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Molecular characterization of seven β-thalassemia mutations in Asian Indians

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To characterize systematically the mutations which produce β-thalassemia in Asian Indians, we first determined the DNA polymorphism haplotype in the β-globin gene cluster of 44 β-thalassemia chromosomes in the ethnic group. Nine different haplotypes were observed. Upon molecular cloning and partial DNA sequencing of one β-gene from each of eight haplotypes and two from the ninth, seven different mutations were found. None of these have been identified in Mediterranean patients, even among the five haplotypes which appeared identical in the two groups. Asian Indian mutations included one nonsense and three frameshift mutations, one deletion affecting an acceptor splice site, and two mutations affecting a donor splice site. The correlation of a specific mutation with a specific haplotype was high but not invariant. Two mutations were associated with more than one haplotype but, in each instance, the mutation spread to a new haplotype could be explained most simply by recombination 5′ to the β-globin gene. In addition, four mutations, one reported here and three others previously reported, have been observed on two chromosome backgrounds that are identical except for the status of a polymorphic *Hinf*I site 5′ to the β gene. This *Hinf*I site does not show significant linkage disequilibrium with markers both 5′ and 3′ to it, suggesting that it lies within a region of relative sequence randomization.
Key words: frameshift mutation/haplotype analysis/nonsense mutation/population genetics/splice junction mutation

Introduction

β-Thalassemia is a heterogeneous inherited disorder of β-globin synthesis characterized typically by transfusion-dependent anemia (Weatherall and Clegg, 1981). In thalassemic individuals various mutations have been defined that affect transcription, translation or RNA processing (Spritz and Forget, 1983; Orkin *et al.*, 1983a). The continued characterization of these mutations offers the opportunity of identifying nucleotide sequences critical for normal gene expression, discovering the extent of genetic heterogeneity of a single gene disorder and exploring general aspects of linkage disequilibrium and genomic recombination.

We have previously detected a strong association between DNA polymorphism haplotypes in the β-globin gene cluster and specific β-thalassemia mutations in Mediterraneans (Antonarakis *et al.*, 1982a; Orkin *et al.*, 1982). In that ethnic group we initially discovered eight different mutations within nine haplotypes. In Black American and Chinese individuals other mutations, apparently distinctive for these ethnic groups, have also been observed (Goldsmith *et al.*, 1983; Chang and Kan, 1979; Antonarakis *et al.*, 1984b; Cheng *et al.*, 1984).

To characterize further the molecular defects associated with β-thalassemia in Asian Indians, we have employed the systematic strategy originally developed in our study of the Mediterranean population (Orkin *et al.*, 1982). DNA polymorphism analysis defined nine chromosome haplotypes associated with β-thalassemia genes. At least one β-globin gene of each haplotype was then cloned and partially sequenced. This approach identified seven mutations in Asian Indians among the 11 genes subjected to sequence analysis. None of these mutations is found among Mediterraneans. These studies strengthen our suggestion that each population group has its own battery of β-thalassemia alleles. Our data strongly supports the association of haplotypes and mutations, and also points to the existence of unexplained sequence randomization 5′ to the β-gene.

Results

Haplotype analysis of β^A and β^{Thal} chromosomes of Asian Indians

Haplotype analysis at nine polymorphic restriction sites in the β-globin gene cluster was performed on 33 β^A and 44 β-thalassemia chromosomes (Table I). β^A globin genes were found associated with 10 haplotypes and β-thalassemia genes with nine. Interestingly the most common β^A haplotype (K) was not represented among the β^{thal} chromosomes. In addition, four other uncommon β^A haplotypes were not found among the β^{thal} chromosomes, while four rare β^{thal} haplotypes were missing from β^A haplotypes. Conversely, 64% of β^{thal} alleles representing two common mutations (see below) were found on a haplotype that was under-represented among β^A chromosomes (18% of the total). One β^{thal} haplotype (E) was differentiated solely on the basis of a rare *Pst*I polymorphism which resulted from a silent nucleotide substitution (C→A) in codon 10 of the β-globin gene.

Five haplotypes, designated B, C, D, F and G in Table I were represented among β-thalassemia chromosomes of Mediterraneans (haplotypes II, III, Va, VII and IX, respectively, of Figure 1, Orkin *et al.*, 1982). As shown below, four different β-thalassemia alleles were found among these five haplotypes in Asian Indians (Table II), and these alleles were not previously found among β-thalassemia genes in Mediterraneans.

β-Thalassemia genes in Asian Indians and their haplotype associations

Upon characterization of the 44 β-thalassemia chromosomes, one β-gene of each haplotype was selected for cloning and DNA sequence analysis. After the first gene chosen from haplotype G was sequenced and later shown by restriction

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analysis with *Fnu*4H to represent a mutant allele unique to the entire panel, a second β-thalassemia gene from this haplotype was analyzed. In all, 11 β-thalassemia genes were examined and seven new mutations were identified (Table II).

The most common mutation, a G→C substitution at nucleotide 5 of the donor splice site consensus sequence of IVS-1, was found as the exclusive non-deletion mutation of haplotype F (see below), but also within the rare haplotypes H and I. The effect of this point mutation on RNA processing has already been described (Treisman *et al.*, 1983). A 4-bp deletion (TCTT) in codons 41 and 42 was found associated with two rare haplotypes, C and D. As is the case with several other small deletions (Efstratiadis *et al.*,1980; Kazazian *et al.*, 1983b), this tetranucleotide deletion occurs in a region of short direct repeats within the β-gene sequence. In fact, the loss of TCTT could occur in any one of three ways. Other mutations observed are: (i) a 25-bp deletion that includes the IVS-1 acceptor site and can be detected directly by restriction analysis with the endonuclease *Fnu*4H (Orkin *et al.*, 1983c); (ii) a G→T substitution at IVS-1 position 1 that affects RNA processing adversely in a manner identical to that of a G→A substitution at the same position (Treisman *et al.*, 1983; S.H. Orkin, unpublished data); (iii) a nonsense mutation (G→A) in codon 15; (iv) a single nucleotide deletion (−C) in codon 16; and (v) a single nucleotide insertion (+G) between codons 8 and 9. In addition, we found that the partially deleted β-gene of some Indian β°-thalassemics (Orkin *et al.*, 1979; Spritz and Orkin, 1982) is uniquely associated with haplotype F (see Table I).

We have previously found that normal β-globin genes have DNA polymorphisms that define three common sequences, designated frameworks, in various ethnic groups (Orkin *et al.*, 1982; Antonarakis *et al.*, 1982b). Haplotypes F, H and I which are associated with the IVS-1 position 5 mutation contain the same β-gene framework, designated 3 Asian (Antonarakis *et al.*, 1982b) (Table II). Likewise, haplotypes C and D which are associated with the deletion in codons 41 and 42 both contain framework 2 β-genes. Although an independent origin of mutations in different haplotypes is possible, these findings are more compatible with recombination 5′ to the β-globin gene. In this manner, the mutant β-globin gene and the polymorphisms in the 3′ portion of the cluster are reassociated with different 5′ haplotypes. Such recombination is likely to occur within a 9-kb region just 5′ to the β-gene (Antonarakis *et al.*, 1982a; H.H. Kazazian, unpublished results).

The distribution of the IVS-1 position 5 mutation within the panel of Indian β-thalassemic samples was assessed directly by oligonucleotide hybridization. The 19-mer probe corresponded to the mutant sequence (see Materials and methods). All 13 available non-deletion β-thalassemia DNAs containing a haplotype F chromosome hybridized with the probe. Thus, it appears that haplotype F is exclusively associated with either the IVS-1 position 5 mutation or the 3′ deletion gene. Of the 44 β-thalassemia chromosomes available to us for analysis in the Asian Indian group, the specific mutation could be definitively assigned in 36 (82%) (see Table II).

Sequence randomization neighboring the HinfI site 5′ to the β-gene

As noted above, the IVS-1 position 5 mutation was associated with three different haplotypes, F, H and I. These are identical from the 5′ end of the β-gene extending 18 kb in the 3′ direction, but differ in the portion of the cluster 5′ to the β-gene. In addition, we have observed the IVS-1 position 5

Table I. Haplotypes of β^A and β^{thal} chromosomes in Asian Indians

5′ — ε — Gγ Aγ — ψβ₁ — δ — β — 3′

Haplotype	1 (ε)	2 (Gγ)	3 (Aγ)	4 (ψβ₁)	5 (ψβ₁)	δ	6 (β)	7 (β)	8	9	No. with haplotype β^A	β^{thal}
A	+	−	−	−	−		+	+	−	+	−	3
B	−	+	+	−	+		+	+	+	+	1	1
C	−	+	−	+	+		+	+	+	−	−	1
D	+	−	−	−	−		+	+	+	−	6	2
E	+	−	−	−	−	*Pst*I	+	+	+	−	−	3
F	+	−	−	−	−		−	−	−	+	6	28
G	−	+	−	+	+		+	+	−	+	1	4
H	−	+	+	−	+		−	−	−	+	1	1
I	−	+	−	−	+		−	−	−	+	−	1
J	−	+	−	+	+		−	−	−	+	4	−
K	+	−	−	−	−		+	+	+	+	10	−
L	−	+	−	+	+		+	+	+	+	2	−
M	−	+	+	−	+		+	+	−	+	1	−
N	+	+	+	−	+		+	+	+	+	1	−
										Totals	33	44

Polymorphic sites, designated 1 to 9, were identified by the following restriction endonucleases: 1,4,5-*Hinc*II; 2,3,8-*Hind*III; 6-*Hgi*A I; 7-*Ava*II and 9-*BamH*I. (+) indicates the presence of cleavage and (−) indicates absence of cleavage at a site.

Table II. β-thalassemia alleles in Asian Indians

Haplotype (Mediterranean haplotype)	β-gene framework	No. with haplotype	Mutation	No. with mutation documented
A	1	3	Frameshift β^{8-9} (+G)	1
B (II)	1	1	Nonsense codon 15 (TGG→TAG)	1
C (III)	2	1	Frameshift β^{41-42} (−TCTT)	1
D (Va)	2	2	Frameshift β^{41-42} (−TCTT)	2
E	2	3	Frameshift β^{16} (−C)	1
F (VII)	3 Asian	28	IVS-1 nt 5 (G→C)	13
			619-bp deletion	13
G (IX)	1	4	25 nt deletion, 3′ end of IVS-1	1
			IVS-1 nt 1 (G→T)	1
H	3 Asian	1	IVS-1 nt 5 (G→C)	1
I	3 Asian	1	IVS-1 nt 5 (G→C)	1
		44		36 (82%)

mutation in a haplotype F chromosome, which differed from other haplotype F chromosomes only by loss of a polymorphic *Hinf*I site 990 bp 5′ to the β-gene. Moreover, the IVS-1 position 5 mutant gene of haplotype H was also associated with the absence of this *Hinf*I site. Likewise, the frameshift mutation of codons 41 and 42 was seen on two haplotypes, C and D, which differed not only in other 5′ polymorphic sites of the cluster, but also at this *Hinf*I site. This *Hinf*I site is furthermore variably associated with thalassemic genes of other ethnic groups. In Mediterraneans we have observed an IVS-2 position 1 mutation on an unusual haplotype III chromosome containing this *Hinf*I site, whereas the seven other haplotype III chromosomes with this mutation lacked the site. Moschonas *et al.* (1982) have previously reported a β-thalassemia gene in Mediterraneans containing a nonsense mutation in codon 39 which lacked this site, while in our panel all of 43 Mediterranean β-thalassemia genes of this type contained the site. In Blacks the β^S mutation has been observed in one of 108 common β^S-bearing chromosomes [see haplotype m of Table I, Antonarakis *et al.* (1984a)], which contained this *Hinf*I site, while the remaining 107 chromosomes of this type (haplotype A) lacked it (Antonarakis *et al.*, 1984a). Thus, these unusual findings have led us to exclude this polymorphic site from our haplotype designations.

In an effort to investigate the DNA region neighboring this *Hinf*I site, we have examined two other sequence polymorphisms 5′ to the β-gene in a variety of alleles (Table III). These sequence polymorphisms are the ATTTT repeat polymorphism located 1400 bp upstream from the β-gene (Spritz, 1981) and the single site polymorphism (T or C) at position −341 (Moschonas *et al.*, 1982). The *Hinf*I polymorphism lies between these two sequence markers and thereby permits assessment of linkage disequilibrium or gene conversion in this segment of DNA 5′ to the β-gene. We examined three different IVS-1 position 5 clones and compared them (Table III) to both the sequence 5′ to the normal β-gene reported by Moschonas *et al.* (1982) and the sequence of two β-thalassemia genes containing a nonsense mutation in codon 39. While the three β-genes containing IVS-1 position 5 mutations are all of the framework 3 Asian type, the normal β-gene and the two genes containing the nonsense codon 39 are each of the framework 1 type.

Our data demonstrate that the status of the *Hinf*I site is not strictly associated with either the number of ATTTT repeats or the polymorphism at −341. When the 5′-flanking regions neighboring the three framework 1 β-genes were compared, a different pattern at the *Hinf*I site and −341 polymorphisms was observed for each. Data on the −341 polymorphism are particularly instructive and strongly suggest that the segment of DNA neighboring the *Hinf*I site at −990 bp represents a region of relative sequence randomization. This region is not strongly associated with either the 5′ or 3′ portions of the β-gene cluster (Kazazian *et al.*, 1983a; H.H. Kazazian and A. Chakravarti, unpublished data).

Table III. Sequence in 5′-flanking regions of β-globin gene

β-Globin gene	No. of ATTTT repeats (−1400 bp)	*Hinf*I site (−990 bp)	C-T polymorphism (−341 bp)
Normal[a]	5	+	C
Nonsense codon 39[a]	5	−	T
Nonsense codon 39	5	+	T
IVS-1 position 5 (Haplotype F)	5	+	C
IVS-1 position 5 (Haplotype F)	5	−	C
IVS-1 position 5 (Haplotype H)	6	−	C

[a]The sequence of the 5′-flanking regions of these β-globin genes was determined by Moschonas *et al.* (1982).

Discussion

In a systematic search we have identified seven new mutations producing β-thalassemia among Asian Indians by haplotype analysis of β-thalassemia chromosomes followed by cloning and sequence analysis of genes associated with different haplotypes. Using this strategy, we have limited our cloning and DNA sequencing to 11 β-thalassemia genes of Asian Indians to obtain seven new mutations. Including our results with Mediterraneans, Chinese and American Blacks (Orkin *et al.*, 1982; Cheng *et al.*, 1984; Antonarakis *et al.*, 1984b), this approach has uncovered 22 new mutations among 28 thalassemia genes directly analysed.

We can make several generalizations. First, specific β-thalassemia mutations are strongly associated with specific haplotypes within an ethnic group, and this is useful in the search for new alleles. While exceptions to this linkage are commonplace, in general, one, or perhaps two, of many possible mutations have been found associated with any given haplotype. While a particular mutation is usually associated strongly with one haplotype, it may be found rarely with one or two others. An example of the former exception is haplotype G in Asian Indians which is associated both with the 25-bp deletion of the 3′ end of IVS-1 and a point mutation at IVS-1 position 1. An example of the latter exception is the IVS-1 position 5 mutation, which is found in three different haplotypes, all of which contain a framework 3 Asian β-gene.

Second, each ethnic group carries its own set of mutations. An exception to this rule is the presence of the IVS-1 position 5 mutation of Asian Indians in a Chinese patient (Cheng *et al.*, 1984). This individual, however, had a haplotype and β-gene framework different from that found in Indian haplotypes F, H and I, suggesting that the Chinese mutation represents a second independent origin of the IVS-1 position 5 substitution.

The four-nucleotide deletion (TCTT) in codons 41 and 42 of the β-gene has been recently reported in a Chinese individual (Kimura *et al.*, 1983). Since (i) haplotype analysis of the pertinent β-thalassemia chromosome was not included in that report and (ii) the TCTT deletion can arise in any one of three ways, we cannot assess whether this allele has spread from one population group to the other or has arisen independently in the two groups.

Our findings have identified the unexpected behavior of a DNA segment neighboring the polymorphic *Hinf*I restriction site 990 bp 5′ to the β-gene. This site appears not to be in linkage disequilibrium with markers within 700 bp both 5′ and 3′ to it, suggesting that it lies within a limited region of sequence randomization. Whether the behavior of this site is related to the phenomenon of apparent recombination 5′ to the β-gene that disperses one mutation to several haplotypes is unknown, but is worthy of consideration.

The molecular heterogeneity of β-thalassemia is quite extraordinary. Including two new mutations in Chinese (Cheng *et al.*, 1984) and four others in Black Americans (Antonarakis *et al.*, 1984b; S.H. Orkin and S.E. Antonarakis,

unpublished data), we are now aware of 30 different mutations in five ethnic groups (Mediterraneans, Asian Indians, Chinese, Black Americans and Kurdish Jews). Since a number of ethnic groups with a high frequency of β-thalassemia are yet to be studied, the expected number of such mutations in the world population may be considerably greater. This observation has significant implications for the expected heterogeneity of mutations in other single gene disorders of man.

Materials and methods

Subjects

Subjects were unselected Asian Indian and Pakistani families referred for prenatal diagnosis of β-thalassemia. Patients were generally immigrants from the regions of India neighboring Bombay, New Delhi, and, to a lesser extent, Calcutta. In general, nuclear families which included both parents and an affected child were studied.

DNA polymorphism analysis

Chromosomes bearing β^A and β^{thal} genes were haplotyped by determining the presence or absence of a number of polymorphic restriction endonuclease sites in the β-globin gene cluster in various family members. The sites used for haplotype analysis were (i) *Hinc*II 5′ to the ε gene, (ii) *Hind*III in IVS-2 of the $^G\gamma$ gene, (iii) *Hind*III in IVS-2 of the $^A\gamma$ gene, (iv) *Hinc*II in the $\psi\beta_1$ gene, (v) *Hinc*II 3′ to the $\psi\beta_1$ gene, (vi) *Hinf*I 5′ to the β gene, (vii) *Hgi*AI in exon 1 of the β gene, (viii) *Ava*II in IVS-2 of the β gene, (ix) *Hind*III 3′ to the β gene, (x) *Bam*HI 3′ to the β gene (Antonarakis *et al.*, 1982a; Jeffreys, 1979; Tuan *et al.*, 1979, 1983; Moschonas *et al.*, 1982; Kan *et al.*, 1980; Kazazian *et al.*, 1984). The ^{32}P-labelled probes used and the technique of Southern analysis on nitrocellulose filters have been described (George *et al.*, 1981).

DNA sequence analysis

β-Thalassemia genes were cloned as 7.5-kb *Hind*III fragments in Charon 28 as previously described (Orkin *et al.*, 1982). Genes were subcloned as 3.7-kb *Bgl*II-*Pst*I in πSVplac (Treisman *et al.*, 1982) and subjected to partial DNA sequencing by the method of Maxam and Gilbert (1980).

Oligonucleotide analysis

A 19 nucleotide (19-mer) probe (5′-GCAGGTTGCTATCAAGGTT-3′) was synthesized corresponding to the sequence surounding the IVS-1 position 5 (G→C) mutation (Wallace *et al.*, 1981). The oligonucleotide was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase to a specific activity of $5-10 \times 10^8$d.p.m./μg. 10 μg genomic DNA samples were digested with *Bam*HI, electrophoresed in agarose, and transferred to nitrocellulose filters as previously described (Orkin *et al.*, 1983b). Hybridization was performed at 47°C and the final wash at 54°C for 2 min (Orkin *et al.*, 1983b).

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