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ARTICLE

Nucleotide changes in the γ-globin promoter and the $(AT)_xN_y(AT)_z$ polymorphic sequence of βLCRHS-2 region associated with altered levels of HbF

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We have studied 31 β-thalassaemia intermedia, 30 β-thalassaemia major patients and 50 normal individuals from Turkey, determining the relationship between the nucleotide variations in β-globin gene cluster, the altered levels of foetal haemoglobin and the relative ratios of β- and γ mRNAs. We have found in β-thalassaemia intermedia patients with high foetal haemoglobin expression that the three nucleotide variations in the 5' sequences of the gamma globin genes, A→G at Gγ – 1396, the T→C at Aγ – 228, and the GA→AG at Aγ – 603/4, are linked to haplotype II in haplotypic homozygotes and the $(AT)_8N_{14}(AT)_7$ motif in βLCR. Conversely, the three single nucleotide substitutions in the 5' sequences of gamma globin genes, the G→A at Gγ – 1225, the A→G at Aγ + 25 and the C→G at Aγ – 369, which have a strong linkage with haplotype I, V or VI in haplotypic homozygotes and the $(AT)_{10}N_{12}(AT)_{12}$ and the $(AT)_9N_{12}(AT)_{12}$ motifs in HS-2 of βLCR are all associated with low foetal haemoglobin levels. The number of nucleotide changes in β-globin gene cluster implied in our study are not the primary cause of the differences in haemoglobin F levels. They perhaps may contribute to the variations in the clinical severity observed among β thalassaemia intermedia and major patients with other yet unknown gene conversions.

Keywords: β-thalassaemia; HPFH; HbF; γ-mRNA; β-globin gene; γ-globin genes; β-globin gene haplotype

Introduction

The clinical manifestations of β thalassaemia (thal) have been found to vary substantially among patients.

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There is general agreement that the inheritance of a mild β-thal mutation,[1] the co-inheritance of an α-thal determinant[2] or the co-inheritance of a gene for hereditary persistence of foetal haemoglobin (HPFH)[3–6] are major determinants of disease severity. Since a high level of foetal haemoglobin (HbF) appears to ameliorate the severity of the disease, the study of molecular basis and potential genetic markers that would distinguish the high HbF responders from the low responders are medically relevant.[7–9] Expression of

foetal haemoglobin (HbF) is under polygenic control[10,11] involving determinants both linked[12,13] and unlinked[14–16] to the β-globin gene cluster on chromosome 11.

HPFH[17] and δβ-thal[18] which result in a clear enhancement of HbF production in adult life are caused by large deletions within the β-gene cluster or, in some cases of HPFH, by mutations in the promoter of the γ-globin genes.[19,20] In these cases, HPFH determinants show a clear Mendelian inheritance with the β-globin gene complex locus.[20] This is in contrast to heterocellular HPFH or Swiss HPFH which are characterised by smaller (< 5%) increases of HbF distributed in an uneven fashion among so-called F cells (FC). Although heterocellular HPFH segregates with the β-globin gene cluster in some families,[21] it can also segregate independently of the β-globin gene cluster implicating the presence of trans-acting regulatory factors.[15]

An X-linked determinant which seems to influence the production of FC in response to anaemia are reported to account for about 40% of the variation in HbF levels among the factors unlinked to the β-cluster. The variation in FC numbers in these patients and normal adults is linked to a locus controlling the F-cell production (FCP), located at Xp22.2–p22.3.[22] Characterisation of another major genetic determinant for HbF production on chromosome 6q 22.3–q23.1 which modifies FC levels provides another approach in the study of the trans components involved in haemoglobin switching.[23] Such loci could code for trans-acting factors, or subunits thereof, which could function either as a repressor or as a transcriptional activator that binds the regulatory regions such as the β-locus control region (LCR) or the γ promoter,[15] or interact with proteins that bind to DNA in these regions.[24] Alternatively, the trans-acting gene could code for a factor that influences the ability of γ-globin to be transcribed during critical periods of the erythroid maturation pathway.[25]

Analyses of pedigrees with variable HbF levels have indicated that HbF is also influenced by some other genetic factors linked to the β-globin region. A polymorphism in the promoter region of the Gγ-globin gene identified by the presence or absence of an Xmn I restriction site are known to modify the level of HbF production.[26] The number of α-globin genes on chromosome 16[2] and sequence polymorphisms within the DNase I-hypersensitive site 2 of the locus control region (LCRHS2) including an AT-rich region has also been suggested to influence the level of HbF.[27]

Based on these findings, we screened putative *cis*-acting determinants modulating γ-globin gene expression by looking for transcription factor binding motifs that may be involved in erythroid expression. They were tested for their linkage with haplotypes and their implication in HbF modulation in homozygous β thal to gain further insights into the clinical and molecular differences between thal intermedia and major patients.

Subjects and Methods

Patients

From 100 individuals with β thal, we chose for detailed studies 61 patients aged 10–45 years with a steady state HbF level, and 50 normal adults were recruited into the study. Patients were divided into three groups according to transfusion requirements and haematological and clinical observations made during the many years of caring for these patients; 31 patients who had raised HbF levels were categorised as having β thal intermedia as they were not dependent on regular blood transfusion. Fifteen patients were transfusion-dependent since infancy and were categorised as having early presentation β thal major; 15 occasionally transfused patients with late presentation β thal major needed the first blood transfusion at maximally 5–6 years of age.

Haematological Data

Haematological parameters were recorded regularly during each clinic visit for a period of 4 years. Foetal haemoglobin (HbF) levels were determined with a high performance liquid chromatographic (HPLC) procedure using a weak cation exchanger (polyCAT) in columns of different sizes. HbF levels of transfusion-dependent patients reported here were obtained under nontransfused steady state condition or corresponded to values before they began their first transfusion regimen. HbF was purified by preparative PolyCAT-HPLC.[28] The Gγ to Aγ ratios were determined by reversed phase HPLC using the Vydac C4 column and H20-acetonitrile-trifluoroacetic acid developers.[29] In the determining of HbF level, none of the subjects in the control group were children, which excludes age as an important factor.

DNA and Haplotype Analyses

DNA was isolated from white blood cells[30] of probands and immediate family members. Haplotypes of the β-globin gene cluster were determined by the method of Orkin *et al*.[31] Fragment of the β-globin gene cluster that contained the polymorphic restriction sites were amplified and subjected to restriction enzyme digestion.[32]

PCR Applications

A 1.6 kb fragment 5' of Gγ-globin gene, a 720 bp fragment 5' of the Aγ-globin gene and a 552 bp fragment of HS-2 from patients and normal individuals were amplified by PCR[33] using the primers shown in Table 1. The conditions of

denaturation, annealing and extention steps were 94°C for 45 s, 55°C for 1 min and 72°C for 1 min. 30 cycles were then followed by a final elongation for 7 min at 72°C.

The highly polimorphic $(AT)_xN_y(T)_z$ motif of HS-2 of βLCR was amplified by a 95°C/30 s denaturation, a 56°C/30 s annealing and a 72°C/45 s extention. After 30 cycles, a final elongation step was performed for 7 min at 72°C.

Mutation Screening Using the Restriction-Endonuclease Fingerprinting Assay (REF)

One hundred ng of amplified DNA from the HS-2 region of LCR and the flanking regions of Aγ- and Gγ-globin genes in each group of patients were digested in separate tubes by three different groups of restriction endonucleases; group I: Alu I, DdeI and Mse I for the Gγ-globin gene; group II: Alu I, Dde I, Hinf I and Hae III for the Aγ-globin gene; group III: Hinf I, Xba I, Mnl I, and Sna B I for the HS-2 of the LCR. The endonucleases in each group were chosen to obtain fragments with an average size of 200 bp. After digestion, the products were mixed and denaturated. Ten nanograms of digested DNA products were 5' end-labelled with T4 polynucleotide kinase. The labelled samples were run on a non-denatured 6% polyacrylamide gel at 4°C.[34]

Sequence Analysis

Templates were double-stranded PCR products obtained by amplification with the primers in Table 1. Amplified DNA was precipated using one volume of 4 M ammonium asetat and 2 volumes of isopropanol. DNA was wash with 70% ethanol; 500 ng of purified template was denatured at 94°C for 10 min in the presence of relevant primer used for amplification of the Gγ, Aγ or HS-2 region (5 pmol) and dimethyl sulfoxide (10%). Annealing was accomplished by direct chilling in dry ice. Sequences were determined using T7 DNA polymerase and the dideoxynucleotide method.[35]

β-Globin Gene Mutations

Identification of β-thal alleles was through the hybridisation of amplified DNA with ^{32}P-labelled oligonucleotide probes complementary to the most common β-thal mutations in the Mediterranean area.[36]

α-Genotype

Absence of deletions in the α-globin gene cluster was ascertained by Bam HI and Bgl I digestions and the occurrence of most frequent Mediterranean non-deletion forms of α-thal was excluded in all of our patients by digestions with Nco I and Hph I. The number of α-globin genes in each of the β-thal patients were also determined by reprobing the blots with α and γ globin gene probes[37] and comparing the intensity of the signals.

RNA Analysis

Total cellular RNA was isolated by the method of Chomczynski.[38] The relative amounts of γ- and β-mRNA were determined by RT-PCR methodology described in detail by Efremov[39] in β thalassaemia major and β-thal intermedia patients.

Bandshifts

Bandshifts with nuclear extracts from K562 cells were as described.[40] The sequence of wild-type and mutant oligonucleotides is shown in Table 1. In competitive experiments, the amount of competitor is expressed as a multiple of the labelled oligonucleotide.

Results

Subjects

Data were obtained for 61 patients (Tables 2 and 3). Of these, there were 15 patients for the IVS-I-6 (T→C) mutation, 13 patients were homozygous for the IVS-I-110 (G→A) mutation, four patients for the codon 39 (C→T) mutation, one for the IVS-II-1 (G→A) mutation, whilst 24 of the remaining 28 patients were compound heterozygotes for two of these four mutations. Two patients had the combination of the IVS-I-110 (G→A) mutation and the IVS-I-1 (G→A) mutation. One patient had the combination of the IVS-I-1 (G→A) and the IVS-I-6 (T→C) mutations. One patient had the combination of the IVS-I-1 (G→A) mutation and the IVS-II-I (G→A) mutation.

Although a number of β-thal intermedia patients in this study had the same mutations as some of the β-thal

Table 1 Amplification primers and oligos for specific mutations

Position	*Sequence*	*Specificity function*
−1475 to −1454	5′-TCTTTCCTTTCTTATTCAACT-3′	Gγ (5′ flanking) direct primer
+74 to +52	5′-TGTCCTCCTCTGTGAAATGAC-3	Gγ (5′ flanking) reverse primer
−720 to −623	5′-ACACTAATCTATTACTGCG-3′	Aγ (5′ flanking) direct primer
+76 to +55	5′-TGTCCTCCTCTGTGAAATGAC-3	Aγ (5′ flanking) reverse primer
10965 to 10940	5′-TAAATAAGCTTCAGTTTTC-3′	HS-2 direct primer
10506 to 10486	5′-CCGCTTCTAGGTATAGAC-3′	HS-2 reverse primer
−1225 Gγ normal oligo	5′-TTTCCAGAGTTTCTGAC**G**TCATAATCTACCAA-3′	
−1225 Gγ mutant oligo	5′-TTTCCAGAGTTTCTGAC**A**TCATAATCTACCAA-3′	
−1396 Gγ normal oligo	TTGTTGCGCAGGTCAAC**A**TGTATCTTTCTGGTCT-3′	
−1396 Gγ mutant oligo	5′-TTGTTGCGCAGGTCAAC**G**TGTATCTTTCTGGTCT-3′	
−228 Ag normal oligo	5′-GGCTATAAAAAAAA**T**TAAGCAGCAGTATCC-3′	
−228 Ag mutant oligo	5′-GGCTATAAAAAAAA**C**TAAGCAGCAGTATCC-3′	

Table 2 Summary of genetic and haematological data in patients with β thal intermedia and β thal major

	Mutation	*HbF* (%)	*Gγ* (%)	*Aγ^{I}* (%)	*Aγ^{T}* (%)	*mRNA* (%) γ (γ+β)
Late presentation β *thalassaemia major* (*n*)						
6	IVS-1-110/IVS-1-110	75±1.74	63.5±3.18	36.5±2.89	0	75.5±3.79
3	IVS-1-6/IVS-1-6	51.7±5.3	61.5±4.7	25.2±4.1	13.3±3.3	62.8±1.87
2	IVS-1-110/IVS-1-6	64.3±3.99	60.4±3.7	26.6±6.5	12.0±2.7	66.1±2.03
1	IVS-1-1/IVS-1-6	79.3	51.8	0	48.2	82.5
1	IVS-1-110/IVS-1-6	78.3	52.2	0	47.8	80.4
1	IVS-1-6/codon 39	80.6	70.9	0	29.1	88.5
1	codon 39/codon 39	90.8	72	0	28	91.3
Early presentation β *thalassaemia major* (*n*)						
7	IVS-1-110/IVS-1-110	81.6±0.93	58.7±1.8	41.2±1.1	0	82.2±3.4
3	IVS-1-10/codon 39	86.3±1.2	60.6±4.5	39.4±3.4	0	88.4±2.1
2	codon 39/codon 39	93.6±0.61	62.4±4.8	37.6±4.8	0	96.6±1.3
1	IVS-II-I/IVS-II-I	81.2	62.2	37.8	0	78.2
2	IVS-1-110/IVS-I-1	94.4±1.95	58.6±5.5	27.8±4.2	13.6±3.1	90.5±4.7
β *thalassaemia intermedia* (*n*)						
7	IVS-1-110/IVS-1-6	85.9±4.3	72.2	0	27.8	87.6±2.6
1	IVS-1-6/codon 39	91.5	41.2	0	58.8	94.0
1	IVS-1-1/IVS-11-1	80	53.3	46.7	0	81.5
12	IVS-1-6/IVS-1-6	72.4±2.6	57.3±3.3	0	42.7±4.1	80.2±4.7
6	IVS-11-1/codon 39	97.2±2.8	68.4±3.0	0	31.6±3.8	96.1±2.4
1	codon 39/codon 39	100	79.7	0	20.3	100
3	IVS-1-110/IVS-1-6	90.1±1.5	71.0±3.3	0	29±2.9	92.8±3.1
Control group (*n*)						
20		0.8±0.1	55	20	15	ND
17		0.9±0.05	59	41	0	ND
10		0.5±0.04	57.6	42.4	0	ND
3		0.9±0.3	58.7	23.6	17.7	ND

ND = not determined, *n* = number of patients in each row.

major patients, they showed a discordant phenotype. We therefore identified each of their β-globin haplotypes since variations in clinical severity observed between thal major and intermedia patients have been attributed to the variable expression of γ-globin genes. Table 3 summarises the result of all polymorphisms in β-globin gene cluster and the correlation between haplotype and thal major or intermedia phenotype. The results show that although a milder phenotype is often seen in patients that are heterozygous or homozygous for the IVS-I-6 mutation, any correlation between mild transfusion-independent phenotype and concomitant α-thal or – 158 Gγ polymorphism was apparent, indicating the involvement of other ameliorating determinants. The relative amounts of γ-mRNA in all patients except the IVS-I-6 homozygotes were almost the same as the levels of HbF. The relative γ-mRNA level in the IVS-I-6 homozygotes was found to be slightly higher than the corresponding HbF level.

Identification of the Nucleotide Sequence in the Promoter Region of the γ-Globin Genes and HS-2 of the LCR We used restriction endonuclease fingerprinting (REF)[34] to search for nucleotide changes in the promoter regions of the γ-globin genes and the HS-2 region of the LCR. REF is a modification of single strand confirmation polymorphism (SSCP) analysis. Representative results of these experiments for the region 1.6 kb upstream of Gγ, 720 bp upstream of Aγ and 552 bp HS-2 fragment are shown in Figure 1. A letter above each lane indicates a group of patients with the same mutations. The bands in each lane were compared with the bands in control digests to determine which fragment had a mutation. The controls contained digests with a single enzyme or the combination of all the enzymes minus one. Mutated fragments were identified by the altered mobility (or absence) of fragments, or the presence of additional fragments. Figure 1 shows that each of the three regions

Table 3 DNA sequence polymorphisms of the human β-globin gene cluster

	Mutation	*Haplotype*	*Nucleotide variation in Gγ globin gene promoter*	*Nucleotide variation in Aγ globin gene promoter*	*Nucleotide variation in βLCRHS-2*
Late presentation β thalassaemia major (n)					
6	IVS-1-110/IVS-1-110	I/I	G→A at −1225	—	$(AT)_{10}N_{12}(AT)_{12}$
3	IVS-1-6/IVS-1-6	VI/VI	—	C→G at −369	$(AT)_{10}N_{12}(AT)_{12}$
2	IVS-1-110/IVS-1-6	I/VI	—	C→G at −369	$(AT)_{10}N_{12}(AT)_{12}$
1	IVS-1-1/IVS-1-6	II/II	—	T→C at −228	$(AT)_{9}N_{12}(AT)_{12}$
1	IVS-1-110/IVS-1-6	II/II	—	T→C at −228	$(AT)_{9}N_{12}(AT)_{12}$
1	IVS-1-6/codon 39	II/II	A→G at −1396	—	$(AT)_{9}N_{12}(AT)_{12}$
1	codon 39/codon 39	II/II	A→G at −1396	—	$(AT)_{9}N_{12}(AT)_{12}$
Early presentation β thalassaemia major (n)					
7	IVS-1-110/IVS-1-110	I/I	G→A at −1225	—	$(AT)_{10}N_{12}(AT)_{12}$
3	IVS-1-110/codon 39	I/I	G→A at −1225	—	$(AT)_{10}N_{12}(AT)_{12}$
2	codon 39/codon 39	I/I	G→A at −1225	—	$(AT)_{10}N_{12}(AT)_{12}$
1	IVS-II-I/IVS-II-I	V/V	—	A→G at +25	$(AT)_{9}N_{12}(AT)_{12}$
2	IVS-1-110/IVS-I-1	II/V	—	A→G at −340	$(AT)_{9}N_{12}(AT)_{12}$
β thalassaemia intermedia (n)					
7	IVS-1-110/IVS-1-6	II/II	A→G at −1396	—	$(AT)_{8}N_{14}(AT)_{7}$
1	IVS-1-6/codon 39	II/II	—	T→C at −228	$(AT)_{8}N_{14}(AT)_{7}$
1	IVS-1-1/IVS-11-1	V/V	—	T→C at −228	$(AT)_{9}N_{12}(AT)_{12}$
12	IVS-1-6/IVS-1-6	VI/VI	—	GA→AG at −603/604 −T at −611 (7)	$(AT)_{10}N_{12}(AT)_{12}$
6	IVS-11-1/codon 39	II/II	—	GA→AG at −603/604	$(AT)_{8}N_{14}(AT)_{7}$
1	codon 39/codon 39	II/II	A→G at −1396	—	$(AT)_{8}N_{14}(AT)_{7}$
3	IVS-1-110/IVS-1-6	II/II	A→G at −1396	—	$(AT)_{8}N_{14}(AT)_{7}$
Control group (n)					
20			—	C→G at −369	—
17			—	A→G at −340	—
10			—	A→G at +25	—
3			—	−T at −611	—

ND = not determined, Haplotypes = determined according to Orkin *et al*[31]; *n* = number of patients in each row.

has aberrant fragments in a number of patients. The DNA from patients was selected for sequence analysis of the relevant Gγ, Aγ or HS2 fragment. The DNA was amplified using the primers described in Table 1 and the resulting PCR products were sequenced and compared in parallel to a fragment derived from a normal individual. The sequence analysis of the 552 bp HS2 fragment showed only known polymorphisms[27] that occur in three different sequence configurations of the $(AT)_X N_Y (AT)_Z$ motif, $(AT)_8 N_{14} (AT)_7$, $(AT)_9 N_{12} (AT)_{12}$, $(AT)_{10} N_{12} (AT)_{12}$, specific to each of the haplotypes (data not shown).

The sequence analysis of the 5' flanking region of the Aγ-globin gene showed five different polymorphisms. A T→G substitution at −228 bp of Aγ-globin gene is located in an AATTAA sequence (data not shown). This mutates a putative binding sequence for homo-domain proteins, known to control the expression of developmentally expressed genes in other systems.[41] The mutant −228 G (AATGAA) exhibited a very strong affinity for nuclear proteins compared with the wild type −228 T (AATTAA) (data not shown). This nucleotide change was found both in two β-thal intermedia patients in the homozygous state and two late presentation β-thal major patients in the heterozygous state. One β-thal intermedia patient with homozygosity for the $(AT)_8 N_{14} (AT)_7$ polymorphic pattern and haplotype II has moderately increased HbF and γ(γ + β) levels when compared with other one β-thal intermedia and two late presentation β-thal major patients having the same mutation (Tables 2 and 3).

The C→G mutation at Aγ −369 and the A→G mutation at Aγ +25 published previously by Lancos *et al*[42] were found predominantly in five late presentation

β-thal major patients in the heterozygous state and one early presentation β-thal major patients in the homozygous state respectively. The A→G mutation at Aγ −340 was found in two early presentation β-thal major patients (Table 3).

The GA→AG substitution at −603/4 bp upstream from cap site of Aγ-globin gene was accompanied by a deletion of timine at −611[42] in seven of 12 β-thal intermedia patients with homozygosity for haplotype VI shown to be linked by the $(AT)_{10}N_{12}(AT)_{12}$ motif. The GA→AG substitution at −603/4 was also found in six β-thal intermedia patients with high levels of HbF and γ-mRNA. They are all homozygous for haplotype II and $(AT)_8N_{14}(AT)_7$ motif (Tables 2 and 3).

Most of the nucleotide changes in the promoter region of Aγ-globin gene were also found in normal individuals with the exception of the changes at −228 (T→C) and −603/4 (GA→AG). Hence, we postulate

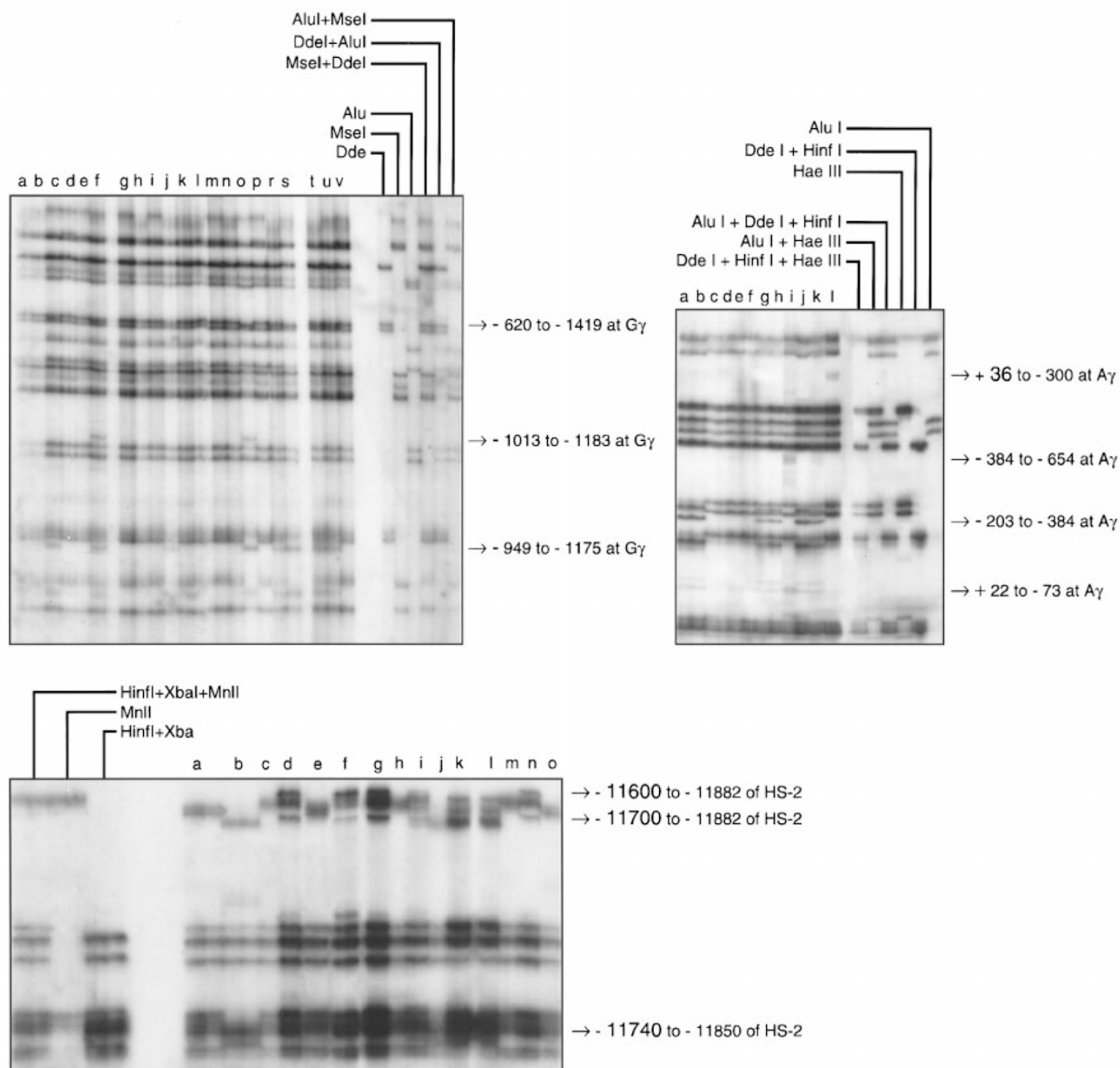

Figure 1 *Restriction enzyme fingerprinting autoradiographs of: Left panel, the promoter region of the Gγ-globin gene. Middle panel: the promoter region of the Aγ-globin gene. Right panel: the 0.552 kb fragment including the highly polymorphic $(AT)_XN_Y(T)_Z$ motif of HS-2 of the LCR. The letter above the lanes indicates a group of patients with the same mutations. The lanes on the right or the left are control DNA samples digested with the restriction enzymes indicated. The positions of fragments with mutations are shown on the right.*

that these nucleotide variations of the 5' flanking region of Aγ-globin gene result in an elevated expression of HbF.

Sequence analysis of the promoter of the Gγ-globin gene showed the G→A nucleotide change in a putative CRE (cyclic AMP responsive element) binding site at –1225 bp[43] in 18 β-thal major patients out of 30. All these patients with homozygosity for haplotype I and the $(AT)_{10}N_{12}(AT)_{12}$ motif have low levels of HbF and γ-mRNA (Tables 2 and 3). The –1225 G/–1225 A CRE motif appears to bind two protein complexes, present in K562 nuclear extract. The mutant CRE site –1225 A preferentially binds complex 2, whilst the wild type –1225 G CRE motif preferentially binds complex 1 (Figure 2A). Strong protein binding at wild type CRE site is associated with the high HbF level in β-thal intermedia patients.

The second polymorphism in the promoter region of Gγ-globin gene, which was found in homozygosity, affects a motif of unknown function at –1396 bp in 11 out of 31 β-thal intermedia patients homozygous for haplotype II and $(AT)_8N_{14}(AT)_7$ sequence pattern (data not shown). In addition to β-thal intermedia patients with the elevated levels of HbF and γ-mRNA, two late presentation β-thal major patients with homozygosity for the $(AT)_9N_{12}(AT)_{12}$ motif and haplotype II were also shown to have the A→G change at Gγ –1396 in the heterozygous state (Tables 2 and 3). The affected sequences resembles a YY1 binding site, ACATGT.[44] The mutant –1396 G motif exhibited a very low affinity for nuclear proteins from K562 nuclear extracts compared with the wild type –1396 A motif. PhosphorImager scanning revealed that the wild-type –1396 oligo was competed 30–40 fold more efficiently than mutant –1396 motif (Figure 2B). Higher γ-mRNA values for the T→C changes at Aγ –228, the GA→AG substitution at Aγ –603/4 and the A→G changes at Gγ –1396 were present in their heterozygous parents, suggesting that the factors affecting the γ-mRNA levels are genetically determined.

Discussion

The present study of 31 patients with β-thal intermedia and 30 patients with β-thal major was undertaken in an attempt to improve our understanding of the molecular differences between phenotypes, ie the severe, transfusion-dependent type and the intermediate type, where transfusions are only occasionally required. Except for transfusion requirement, the most striking difference between the two groups of patients is that a number of β-thal intermedia patients appears to produce sufficiently high levels of haemoglobin F. Re-activation of the foetal globin genes is the most realistic approach to correct the deranged pathophysiology of the haemoglobinopathies because the presence of gamma-chains can neutralise the toxic effects of the unbound alpha-globin chains in the beta-thalassaemias.[45] The relationships between anaemia, HbF and total erythropoiesis in thalassaemia are more complex than expected. Further studies of subjects with high HbF and benign conditions, such as HPFH, could be of help in clarifying this point, with the aim of safely increasing HbF in thal intermedia.

The patients in this study were not carriers of α-thal or of –158 T mutation in the Gγ-promoter. This promoter mutation is found with a higher frequency in intermedia patients who have a mild mutation in combination with a severe mutation.[46] It is linked to the haplotypes III and IX in β-thal patients with increased amounts of HbF and a high ratio of Gγ-globin chain synthesis. However the increased Gγ-ratio linked to this –158 C→T polymorphism has also been found in normal and in thal chromosomes without an increase of the HbF levels.[47] Thus this nucleotide change alone may not be sufficient to raise the level of HbF. Interestingly, a strong linkage has been shown between this Gγ-mutation and the $(AT)_9(T)_5$ motif in the promoter of the β-globin gene. The latter results in an increased binding affinity for the repressor factor, BP-1.[48] Moreover, the observed association between Xmn I polymorphism and HbF expression has been suggested very likely to be due to linkage disequilibrium with LCRHS2 sequences.[49]

HS-2 Changes

Recently, it has been shown that specific nucleotide variations occur in the AT-rich segments of HS-2 of the βLCR. This region may be responsible for different expression levels of the γ-globin genes and it has been postulated that the abnormally high levels of HbF observed in some homozygous sickle cell anaemia patients with the Benin haplotype is caused by AT sequence changes in HS-2.[27] This would be in agreement with our unpublished data (Talbot and Grosveld, 1995) which show that the AT stretch in HS-2 behaves as a negative regulator in transgenic mice. By using REF and direct sequencing, we have characterised three distinct polymorphic patterns, $(AT)_{10}N_{12}(AT)_{12}$, $(AT)_9N_{12}(AT)_{12}$ and $(AT)_8N_{14}(AT)_7$, in HS-2 of βLCR both in thal major and intermedia patients. The later

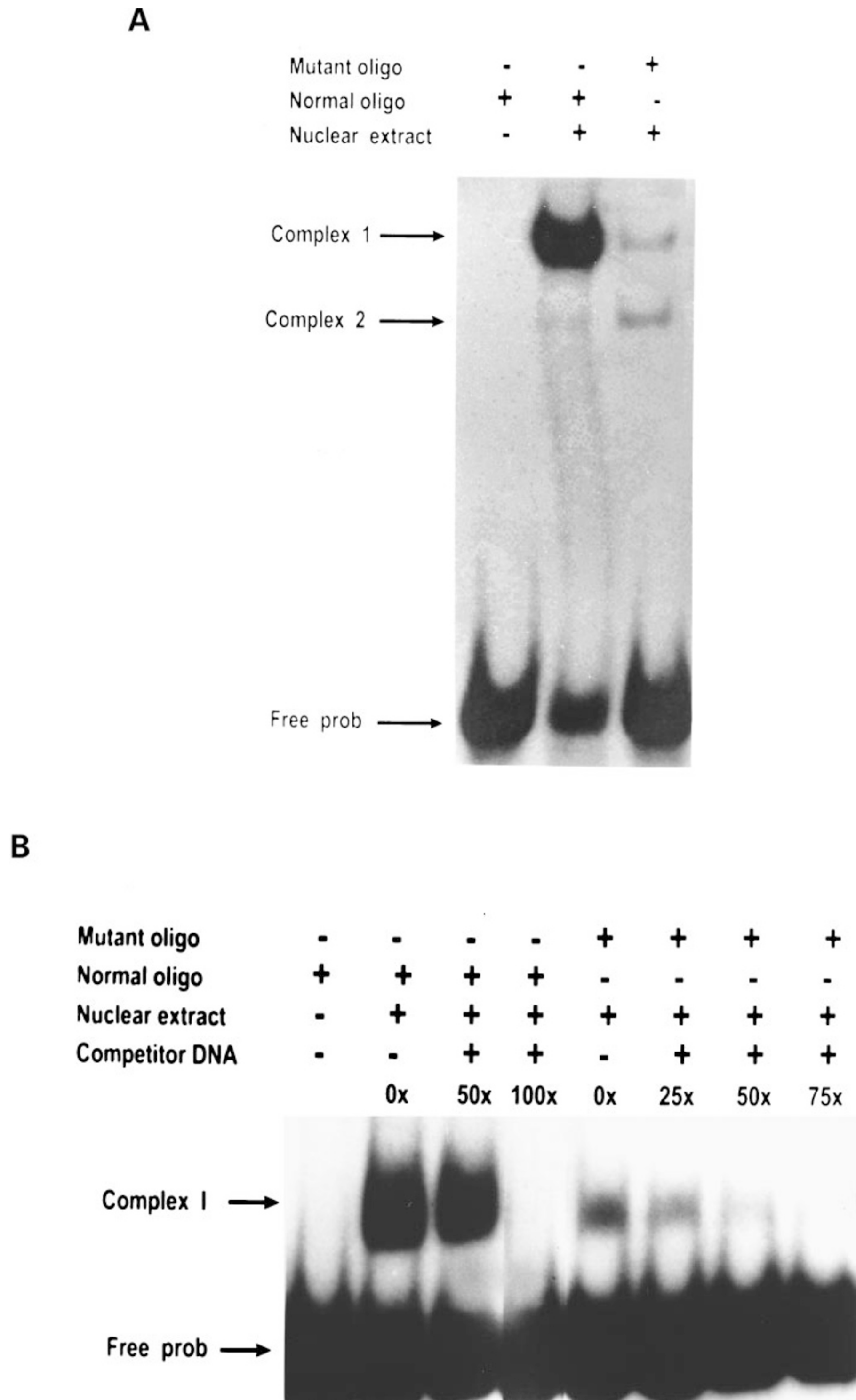

Figure 2 *Gel retardation assay with the −1225 CRE motif and the −1396 polymorphic site.* **A** *Binding activity of the native −1225 G and mutant −1225 A CRE motifs. Two complexes are seen with K562 nuclear extracts. The binding affinities of the two motifs were different: complex 1 bound more strongly to −1225 G and complex 2 to −1225 A.* **B** *Binding activity of K562 nuclear proteins to the native −1396 A and the mutant −1396 G motifs. Cross-examination experiments permit us to estimate the affinity of the −1396 motif to be 30-fold higher than the −1396 G motif.*

motif is usually linked to the haplotype II and three different nucleotide variations in the flanking regions of gamma globin genes, the Gγ(A→G) at –1396, the Aγ(T→C) at –228 bp and Aγ(GA→AG) at –603/604 in the β-thal intermedia patients with an abnormally high level of γ-mRNA and HbF expression. We therefore conclude that this polymorphic motif plays no role in the specific regulation of γ-globin expression in these thalassaemias although they may play a general role in the expression of the locus as a whole.

Gγ-Mutations

The first polymorphism, at –1396 bp with respect to Gγ cap site, was detected in β-thal intermedia patients homozygous for haplotype II and the $(AT)_8N_{14}(AT)_7$ polymorphism. Their HbF levels and Gγ/Aγ ratios are significantly increased when compared with β-thal major patients and to normal adults in the control group. This polymorphism is present in a YY1-like binding site ACATGT which suggests that YY1 may act as a suppressor factor.[44] Its inability to bind to this sequence would result in the elevation of HbF levels in a number of β-thal intermedia patients with haplotype II. We also found in two patients with late presentation β-thal major patients that the A→G mutation at Gγ –1396 is linked to the $(AT)_9N_{12}(AT)_{12}$ motif and haplotype II. Both require only four or five transfusions per year. Thus, these patients have the least severe phenotype of all the β-thal major patients. These data suggest that this polymorphism is sufficient for substantially higher HbF expression.

Although the cyclic AMP regulated factors have never been demonstrated to be involved in globin gene expression, the analysis of the promoter sequence of the Gγ-globin gene identified a CRE site that could mediate a transcriptional response to changes in the levels of intracellular cAMP.[43] Normally, a CRE site is bound by proteins, such as ATF, CREB, CREMι and AP-1. AP-1 is a complex of two proteins: c-fos and c-jun. We showed that the original TGAC**G**TCAT motif is changed to the TGAC**A**TCAT at –1225 bp with respect to Gγ cap site in 18 of the 30 β-thal major patients, but not in any of the β-thal intermedia patients. Since c-fos is known to be involved in the signal transduction of erythropoiesis and erythropoietin is known to affect the cAMP levels in the cell,[50] this may explain why patients with β-thal major who had a mutation in a possibly positively acting element on foetal globin gene expression may not have an effective response to treatment with erythropoietin. The results of our study confirmed that recombinant erythropoietin seems to be an effective treatment for anaemia of β-thal intermedia with bone marrow reserve sufficient to respond to the myelotoxicity of this agent with the evolution of a population of high HbF-producing erythroblasts. But longer term randomised trials with recombinant erythropoietin need to improve the clinical presentation and haematological parameter values in some selected transfusion-dependent patients with β-thal major. Selection criteria for such patients should include a mild beta-genotype of co-inheritance of a gene for hereditary persistence of foetal haemoglobin (HPFH) or co-inheritance of alpha-thalassaemia, splenectomy and pretreatment reticulocyte response of the patients as well as the patients' compliance.[51]

Aγ-Mutations

Developmental regulation of globin gene expression may be controlled by developmental stage specific nuclear proteins that influence interactions between the locus control region and local regulatory sequences near individual globin genes. Although the binding of erythroid and ubiquitous factors to sequences present in γ-globin promotor and the human β-globin 3' enhancer have been described, no developmental specific factor binding to the human globin gene has been clearly defined. Recently, De Simone and colleague described a protein of 87 kDa in K562 and foetal baboon bone marrow nuclear extracts that specifically binds to AT rich sequences in the 3' Aγ-enhancer and γ-globin promotor and LCR. The presence of this protein in foetal globin expressing cells and its absence in adult globin expressing cells suggested that it may be a developmental stage specific factor. This protein, which has a homeodomain homologous to the *Drosophila antennapedia* protein, was shown to be a product of HOX B2 locus.[52] In the study described here, we have found a T→G substitution at the –228 position of the Aγ-globin gene in two patients with β-thal intermedia and in two patients with late presentation β-thal major. This is in an AATTAA sequence which has been implicated as a putative binding site for homeodomain proteins. Of all patients having this mutation, only one β-thal intermedia patient with haplotype II has the $(AT)_8N_{14}(AT)_7$ polymorphism in βLCRHS-2 and higher HbF expression. This polymorphism was not found in normal individuals and we therefore suggest that it has a positive effect on γ-globin expression in this patient with the $(AT)_8N_{14}(AT)_7$ polymorphism. By inference it suggests that this mutated sequence is important for the developmental-specific activation of γ-globin gene expression by promoting an interaction

between the locus control region and local regulatory sequences near individual globin genes.

Whereas Lancos *et al*[42] found the GA→AG substitution at −603/4 bp linked to the T deletion at −611 bp in the 5' flanking region of the Aγ-globin gene of the β^s-chromosomes from sickle cell anaemia patients with a low level of HbF, we only found this mutation in 12 β-thal intermedia patients with homozygosity for IVS-I-6 mutation and haplotype VI. Since these patients are the mild splicing mutants, the β-mRNA output from this allele is high, and these patients are therefore likely to show the intermediate severity of the disease. However, the GA→AG substitution at Aγ −603/4 bp without linking to the deletion at Aγ −611 bp was also found in the second group which contains six β-thal intermedia patients with compound homozygosity for the IVS II-1/codon 39 mutation. The most striking difference between the two groups of β-thal intermedia patient with this mutation is that the second group of patients with high foetal haemoglobin expression are observed in strong linkage disequilibrium with haplotype II and the $(AT)_8N_{14}(AT)_7$ motif in βLCRHS-2. We therefore postulate that different foetal haemoglobin levels are associated with haplotype II which may be a marker for the $(AT)_8N_{14}(AT)_7$ motif in βLCRHS-2 and consequently the clinical course of thal disease.

In our study, the clinical phenotype of some patients remains unexplained even after exclusion of all known ameliorating factors. Most puzzling are those patients who have variations in clinical course despite the fact that they have the same β-thal mutations and sequence variations within regulatory elements of the β-globin gene cluster. There is considerable evidence that much of the observed clinical spectrum of β-thal can largely be explained by the ubiquitin proteolytic pathway which is involved in the degradation of precipitated α-globin chains in β-thal,[53] resulting in the decrease of the extent of ineffective erythropoiesis and peripheral haemolysis and consequently an improving clinical course in β-thal. The data presented here suggest that it would be of interest to establish whether this indirect mechanism may be associated with the phenotypical variability observed between identical mutations.

Perspective

In conclusion, we have identified a number of nucleotide changes that appear to modulate gamma globin expression levels. In this study we analysed about 100 individuals with REF; it is therefore likely that there is a number of other, as yet unknown, sequence changes linked to HbF elevation. Future studies will be aimed at the identification of such changes in order to provide a comprehensive database of naturally occurring mutations influencing γ-globin expression in thalassaemia. The availability of such a database would be important for the rational development of compounds eliciting elevated γ-globin levels, and would provide a guide to the treatment of patients, for instance in the case of erythropoietin administration.

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