated by electrophoresis in thin polyacrylamide-urea gels (21), and autoradiographed with intensifying screens at -70°C . All enzymes were obtained from Bethesda Research Laboratories, Incorporated, New England Biolabs, and Boehringer Mannheim. Deoxy- and dideoxy-nucleotide-triphosphates were obtained from PL Biochemicals.

RESULTS

Restriction endonuclease mapping of the cloned β globin gene fragments:

Restriction endonuclease mapping of β^+ 5.2 and β 12 was performed with a number of frequently and infrequently cutting enzymes. In each case, the digestion patterns of the thalassemic β genes were normal (data not shown), indicating

that there have been no detectable rearrangements, deletions, or other changes, involving the thalassemic genes.

Nucleotide sequence of the thalassemic β globin genes:

The nucleotide sequence of the β globin allele from the Moroccan abortus (β^+ 5.2) was determined from a point within codon 27, 5' to IVS 1, to 82 nucleotides into IVS 2. The nucleotide sequence of the β globin gene from the Scottish-Italian patient (β 12) was determined from 50 bases 5' to the cap site of β globin mRNA to 30 nucleotides 5' to the poly (A) addition site. Comparison of the sequences determined with the known sequence of a normal β globin gene (11) revealed only a single nucleotide change in each case. At codon 39, at which site the glutamine codon CAG is normally present, a C→T transition mutation causes an amber (TAG) termination signal (Figure 1). The remainder of the nucleotide sequences determined are identical to those of a normal human β globin gene (11) (Figure 2).

DISCUSSION:

In this study, we report the nucleotide sequence analysis of β globin genes from two individuals with diverse ethnic backgrounds. Each individual had the β^+ thalassemia phenotype, and one of the two β globin genes in each has a mutation at codon 39, at which site the CAG codon (glutamine), which is normally present, has been mutated to TAG (amber) (Figure 1). Furthermore, the single base change in IVS 1, found in two other β^+ thalassemia genes (14, 15), is absent from both of these genes (Figure 2). No other nucleotide changes have been detected, and all splice junctions are identical to those in a normal β globin gene junction (11).

There are several sites in the β globin gene at which a single base change would result in the generation of a termination codon in the mRNA. A previously reported β^0 thalassemia patient with detectable mRNA was shown to have an A→T mutation at codon 17, changing the normal lysine codon to read amber (8). This mutation completely eliminates β globin production. The same mutation described here in genomic DNA has also been seen in an analysis of cDNA from phenotypic β^0 thalassemia patients of Sardinian extraction (9). Thus, the codon 39 nucleotide

Figure 1:

An amber mutation at codon 39:

A Sau 3A fragment of the β globin gene from the Moroccan patient was 3' end-labelled, recut with Hgi AI, and sequenced by the Maxam-Gilbert method. This autoradiogram of the coding DNA strand shows the same defect at codon 39 seen in the Scottish-Italian.

change to TAG is associated with a β^0 thalassemia phenotype in at least the Sardinian population. It, therefore, appears most likely that these two individuals with phenotypic β^+ thalassemia with apparently different ethnic origins (Scottish-Italian and Moroccan), are doubly heterozygous for β^+ and β^0 thalassemia genes, and that we have sequenced the β^0 thalassemia genes.

The β^+ thalassemia genes remain to be isolated and characterized. However, it remains formally possible that expression of a β^+ thalassemia phenotype could result from the presence of amber suppressor tRNAs in individuals, such as those described here, carrying the $\beta 39$ CAG→TAG allele. A tyrosine

Figure 2:
Sequence analysis of IVS 1. This figure shows a Maxam-Gilbert gel autoradiogram of a 5' end labelled Ava II/Hae III fragment from the Scottish-Italian patient, containing the IVS 1. The region around the reported β^+ thalassemia defect (14, 15) is indicated. This sequence (coding DNA strand) is identical to that found in a normal β globin gene (11).

amber suppressor, for example, could insert a tyrosine instead of the normal glutamine at codon 39, with no change in the charge of the resulting protein. We are currently looking for such a suppressor tRNA in the Scottish-Italian patient. The remaining β genes from these patients are, of course, of great interest, and are currently being sought from genomic libraries. These other genes are obligate β^+ thalassemia genes if the genes we have sequenced are β^0 thalassemia genes.

The homozygous β^0 thalassemia patients from Sardinia with the codon 39 defect have been shown to have markedly decreased levels of β globin mRNA (9). This could be due to decreased

transcription, processing, stability, or transport of nuclear β globin mRNA precursors or decreased stability of cytoplasmic β globin mRNA. However, transcription of both of the alleles described here has been previously shown to be normal *in vitro* (23, 24). Abnormal accumulation of nuclear β globin mRNA precursors has been demonstrated in some β^+ thalassemia patients, suggesting abnormalities of RNA processing in these patients (25-27). It remains to be determined whether the decreased β mRNA in the codon 39 patients described here is due to abnormal β globin mRNA metabolism in the nucleus, or to decreased stability of cytoplasmic β globin mRNA.

NOTE ADDED IN PROOF

Moschonas, N., deBoer, E., Grosveld, F. G., Dahl, H. H., Wright, S., Shewmaker, C., and Flavell, R. A., have independently obtained similar results (Nucl. Acids Res. 9, 4391-4401, 1981).

ACKNOWLEDGMENTS

We would like to thank Ms. Maryann Donovan-Peluso, Ms. Helen Schreiner, and Ms. Mary Beth McCormick, for their technical assistance. This work was made possible by NIH grants AM-25274 and AM-16666; National Science Foundation grant 24120; Cooley's Anemia Foundation grant 312-2581; March of Dimes Birth Defects Foundation grant NF-1-664; and New York Community Trust grant 6-40664. Y. W. K. is an Investigator of the Howard Hughes Medical Institute.

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REFERENCES

1. Bank, A., Mears, J. G., and Ramirez, F. (1980) Science 207, 486-493.
2. Maniatis, T., Fritsch, E. F., Lauer, J., and Lawn, R. M. (1980) Ann. Rev. Genet. 14, 145-178.
3. Orkin, S. H., Kolodner, R., Michaelson, A., and Husson, R. (1980) Proc. Natl. Acad. Sci. 77, 3558-3562.
4. Flavell, R. A., Bernards, R., Kooter, J. M., and deBoer, R. (1979) Nucl Acids Res. 6, 2749-2760.
5. Mears, J. G., Ramirez, F., Leibowitz, D., and Bank, A. (1978) Cell 15, 15-23).

6. Bank, A., Mears, J. G., Ramirez, F., Burns, A. L., and Feldenzer, J. (1979) in *Eucaryotic Gene Regulation*, Axel, R., Maniatis, T., and Fox, F. C., Eds., pp. 355-366 Academic Press, New York.
7. Temple, G. F., Chang, T. C., and Kan, Y. W. (1977) *Proc. Natl. Acad. Sci.* 74, 3047-3051.
8. Chang, T. C. and Kan, Y. W. (1979) *Proc. Natl. Acad. Sci.*, 76, 2886-2889.
9. Trecartin, R. F., Liebhaber, S. A., Chang, J. C., Lee, K. Y., Angus, A., Furbetta, M., and Cao, A. (in press) *J. Clin. Invest.*
10. Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G., and Maniatis, T. (1978) *Cell* 15, 1157-1174.
11. Lawn, R. M., Efstratiadis, A., O'Connell, C., and Maniatis, T. (1980) *Cell* 21, 647-651.
12. Baird, M., Driscoll, M. C., Schreiner, H., Sciaratta, G. V., Sansone, G., Niazi, G., Ramirez, F., and Bank, A. (1981) *Proc. Natl. Acad. Sci.*, 78, 4218-4221.
13. Shander, M., VandeWoude, S., Proudfoot, N., and Maniatis, T. (1981) *J. Supramol. Structure Cel. Biochem. supplement* 5, p. 229.
14. Spritz, R. A., Jagadeeswaran, P., Choudary, P. V., Biro, P. A., Elder, J. T., DeRiel, J. K., Manley, J. L., Gefter, M. L., Forget, B. G., and Weissman, S. M. (1981) *Proc. Natl. Acad. Sci.*, 78, 2455-2459.
15. Westaway, D. and Williamson, R. (1981) *Nucl. Acids Res.* 9, 1777-1788.
16. Blin, N. and Stafford, D. W. (1976) *Nucl. Acids Res.* 3, 2303-2308.
17. Polsky, F., Edgell, M. H., Sardinian, J. G., and Leder, P. (1978) *Anal. Biochem.*, 87, 397-410.
18. Burns, A. L., Spence, S., Kosche, K., Ramirez, F., Mears, J. G., Schreiner, H., Miller, C., Baird, M., Leibowitz, D., Giardina, P., Markenson, A., and Bank, A. (1981) *Blood* 57, 140-146.
19. Schreier, P. H. and Cortese, R. (1979) *J. Mol. Biol.*, 129, 169-172.
20. Maxam, A. M. and Gilbert, W. (1977) *Proc. Natl. Acad. Sci.* 74, 560-564.
21. Sanger, F. and Coulson, A. R. (1978) *FEBS Letter* 82, 314-317.
22. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., and Roe, B. A. (1980) *J. Mol. Biol.* 143, 161-178.
23. Parker, C. S., Goossens, M., and Kan, Y. W. (1980) *Clin. Res.*, 28, 488A (Abstract).
24. Proudfoot, N. J., Shander, M. H. M., Manley, J. L., Gefter, M. L., and Maniatis, T. (1980) *Science* 209, 1329-1336.
25. Maquat, L. E., Kinniburgh, A., Beach, L. R., Honig, G. R., Lazerson, J., Ershler, W. B., and Ross, J. (1980) *Proc. Natl. Acad. Sci.* 77, 4287-4291.
26. Kantor, J. A., Turner, P. H., and Nienhuis, A. W. (1980) *Cell* 21, 149-157.
27. Benz, E. J., Scarpa, A. L., deRiel, J. K., Tonkonow, B. L., Ritchey, A. K., and Forget, B. G. (1980) in *Organization and Expression of Globin Genes*, Stamatoyannopoulos, G. and Nienhuis, A. W., Eds., pp. 241-250, Alan R. Liss, New York.