HUMAN MUTATION 24:338–349 (2004)

METHODS

Sequence Variations of the α-Globin Genes: Scanning of High CG Content Genes With DHPLC and DG-DGGE

Giuseppina Lacerra,[1] Mirella Fiorito,[1] Gennaro Musollino,[1] Francesca Di Noce,[1] Maria Esposito,[2] Vincenzo Nigro,[2,3] Carlo Gaudiano,[4] and Clementina Carestia[1]*

[1]Istituto di Genetica e Biofisica "Adriano Buzzati Traverso," Consiglio Nazionale delle Ricerche, Napoli, Italy; [2]Telethon Institute of Genetics and Medicine, Napoli, Italy; [3]Dipartimento di Patologia Generale, Seconda Università degli Studi, Napoli, Italy; [4]Centro per la lotta alle Microcitemie, Ospedale Madonna delle Grazie, ASL4, Matera, Italy

Communicated by Jurgen Horst

The α-globin chains are encoded by two duplicated genes (HBA2 and HBA1, 5′–3′) showing overall sequence homology >96% and average CG content >60%. α-Thalassemia, the most prevalent worldwide autosomal recessive disorder, is a hereditary anemia caused by sequence variations of these genes in about 25% of carriers. We evaluated the overall sensitivity and suitability of DHPLC and DG-DGGE in scanning both the α-globin genes by carrying out a retrospective analysis of 19 variant alleles in 29 genotypes. The HBA2 alleles c.1A>G, c.79G>A, and c.281T>G, and the HBA1 allele c.475C>A were new. Three pathogenic sequence variations were associated *in cis* with nonpathogenic variations in all families studied; they were the HBA2 variation c.2T>C associated with c.–24C>G, and the HBA2 variations c.391G>C and c.427T>C, both associated with c.565G>A. We set up original experimental conditions for DHPLC and DG-DGGE and analyzed 10 normal subjects, 46 heterozygotes, seven homozygotes, seven compound heterozygotes, and six compound heterozygotes for a hybrid gene. Both the methodologies gave reproducible results and no false-positive was detected. DHPLC showed 100% sensitivity and DG-DGGE nearly 90%. About 100% of the sequence from the cap site to the polyA addition site could be scanned by DHPLC, about 87% by DG-DGGE. It is noteworthy that the three most common pathogenic sequence variations (HBA2 alleles c.2T>C, c.95+2_95+6del, and c.523A>G) were unambiguously detected by both the methodologies. Genotype diagnosis must be confirmed with PCR sequencing of single amplicons or with an allele-specific method. This study can be helpful for scanning genes with high CG content and offers a model suitable for duplicated genes with high homology. Hum Mutat 24:338–349, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: CG high content; mutation detection; DHPLC; DG-DGGE; alpha thalassemia; HBA1; HBA2

DATABASES:
HBA@ (alpha globin region) – GenBank: NG_000006.1
HBA1 – OMIM: 141800; GenBank: NM_000558.3
HBA2 – OMIM: 141850; GenBank: NM_000517.3
http://globin.cse.psu.edu (Globin Gene Server)

INTRODUCTION

The human HBA2 (α2) (MIM#141850) and HBA1 (α1) (MIM#141800) genes, 5′–3′, encoding for the α-globin chains that are subunits of the human hemoglobin (α2β2), belong to the class of genes with high CG content and are among those with the highest percentage of CG content [Aissani and Bernardi, 1991]; in fact, they have average CG content >60%, and up to 80% in short segments. This percentage is much higher than the average of human genome or of the HBB (β) globin gene (about 40%) [Fischel-Ghodsian et al., 1987]. Moreover, the α-globin genes show duplicated genomic structures [Higgs et al., 1989]. They have a length of 833 bp (α2) or 841 bp (α1) and are both made of two introns and three exons (Fig. 1A). The two genes are located in tandem on chromosome 16 and are contained in the

Received 30 December 2003; accepted revised manuscript 11 May 2004.

*Correspondence to: Clementina Carestia, Istituto di Genetica e Biofisica "A. Buzzati Traverso" – CNR, Via Guglielmo Marconi 12, 80125 Napoli, Italy. E-mail: carestia@igb.cnr.it

Grant sponsor: Ministero Istruzione, Università e Ricerca (MIUR); Grant number: Legge 488/92 (Cluster C02, Project 2); Grant sponsor: Telethon; Grant numbers: TIGEM-11; TIGEM-C20B.

DOI 10.1002/humu.20088
Published online in Wiley InterScience (www.interscience.wiley.com).

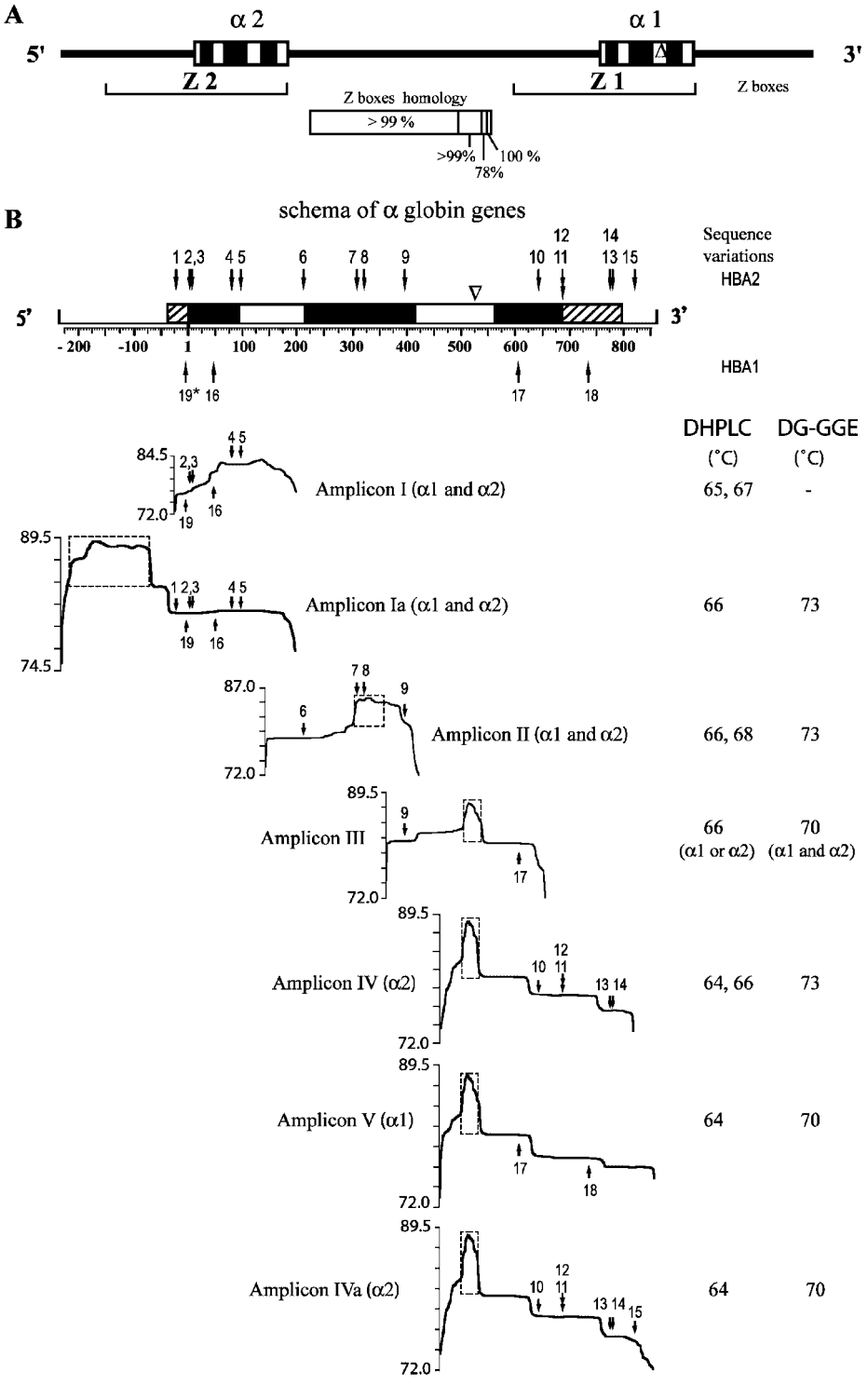

FIGURE 1. **A:** Duplicated Z2 and Z1 DNA boxes containing HBA2 (α2) and HBA1 (α1) globin genes, respectively. They can be divided into three segments of 1436, 171, and 46 bp (5′–3′), with homology of 99, 99, or 100%, respectively. The first and second segments are separated in the HBA1 gene by the insertion of 7 bp (Δ) at IVS2 nt 114. The second and the third segments are separated by a short sequence of 65 bp in α2 and 66 bp in α1, showing only 78% homology. **B:** Schema of the α2- and α1-globin genes and melting profiles of the seven DNA amplicons designed for the scanning of sequence variations. Gene sequence positions were numbered from the ATG (GenBank: NM_000006.1). Sequences enclosed in rectangles in exon2, IVS2, and in the region 5′ to the cap site showed very high TM domain. Amplicons I, Ia, and II were amplified with primers common to both the loci; Amplicons IV, IVa, and V were specific for α2 or α1; Amplicon III was designed in both forms. The analytical temperature used for each amplicon and for each method is reported on the right. Sequence variations are numbered according to Table 3 and are indicated by an arrow up (HBA2) and down (HBA1) the gene schema and the melting profiles. * indicates a sequence variation of a hybrid gene [HBA2:c.-3_-2delAC; HBA2: c.444_HBA1:c.443-del].

3′ extremities of two duplicated boxes (named Z2 and Z1) with >96% sequence homology (Fig. 1A) [Higgs et al., 1984]. The high CG content, which causes high TM, and the particular genomic structure strongly hinder the detection of sequence variations.

α-Thalassemia is the most common worldwide single-gene disorder [Weatherall and Clegg, 1986; Weatherall, 2001]. It is a hereditary anemia characterized by reduction of red blood cell volume (microcytosis) and of the amount of hemoglobin per cell (hypochromia), due to reduced or absent synthesis of the α-globin chain or, in a few cases, to unstable α-globin variants. A large spectrum of hematological/clinical phenotypes due to α-thalassemia has been observed. In fact, the normal genotype has four functional genes (α2 α1/α2 α1) and it is possible to find abnormal genotypes with a different number of functional genes: three, two, one, or zero, causing mild microcythemia (α-thal 2) or severe microcythemia (α-thal 1), hemoglobin H (Hb H) disease, or lethal fetal hydrops, respectively [Weatherall and Clegg, 1986].

Sequence variations account for about 25% of all α-thalassemia defects in our area, while long deletions account for 75%. An unexhaustive panel of 24 sequence variations has been reported to account for molecular basis of α-thalassemia worldwide (http://globin.cse.psu.edu/hbvar/menu.html) [Hardison et al., 2001, 2004].

Several methods have been set up for the detection of a limited number of variant alleles: restriction analysis with enzymes NcoI and HphI for the two most common pathogenic alleles, HBA2:c.95+2_95+6del [Orkin et al., 1981] and HBA2:c.2T>C [Pirastu et al., 1984], respectively; reverse dot-blot [Chan et al., 1999; Foglietta et al., 2003] for a limited number of sequence variations; and multiplex allele refractory mutation system (ARMS) for sequence variations common in Southeast Asia [Eng et al., 2001]. A combined strategy with denaturing gradient gel electrophoresis and single strand conformation analysis (DGGE/SSCA) has been previously established [Harteveld et al., 1996].

Methods such as denaturing high pressure liquid chromatography (DHPLC) [Underhill et al., 1997] and double gradient-denaturing gradient gel electrophoresis (DG-DGGE) [Cremonesi et al., 1997], which are based on the detection of homoduplex and heteroduplex PCR molecules, allow rapid and reliable gene scanning. These methods have been successfully applied to the scanning of other genes, one of which was the β-globin gene (MIM# 141900) [Ghanem et al., 1992; Colosimo et al., 2002]. Both of the methods show high sensitivity, which makes them attractive for gene scanning and detection of sequence variations.

We set up experimental conditions for PCR amplification of the α-globin gene sequences and for the detection of variant alleles with both DHPLC and DG-DGGE. Moreover, we compared sensitivity, reproducibility, and the overall suitability of the two methodologies in a retrospective analysis of 19 variant alleles previously identified by us in carriers (46), homozygotes (7), compound heterozygotes (7), and compound heterozygotes for a hybrid gene (6), for a total of 29 different genotypes. DHPLC showed 100% sensitivity, while DG-DGGE recognized 17 out of 19 sequence variations and 27 out of 29 genotypes (about 90%), but both the methodologies were reliable, suitable, and extremely useful for scanning these genes, as well as other genes with high CG content.

MATERIALS AND METHODS

Screening of Carriers and Patients

Carriers were selected by standard hematological screening methods. Homozygous or compound heterozygous patients were collected from those who had required genotype analysis in the past. Normal subjects, not carrying alpha thalassemia genes, were collected among the members of the families under study. Informed consent was obtained in accordance with Italian laws.

DNA Preparation and α-Globin Genotype Characterization

DNA was purified from peripheral blood leukocytes by a salting-out procedure [Miller et al., 1988]. Carriers and patients had previously been screened for deletions of the α-globin gene cluster with restriction mapping of genomic DNA [Lacerra et al., 1991] or with gap-PCR [Dodé et al., 1993; Bowden et al., 1992]. Identification of variant alleles was carried out by restriction analysis with HphI and NcoI enzymes [Orkin et al., 1981; Pirastu et al., 1984], manual PCR–direct-sequencing of DNA [Lacerra et al., 1991], or DNA sequencing with automated cycle sequencing (3100 Genetic analyzer; Applied Biosystems, Foster City, CA). Oligonucleotides utilized were those reported in Table 1.

Mutation Nomenclature and Data Bank

Two genes, HBA2 and HBA1, 5′–3′, were involved. Reference sequences were from GenBank: NG_000006.1 (genomic, alpha globin region); NM_000517.3 (HBA2 mRNA); and NM_000558.3 (HBA1 mRNA). The mutation nomenclature used conformed to the format suggested by the Human Genome Variation Society (HGVS) [den Dunnen and Antonarakis, 2000, 2001; den Dunnen and Paalman, 2003]. Corresponding traditional nomenclature was from the Globin Gene Server (http://globin.cse.psu.edu).

Primers and PCR-Amplification of α-Globin Genes

Primers for the synthesis of seven DNA amplicons were designed after the melting profile study of the two genes (Table 1; see also the Results section). We performed all PCR synthesis in a thermocycler (GMI, Albertville, MN or Hybaid, Ashford, UK) without mineral oil, which is harmful to the DHPLC column. We used an original buffer we specifically developed for the α-globin gene amplification. Genomic DNA (0.3 μg) was amplified in a 50-μl reaction volume containing 0.6 μM/L of each of the primer, 16.6 mmol/L $(NH_4)_2SO_4$, 67 μmol/L Na_2 EDTA, 67 mmol/L Tris-HCl (pH 8.75), 2.5 mmol/L $MgCl_2$, 10 mmol/L β-mercaptoethanol, 160 μg/ml bovine serum albumin (BSA), and 10% dimethyl sulfoxide (DMSO). After heating for 10 min at 95°C, 1.25 units of ampli-Taq polimerase (Perkin Elmer Cetus, Norwalk, CT) and 8 μl of 1.25 mmol/L dNTP solution (200 μM of each dNTP) were added at 60°C and the samples were subjected to 30 cycles. Each cycle was: 50 sec denaturation at 94°C; 50 sec annealing at 60°C; and 60 sec extension at 72°C. Annealing temperature of 55°C was used for the synthesis of Amplicons I and IVa. Heteroduplex molecules were generated at the end of each PCR session by 60 sec at 95°C, 5 min at 56°C, and 5 min at 4°C. Comparative PCR amplifications were carried out with the proofreading Platinum Pfx DNA polymerase (Invitrogen, Paisley, UK), as reported in the Results section. DNA samples for DHPLC

TABLE 1. Primers and DNA Amplicons Designed for Scanning the HBA2 and HBA1 Globin Genes*

Amplicons: location, length (bp)	Loci	Name, position, and sequence (5′→3′) of the primers	
		Forward	Reverse
Amplicon I -33/IVS1 nt 107 235 bp	HBA2, HBA1	A (-33/-14) TTCTGGTCCCCACAGACTCA	B (+183/+202) AAGCAGAGTGAGGGGTGGGG
Amplicon Ia –239/IVS1 nt 107 441 bp	HBA2, HBA1	A1 (-239/-220) TGGAGGGTGGAGACGTCCTG	B (+183/+202) AAGCAGAGTGAGGGGTGGGG
Amplicon II IVS1 nt 51/IVS2 nt 5 277 bp	HBA2, HBA1	C (+146/+167) ACAGGCCACCCTCAACCGTCCT	D (+403/+422) CTCACCTTGAAGTTGACCGG
Amplicon III Ex2 cod 51/Ex3 cod 32 295 bp HBA2 302 bp HBA1	HBA2, HBA1	E (+363/+384) CGCCCTGAGCGACCTGCACGCG	F (+657/+637, HBA2) (+664/+645, HBA1) ACAGAAGCCAGGAACTTGTC
Amplicon IV IVS2 nt 47/53 bp 3′ polyA; 372 bp	HBA2	G (+464/+487) CCTTCCTCGCAGGGCAGAGGATCA	H (+835/+814) ACATTCCGGGACAGAGAGAACC
Amplicon V IVS2 nt 47/85 bp 3′ polyA; 414 bp	HBA1	G (+464/+487) CCTTCCTCGCAGGGCAGAGGATCA	L (+877/+853) TGTGTGTCCCAGCTGCTGTCCACGC
Amplicon IVa IVS2 nt 47/87 bp 3′ polyA; 403 bp	HBA2	G (+464/+487) CCTTCCTCGCAGGGCAGAGGATCA	M (+867/+843) GTCTGAGACAGGTAAACACCTCCAT

IVS, intron; Ex, exon.
*Position of the primers was numbered in relation to the ATG in the cDNA reference sequence (NM_000558.3 for HBA1 and NM_000517.3 for HBA2). Intronic sequences were derived from the genomic reference sequence NG_000006.1. Locations of the amplicons were referred to gene functional regions. The underscored bases were non-pathogenic sequence variations.

were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) under conditions recommended by the manufacturer in order to remove BSA, which clogs the DHPLC column. DNA from 100 μl of PCR reaction was resuspended in 40 μl of redistilled water.

DHPLC Analysis

DHPLC analysis was carried out using two WAVETM DNA Amplicon Analysis Systems (Mod 3500 HT; Transgenomic, Omaha, NE). The amplified DNA samples (5–8 μl) were injected onto a preheated C18 reversed-phase column with nonporous poly(styrene/divinyl-benzene) particles (DNASepTM column; Transgenomic). For each amplicon, the empirical gradient conditions were achieved starting from different percentages of buffer A (100 mmol/L triethylamine acetate (TEAA), 1% acetonitrile (ACN), pH 7.0) and increasing the Buffer B (100 mmol/L TEAA, 25% ACN, pH 7.0) by 1% per minute for 10 min at a flow rate of 0.9 ml/min. DNA was detected at 260 nm. The theoretical temperature was predicted using the Navigator software (Transgenomic), or submitting the sequences to the DHPLC Melt Program at the Stanford Genome Technology Center, Stanford University (http://insertion.stanford.edu/melt.html). The empirical temperature was established as reported in the Results section. A comparison of the retention time at different temperatures was performed using control amplicons. The optimal conditions for DHPLC analysis of each amplicon are reported in Table 2.

DG-DGGE Analysis

The gel apparatus was the DGGE-2401 system (C.B.S. Scientific, Del Mar, CA). The electrophoretic conditions were those described in Myers et al. [1987]. The MELT87 and the SQHTX programs [Lerman and Silverstein, 1987] indicated that temperatures required were >60°C. We empirically found the optimal temperature for each amplicon (Table 2) by carrying out experiments in the range of 65–75°C by increasing 1 or 2°C. PCR product (10–12 μl) with 4 μl of dye solution was loaded for each sample. The gel slabs were 1.0-mm thick, 18-cm wide, and 22-cm long. All the gels were run in 1 × TAE buffer (1 L 50 × TAE [pH 7.6]: 2 M Tris, 1 M NaOH, 50 mmol/L Na_2 EDTA, and 162 ml glacial acetic acid), at a constant 80 V, at 70–73°C and overnight for 9–15 hr (Table 2). Higher voltage (about 200 V) and shorter electrophoresis time (about 4–5 hr) gave similar results. Initial and final percentage of acrylamide and denaturant (100% denaturant=7 M urea/40% formamide (vol/vol)) were determined empirically and were specific for each amplicon (Table 2). After electrophoresis, the gels were stained with ethidium bromide (0.5 mg/ml) for 10 min, washed in water for 10 min, and visualized with an ultraviolet (UV) transilluminator.

RESULTS

Variant Alleles and Genotypes Analyzed

A total of 19 variant alleles (15 of HBA2, three of HBA1, and one of the hybrid gene HBA2:c.444_HBA1:c.443del) were analyzed; two were characterized by point deletions, and 17 by single-base substitutions (Table 3). Three alleles showed nonpathogenic sequence variations (Table 3; genotypes 1, 15, and 18); the remaining 16 were associated with α-chain defects. The HBA2 alleles c.1A>G, c.79G>A [Lacerra et al., 1997],

TABLE 2. Optimal Experimental Conditions for DHPLC and DG-DGGE

	DHPLC			DG-DGGE			
PCR amplicons	°C	A start/end	Time (min)	°C	Denaturant %	Acrylammide %	Time (hrs)
Amplicon I	65, 67	53–37	10′	–	–	–	–
Amplicon Ia	66	48–32	10′	73	42–72	6.5–12.0	13
Amplicon II	66, 68	52–46	4′	73	30–80	6.5–12.0	9
Amplicon III	66	53–37	10′	70	30–80	6.5–12.0	13
Amplicon IV	64, 66	48–32	10′	73	42–72	6.5–12.0	15
Amplicon V	64	48–32	10′	70	42–72	6.5–12.0	13
Amplicon IVa	64	48–32	10′	70	30–80	6.5–12.0	13

and c.281T>G, and the HBA1 allele c.475C>A [Lacerra et al., 1997] were new, as well as the association *in cis* of three pathogenic sequence variations with nonpathogenic base substitutions in all families studied. These variations were HBA2:c.2T>C (associated with c.–24C>G) and HBA2:c.391G>C and HBA2:c.427T>C (both associated with c.565G>A) (Table 3; genotypes 3, 10, and 12). One allele ([HBA2:-3_-2delAC; HBA2:c.444_HBA1:c.443del]; Table 3; genotype 19) was a hybrid gene, carrying a dinucleotide deletion. The functional hybrid gene is due to a deletion removing 3.7 kb (from the α2 up to the same point of the α1 globin gene), showing breakpoints in sequences with high homology. This deletion is the most prevalent worldwide α-thalassemia defect and for this the rearranged gene can be frequently found *in trans* to sequence variations.

The sequence variations were not homogeneously distributed along the genes and the amplicons (Fig. 1B). Markers were not available for the short high melting domain in IVS2 (see below).

The overall number of abnormal genotypes was 29 (Table 3). We studied five subjects from unrelated families for genotypes with high prevalence in our area and only the available subjects for rare genotypes. In all, 76 subjects were studied (Table 3). Out of them, 10 were normal, 46 were heterozygotes, seven were homozygotes for a variant allele, and seven were compound heterozygotes with sequence variations within the same amplicon. The remaining six patients showed sequence variations *in trans* to the hybrid gene HBA2:c.301-28_HBA1:c.301-35del.

Design of DNA Amplicons for DHPLC and DG-DGGE

We split the genes in four regions from −239 to +877 from the ATG (reference sequence NM_000006.1) on the basis of the TM profiles and designed seven overlapping amplicons with lengths from 235 to 441 bp (Table 1). Their location along the two genes, locus specificity, TM profiles, and utilization for DHPLC or DG-DGGE are reported in Figure 1B. GC clamps were not added, but each amplicon had at least two TM domains, the higher of which substituted for the GC clamp function. Amplicons Ia and IVa were the more extended version of Amplicon I (at 5′ from the cap site) or of Amplicon IV (at 3′ from the polyA addition site), respectively. The TM profiles obtained with the MELT87 software indicated that the designed amplicons allowed us to achieve good conditions for the analysis of 87% of the gene sequence from the cap site to the polyA addition site. The remaining 13% was made of two short segments with extremely high TM domains, most likely requiring extreme analysis conditions (Fig. 1B). The first region, 78-bp long, located in exon 2 codons 63–88, showed 73% of CG content (Amplicon II). The second, 37-bp long, located in the IVS2 nt 87–123, showed 80% of CG content (Amplicons III-IV-IVa-V). Moreover, a 183-bp region (from −239 up to −57) with 88% of CG was present at the 5′ end of Amplicon Ia (Fig. 1B).

Amplicons I, Ia, II, and III were amplified from both α2 and α1 globin genes and, when present, from the hybrid gene HBA2:c.301-28_HBA1:c.301-35del. Amplicons IV, IVa, and V were selectively amplified from α2 (IV and IVa), α1 (V), or the hybrid gene (V) by means of the nucleotide differences between the 3′ untranslated regions (UTRs). Amplicon III amplified with primers common to both genes showed two different lengths because of the insertion of 7 bp in the IVS2 of the α1 gene (Fig. 1A; Table 1) and could be used only for DG-DGGE; instead, Amplicon III generated by nested PCR following α2- or α1-specific amplification was used for DHPLC in order to avoid interference with the analysis.

Gene Scanning by DHPLC

The theoretical temperatures (more than one for each amplicon) suggested for the optimal heteroduplex separation by the Navigator software (Transgenomic) were in the range 64–67°C. To establish the best empirical temperatures, an extensive study of one subject for each abnormal genotype and of two subjects with normal α2α1/α2α1 genotype was carried out by analyzing all the amplicons in the range 63–68°C by varying the temperature by 1°C. Amplicons were not prehybridized with normal DNA sequences, with the exception of the Amplicons IV and IVa in homozygous and hemizygous patients (Table 3; genotypes 22, 23, and 29). All sequence variations analyzed in different genotypes were detectable in the range of 3 or 4°C, with some exceptions (Table 4). Some variations were detected only at the highest oven temperature because they were located in high TM regions (HBA2:c.79G>A, Amplicon I, 67°C; and HBA2:c.193G>A and HBA2:c.237C>G, Amplicon II,

TABLE 3. Genotypes and Number of Subjects Analysed

Genotypes	HGVS nomenclature (www.hgvs.org.mutnomen.html) DNA	Protein	Traditional nomenclature (http://globin.cse.psu.edu)	No. of subjects (tot 76)
Heterozygotes	(HBA2, HBA1,α-3.7)			
	HBA2:c.-24C>G[a]		α_2+14C>G	2
	HBA2:c.1A>G[b]	p.0	α_2ATG>GTG	2
	HBA2:[c.-24C>G; c.2T>C][c]	p.0	α_2ATG>ACG,+14 C>G	5
	HBA2:c.79G>A[b]	p.Ala26Thr[d]	$\alpha_2$cod 26 GCG>ACG (Hb Caserta)	1
	HBA2:c.95+2_95+6del		α_2IVS-1 (GAGGTGAGG→GAGG)	5
	HBA2:c.96G>C	p.Arg31Ser	$\alpha_2$cod 31 AGG>AGC (Hb Prato)	1
	HBA2:c.193G>A	p.Asp64Asn	$\alpha_2$cod 64 GAC>AAC (Hb Aida)	1
	HBA2:c.237C>G	p.Asn78Lys	$\alpha_2$cod 78 AAC>AAG (Hb Stanleyville II)	3
	HBA2:c.281T>G[b]	p.Val93Gly[d]	$\alpha_2$cod 93 GTG>GGG (Hb Bronte)	2
	HBA2:[c.391G>C; c.565G>A][c]	p.Ala130Pro	$\alpha_2$cod 130 GCT>CCT (Hb Sun Prairie), +861 G>A	5
	HBA2:c.427T>A	p.X142Lys31ins	$\alpha_2$cod 142 TAA>AAA (Hb Icaria)	3
	HBA2:[c.427T>C; c.565G>A][c]	p.X142Gln31ins	$\alpha_2$cod 142 TAA>CAA (Hb Constant Spring), +861 G>A	1
	HBA2:c.521A>G		α_2AATAAA>AATGAA	1
	HBA2:c.523A>G		α_2AATAAA>AATAAG	5
	HBA2:c.565G>A[a]		α_2+861 G>A	2
	HBA1:c.47G>A	p.Gly15Asp	$\alpha_1$cod 15 GTA>ATA (Hb J-Oxford)	2
	HBA1:c.349G>A	p.Phe116Lys	$\alpha_1$cod 116 GAG>AAG (Hb O-Indonesia)	1
	HBA1:c.475C>A[a,b]		α_1+778 C>A	2
	[HBA2:-3_-2delAC; HBA2:c.444_HBA1:c.443del]		$-\alpha^{3.7-AC}$	2
Homozygotes				
	HBA2:[c.-24C>G]+[c.-24C>G]			2
	HBA2:[c.95+2_95+6del]+[c.95+2_95+6del]			2
	HBA2:[c.523A>G]+[c.523A>G]			1
	HBA2:[c.565G>A]+[c.565G>A]			2
Compound heterozygotes				
	HBA2:[c.-24C>G; c.2T>C]+[c.-24C>G]			2
	HBA2:[c.95+2_95+6del]+[c.-24C>G]			2
	HBA2:[c.523A>G]+[c.565G>A]			2
	HBA2:[c.427T>C;c.565G>A]+[c.565G>A]	p.X142Gln31ins	$\alpha_2$cod 142 TAA>CAA (Hb Constant Spring), +861 G>A[c]/α_2+861 G>A	1
Compound heterozygotes for a hybrid gene				
	[HBA2:c.95+2_95+6del]+[HBA2:c.301-28_HBA1:c.301-35del]			5
	[HBA2:c.391G>C]+[HBA2:c.301-28_HBA1:c.301-35del]			1
Normal genotypes				
	HBA2 HBA1/HBA2 HBA1		α2 α1/α2 α1	10

Sequence variations were indicated according to the HGVS rules and numbered from the ATG of each gene on reference sequences GenBank: NG_000006.1 (genomic, alpha globin region); NM_000517.3 (HBA2 mRNA); NM_000558.3 (HBA1 mRNA).

[a]Nonpathogenic sequence variations.

[b]New variant alleles.

[c]Alleles with two sequence variations *in cis* not yet reported.

[d]Theoretically deduced.

TABLE 4. Results of the DHPLC Extensive Analysis of the 29 Available Genotypes at Different Temperatures

		Oven temperature (°C)					
Amplicons	Genotypes	63	64	65	66	67	68
I	[HBA2:-3_-2delAC;HBA2:c.444_HBA1:c.443del]	nt	+	+	+	+	-
	HBA2:c.1A4Gn	nt	+	+	+	+	-
	HBA2:c.2T4Cn	nt	+	+	+	+	-
	HBA1:c.47G4An	nt	-	+	+	+	-
	HBA2:c.79G4An	nt	-	-	-	+	-
	HBA2:c.95+2_95+6del	+	+	+	+	+	-
	HBA2:[c.95+2_95+6del]+[c.95+2_95+6del]	+	+	+	+	+	-
	[HBA2:c.95+2_95+6del]+[HBA2:c.301-28_HBA1:c.301-35del]	+	+	+	+	+	-
Ia	HBA2:c.-24C4G	-	-	-	+	+	-
	HBA2:[c.-24C4G]+[c.-24C4G]	nt	nt	nt	nt	nt	nt
	[HBA2:-3_-2delAC;HBA2:c.444_HBA1:c.443del]	-	-	+	+	+	-
	HBA2:c.1A4G	-	-	-	+	+	-
	HBA2:[c.2T4C;c.-24C4G]	-	+	+	+	+	-
	HBA2:[c.2T4C;c.-24C4G]+[c.-24C4G]	-	+	+	+	+	-
	HBA1:c.47G4A	-	-	-	+	+	-
	HBA2:c.79G4A	-	-	-	+	+	-
	HBA2:c.95+2_95+6del	+	+	+	+	+	-
	HBA2:[c.95+2_95+6del]+[c.95+2_95+6del]	+	+	+	+	+	-
	[HBA2:c.95+2_95+6del]+[HBA2:c.301-28_HBA1:c.301-35del]	+	+	+	+	-	-
	HBA2:[c.95+2_95+6del]+[c.-24C4G]	+	+	+	+	+	-
II	HBA2:c.96G4Cn	nt	+	+	+	+	+
	HBA2:c.193G4An	nt	-	-	-	-	+
	HBA2:c.237C4Gn	nt	-	-	-	-	+
	HBA2:c.281T4Gn	nt	+	+	+	+	+
III	HBA2:c.281T4Gn	nt	-	-	+	+/-	+/-
	HBA1:c.349G4An	nt	+	+	+	+	+
IV	HBA2:c.391G4C	-	-	+	+	+	+
	[HBA2:c.391G4C]+[HBA2:c.301-28_HBA1:c.301-35del]	-	-	+	+	+	+
	HBA2:c.427T4A	-	-	+	+	+	+
	HBA2:c.427T4C(HbC.S.)	-	-	-	+	+	+
	HBA2:c.521A4G	+	+	+	+	-	-
	HBA2:c.523A4G	+	+	+	-	-	-
	HBA2:[c.523A4G]+[c.523A4G]	+	+	+	-	-	-
IVa	HBA2:c.565G4A+	+	+	-	-	-	-
	HBA2:[c.565G4A]+[c.565G4A]	+	+	-	-	-	-
	HBA2:[c.391G4C;c.565G4A]	+	+	+	+	-	-
	HBA2:c.427T4A	-	+/-	+	+	+	-
	HBA2:[c.427T4C;c.565G4A]+[c.565G4A]	-	+/-	+	+	+	-
	HBA2:c.521A4G	+	+	+	-	-	-
	HBA2:c.523A4G	+	+	+	-	-	-
	HBA2:[c.523A4G]+[c.565G4A]	+	+	+	-	-	-
VH	HBA1:c.349G4A	+	+	+	+	-	nt
	HBA1:c.475C4A	+	+	+	+	-	nt

Empirical temperatures selected for gene screening were indicated by the shaded areas. Sequence variation nomenclature as in Table 3. "+" and "-" refer to the detection of the anomalous genotype. nt, not tested.

68°C) or near the high CG region at 5′ from the cap site (HBA2:c.−24C>G, Amplicon Ia, 66–67°C). On the contrary, the sequence variation HBA2:c.565G>A was detected only at lower temperatures (63–64°C) because of its position at the end of the Amplicon IVa.

On the basis of these results, only one temperature for the analysis was established for Amplicons III and Ia (66°C) and for Amplicons IVa and V (64°C); on the contrary, two temperatures were established for Amplicon I (65 and 67°C), Amplicon II (66 and 68°C), and Amplicon IV (64 and 66°C) (Fig. 1B; Tables 2 and 4).

All the other carriers, patients, and normal subjects were analyzed at the fixed temperatures. Chromatographic profile printouts are reported in Figure 2.

In the normal subjects, symmetrical peaks were associated in a few cases with small additional peaks, even if the amplified DNA was run as a unique band on acrylamide gel electrophoresis and the use of a proof-reading DNA polymerase did not give any improvement. No false-positive was observed.

Abnormal genotypes were revealed by anomalous reproducible profiles and no false-negative was observed. In the majority of cases, the normal and variant homoduplexes coeluted in a unique peak as well as the two heteroduplexes; in the remaining cases each species was eluted separately.

In the case of coamplification of both α-globin loci (Fig. 2; Amplicon I and II) the sensitivity of the method allowed the detection of a single variant allele in the carriers, although the ratio between the variant and normal alleles was 1:3. The heterozygotes clearly showed the homoduplex and heteroduplex peaks. Also, the homozygotes were characterized by a two-peak profile, but a relative increase of the heteroduplex peak in comparison with the heterozygotes was observed (Fig. 2; Amplicon I, lanes 6 and 7). A similar pattern was

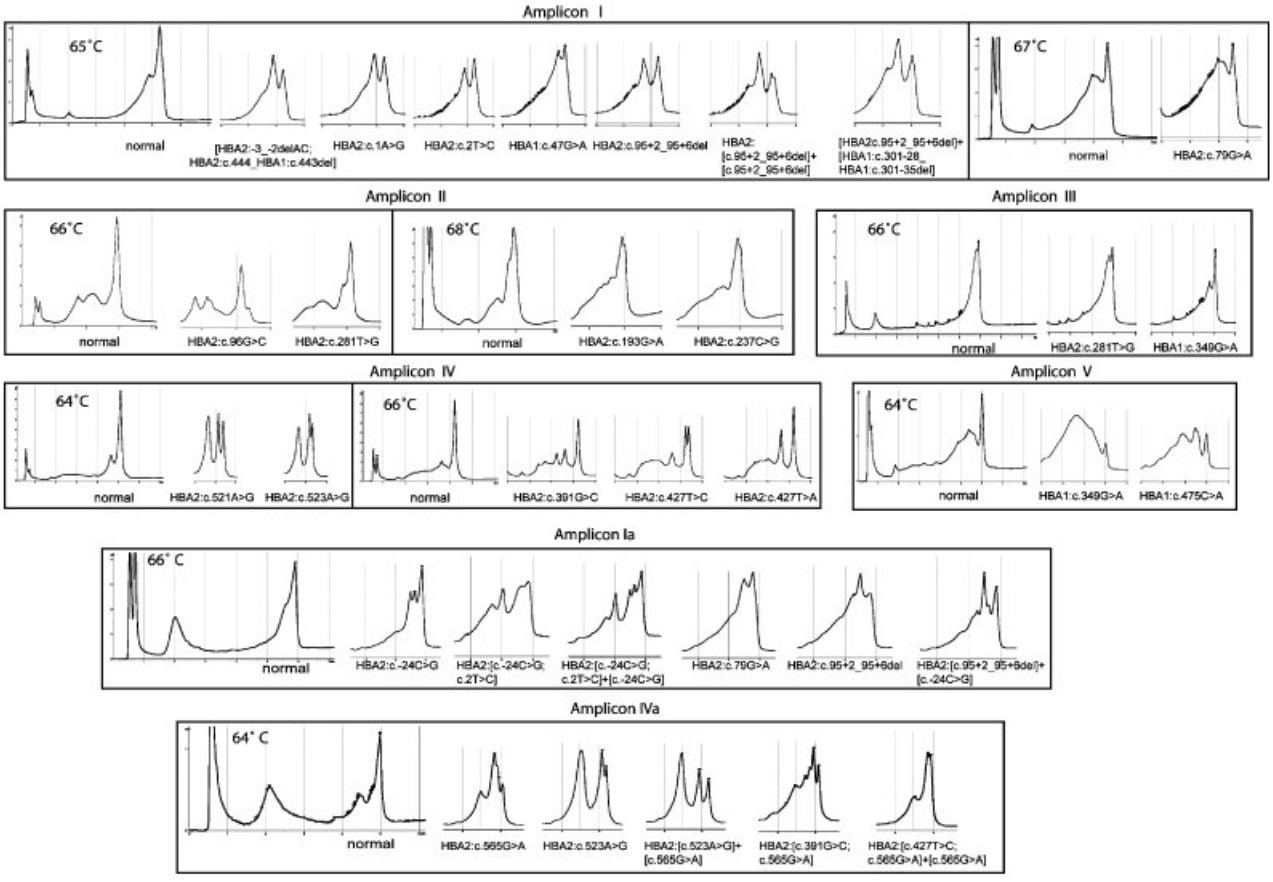

FIGURE 2. **DHPLC chromatograms in abbreviated form, subdivided into groups according to the amplicons and temperatures used. Analysis conditions were reported in Table 2. The profiles were those of the heterozygotes for the 19 investigated alleles and of the relevant homozygous or compound heterozygous genotypes. At least one normal subject was reported for each amplicon in order to visualize the position and shape of the normal homoduplex. Sequence variations and genotypes reported were indicated according to the HGVS Mutation Nomenclature. Reference sequences were GenBank: NG_000006.1 (genomic, alpha globin region); NM_000517.3 (HBA2 mRNA); and NM_000558.3 (HBA1 mRNA). For details see the Results section in text.**

detected in compound heterozygotes for the hybrid gene HBA2:c.301-28_HBA1:c.301-35del, which substituted two normal genes (Fig. 2; Amplicon I, lane 8). Heterozygotes for the AC deletion associated with a second type of hybrid gene (HBA2:-3_-2delAC; HBA2:c.444_HBA1:c.443del) had a typical two-peak pattern (Fig. 2; Amplicon I, lane 2). The variant HBA2:c.79G>A was more detectable with Amplicon Ia instead of I (Fig. 2; Amplicon Ia, lane 5, and Amplicon I, 67°C).

In the case of the amplification of a single locus (Fig. 2; Amplicons IV and V), heterozygotes were characterized by two or more peaks. On the contrary, homozygotes or hemizygotes were characterized by a single peak eluted at the position of the normal genotype, but the reanalysis after the prehybridization with normal DNA gave two peaks, a homoduplex and a heteroduplex such as in the heterozygotes. This was the case of the genotypes HBA2:[c.523A>G]+[c.523A>G] and [HBA2:c.391 G>C]+[HBA2:c.301-28_HBA1:c.301-35del] (not shown).

The analysis of nonpathogenic sequence variations (HBA2:c.–24C>G and HBA2:c.565G>A) was extremely useful for testing compound heterozygosis or heterozygosis for alleles with two variations *in cis*. The allele HBA2:[c.−24C>G; c.2T>C] was analyzed in heterozygosis and with HBA2:c.−24C>G variation *in trans* (Fig. 2; Amplicon Ia, lanes 2–4). The HBA2:c.−24C>G variation was analyzed *in trans* to the HBA2:c.95+2_95+6del allele (Fig. 2; Amplicon Ia, lanes 6–7). Several genotypes with the HBA2:c.565-G>A variation were analyzed (heterozygous, homozygous (not shown), compound heterozygous for the HBA2:c.523A>G, and heterozygous for the allele HBA2:[c.391G>C; c.565G>A]) (Fig. 2; Amplicon IVa, lanes 2–5). The allele HBA2:[c.427T>C; c.565G>A] was analyzed in heterozygosis (not shown) and also with the HBA2:c.565G>A *in trans* (Fig. 2; Amplicon IVa, lane 6). In all cases, specific profiles, different from those due to heterozygosis for the single sequence variation, were obtained.

Gene Scanning With DG-DGGE

The optimal temperature, percentage of acrylamide and denaturant, and the run time for the homoduplex

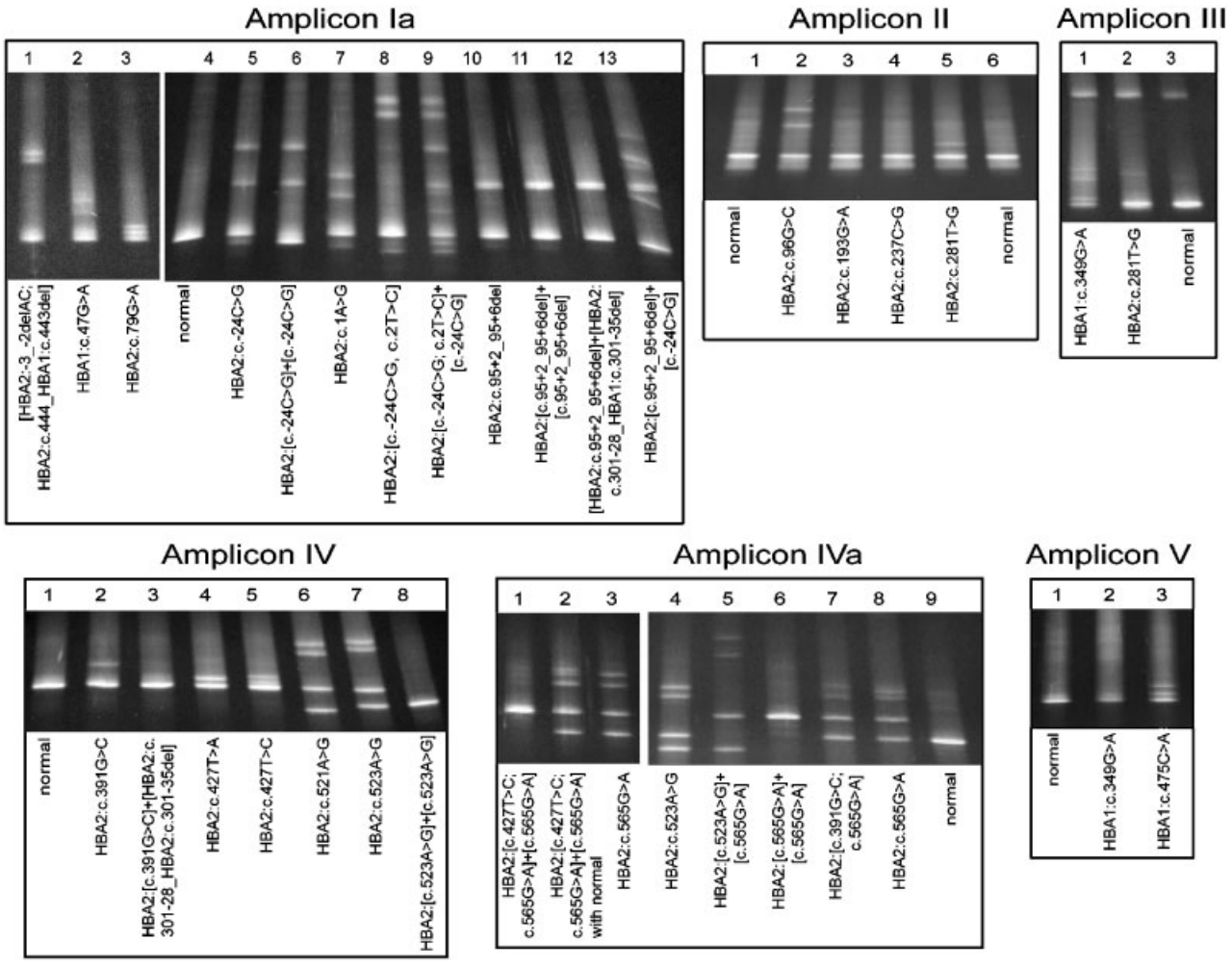

FIGURE 3. **DG-DGGE patterns subdivided into groups according to the amplicons used. Analysis conditions were reported in Table 2. The patterns were those of heterozygotes for the 19 investigated alleles and of the relevant homozygous or compound heterozygous genotypes. At least one normal subject was reported for each amplicon in order to visualize the position of the normal homoduplex. Sequence variations and genotypes reported were indicated according to the HGVS Mutation Nomenclature. Reference sequences were GenBank: NG_000006.1 (genomic, alpha globin region); NM_000517.3 (HBA2 mRNA); and NM_000558.3 (HBA1 mRNA). For details see the Results section in text.**

and heteroduplex separation were empirically determined by testing a range of conditions (Table 2).

Six amplicons were used: Ia, II, III, IV, IVa, and V (Fig. 1B; Table 2). Amplicon I was useless because it had a long segment with homogeneous high TM; it would melt out at the high temperatures used for DG-DGGE. On the contrary, in Amplicon Ia the 183-bp region 5′ to the ATG (−239/−57) with high CG content and high TM was helpful in the stabilization of the partially denatured fragment, and the region corresponding to Amplicon I had homogeneous TM lower than that of the 183-bp region. Amplicon IVa had the same application of Amplicon IV, but in addition it detected the sequence variation HBA2:c.565G>A.

All carriers and patients were analyzed; electrophoretic patterns are reported in Figure 3.

Normal genotypes gave a single homoduplex band in all amplicons with the exception of Amplicon III, which was associated with two bands, one homoduplex and one heteroduplex, because of the 7-bp length difference between the two α-globin genes.

In carriers, anomalous patterns with additional bands (homoduplex and heteroduplex) were detected in the analysis of amplicons with one or two sequence variations *in cis* (Fig. 3; Amplicon Ia, lane 8, and Amplicon IVa, lane 7). Two sequence variations, HBA2:c.193G>A and HBA2:c.237C>G, located in the region of the exon2 with CG content 73% and with high TM domain were not detected at all (Fig. 3; Amplicon II, lanes 3–4). No improvement was obtained by elongating the Amplicon II at the 5′ and 3′ ends or by adding the GC_{45} clamps to the primers, or by increasing the electrophoresis temperature up to 75°C. The HBA1:c.349G>A was not detected with Amplicon V (lane 2) but only with Amplicon III (lane 1), where it was in a lower TM domain at the end of the amplicon (Fig. 1). Sequence variations of the third exon of the α2-globin gene were detectable with Amplicons IV and IVa.

Genotypes homozygous for variant alleles produced patterns with the anomalous homoduplex band alone when a single locus was amplified (Fig. 3; Amplicon IV, lane 8, and Amplicon IVa, lane 6). On the contrary, homozygotes were characterized by patterns with homoduplex and heteroduplex bands if the two loci α2 and α1 were amplified; indeed, the patterns showed an abnormal homoduplex band with a higher intensity than in heterozygotes (Fig. 3; Amplicon Ia, lanes 5–6). In two cases, mutated homoduplex comigrated with the normal one as for the two sequence variations HBA2:c.95+2_95+6del (Fig. 3; Amplicon Ia, lanes 10–12) and HBA2:c.391G>C (Amplicon IV, lane 3). In these cases, the identification of homozygous or hemizygous genotypes required additional analysis such as the amplification of a single locus (Amplicons Ia, II, and III) or reanalysis following prehybridization with normal sequences (Amplicons IV, IVa, and V).

Compound heterozygous genotypes for sequence variations in the same amplicon were revealed by patterns with multiple anomalous homoduplex and heteroduplex bands (Fig. 3; Amplicon Ia, lanes 9 and 13, and Amplicon IVa, lane 5). The exception was the genotype HBA2:[c.427T>C; c.565G>A]+[c.565 G>A], which gave a pattern with a single band typical of homozygosis for the HBA2:c.565G>A; multiple heteroduplex bands were detected after prehybridization with normal sequence (Fig. 3; Amplicon IVa, lanes 1, 2, 3, and 6). Compound heterozygotes for the hybrid gene HBA2:c.301-28_HBA1:c.301-35del behaved as hemizygotes when analyzed with Amplicons IV and IVa, amplified with primers specific for the 3′ UTR sequences of the α2 globin gene (Fig. 3; Amplicon IV, lane 3).

DISCUSSION

We set up the best analysis conditions and evaluated the overall sensitivity and suitability of DHPLC and DG-DGGE in the scanning of the HBA2 (α2) and HBA1 (α1) globin genes, a system of duplicated genes with sequence homology >96%. Furthermore, the two genes had average CG content >60% and showed a complex TM profile.

We split the genes in four regions and designed seven overlapping amplicons to enhance the sensitivity of both methodologies (Fig. 1B). A remarkable improvement of the system was obtained. First, each amplicon contained not more than three TM domains and thus the complexity of the TM profile was reduced. Second, it was possible to analyze some sequence variations in two amplicons showing different TM profiles. Third, the regions in which the profile with the very high TM domains could not be changed by varying the amplicon length were reduced to two short segments, a first one in exon2 with 73% CG content and a second one in IVS2 with 80% CG content. The two segments were about 13% of the entire gene sequence from the cap site to the polyA addition site. Last, we had a unique set of amplicons suitable for both DHPLC and DG-DGGE.

We also chose to coamplify amplicons from both α2 and α1 genes; this was possible only from the cap site to exon3 because of the sequence divergences in the 3′ UTR. The coamplification has the great advantage of contemporaneously scanning the two genes and reducing the number of PCR amplifications and of the DHPLC or DG-DGGE analyses by >50%; in addition, it provides the sequences of other alleles (normal or mutated) necessary for the formation of the heteroduplex in deletion carriers or in homozygous patients.

Both the methodologies gave reproducible results and no false-positive was observed.

DHPLC showed 100% sensitivity for the tested sequence variations; no marker was available for the 37-bp segment with high TM in IVS2. Thus, at least 96% of the sequence of the two genes from the cap site to the polyA addition site could be reliably scanned by DHPLC. Indeed, the high resolving power shown by DHPLC led us to foresee that 100% of the sequence could most probably be successfully explored. No previous studies of these mutants with DHPLC were available and no comparison can be carried out.

DG-DGGE gave no false-positive results, but showed about 90% sensitivity because it detected 17 out of 19 alleles and 27 out of 29 genotypes. The two sequence variations HBA2:c.193G>A and HBA2:c.237C>G gave false negative results. The principal reason for missing these variant alleles was most likely their placement in the region with high TM in exon2. Considering also that the sequence variations in the region with high TM domain in IVS2 (not available) will most likely escape detection, it can be assumed that about 13% of the two genes could not be explored with DG-DGGE. This result is significantly more advanced than the previous results with DGGE [Harteveld et al., 1996]; in fact, the α-globin gene sequence that can be explored increased from the previous value of 68% to the present value of 87%. Sequence variations undetected with DG-DGGE fall in a region that remained unresolved in the DGGE system, which also resulted in the detection experiments we carried out using amplicons and conditions previously reported [Harteveld et al., 1996].

The DG-DGGE and the DHPLC patterns were specific and in most cases we were able to identify the genotype. Nevertheless, a confirmation of the mutation or its correct identification, the assignment to one of the two genes (α2 or α1) involved, and the definition of the α-globin genotype (heterozygous, homozygous, or compound heterozygous) required a subsequent "direct" molecular test. There are several reasons for this step. First, in the analysis of the region from the cap site to exon3, the Amplicons I, Ia, II, and III are coamplified from both α2 and α1 genes; this coamplification eliminates the gene specificity and in addition could mask homozygosis. Second, there are complex genotypes in which one mutation can be completely masked. Third, the genome variability

may impair specificity of patterns by producing sequence variations with DHPLC or DG-DGGE patterns most similar to other patterns. Last, there are variant alleles involving the same base change in the same position but in different genes (α2 or α1) as the new HBA2 variant (reported in this article) of the common HBA1:c.1A>G (previously reported by Pirastu et al. [1984]). The gold-standard method for the mutation confirmation is PCR DNA sequencing of the amplicon with anomalous pattern, which can be carried out in the proband(s) of each family. However, setting up ARMS conditions [Old et al., 1990] for the confirmation of the three most frequent pathogenic variant alleles could be of great help, as has already been realized for some α-thalassemia pathogenic alleles common in Southeast Asia [Eng et al., 2001].

Utilization of DHPLC or DG-DGGE allows the adoption of a fast and low cost strategy for the identification of alleles of the α-globin genes. Considering the location and distribution of known sequence variations along the genes, scanning can start with the 5′ and 3′ regions of the two genes, followed only in the negative cases by the study of other regions. PCR sequencing for confirmation of common sequence variations or for the identification of rare or new ones is restricted only to one amplicon showing an anomalous pattern amplified from HBA2 and/or HBA1. On the contrary, methods available to date, such as reverse dot-blot or multiplex ARMS, only address the identification of carriers of the most common sequence variations; in all the remaining subjects, PCR sequencing of both the two α-globin genes is needed.

Three nonpathogenic sequence variations, HBA2:c.−24C>G, HBA1:c.475C>A, and HBA2:c.565G>A were easily detected with DHPLC and DG-DGGE. Preliminary results indicate that the carrier frequency ranges from 5 to 15% in families from Southern Italy (unpublished results). The assessment of their state (±) is a most important tool in the studies of population genetics and in studies of the origin and spreading of variant alleles. In addition, they are excellent markers for deletions *in trans* in the family studies. This is the case of a family under study for a new deletion: the mother was heterozygous, while the father and the two children were apparently homozygous, but they were actually hemizygous for HBA2:c.565 "G" (father) and "A" (children).

In conclusion, DHPLC had the power to detect 100% sequence variations in the α-globin gene in spite of the extremely high CG content and the complex duplicated structure of this system. DG-DGGE showed a lower sensitivity (about 90%). Indeed, both the methodologies are suitable and reliable and can strongly contribute to research on sequence variability of the α-globin genes as well as to molecular diagnosis of α-thalassemia or of the Hb α-variants. Moreover, this study offers a suitable model for duplicated genes with high homology and can be helpful in the procedures for scanning genes with high CG content.

ACKNOWLEDGMENTS

We thank the families for their consent to this study and for their collaboration. We thank Mr. Romeo Prezioso, from Istituto di Genetica e Biofisica "Adriano Buzzati Traverso," CNR, Napoli, for his skillful aid in collecting and transferring experimental results and family pedigree data in an electronic database, and in the elaboration of a specific software.

REFERENCES

Aissani B, Bernardi G. 1991. CpG islands, genes and isochores in the genomes of vertebrates. Gene 106:185–195.

Bowden DK, Vickers MA, Higgs DR. 1992. A PCR-based strategy to detect the common severe determinants of alpha thalassaemia. Br J Haematol 81:104–108.

Chan V, Yam I, Chen FE, Chan TK. 1999. A reverse dot-blot method for rapid detection of non-deletion alpha thalassaemia. Br J Haematol 104:513–515.

Colosimo A, Guida V, De Luca A, Cappabianca MP, Bianco I, Palka G, Dallapiccola B. 2002. Reliability of DHPLC in mutational screening of beta-globin (HBB) alleles. Hum Mutat 19:287–295.

Cremonesi L, Firpo S, Ferrari M, Righetti PG, Gelfi C. 1997. Double-gradient DGGE for optimized detection of DNA point mutations. Biotechniques 22:326–330.

den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 15:7–12. [Erratum: Hum Mutat 20:403.]

den Dunnen JT, Antonarakis SE. 2001. Nomenclature for the description of human sequence variations. Hum Genet 109:121–124.

den Dunnen JT, Paalman MH. 2003. Standardizing mutation nomenclature: why bother? Hum Mutat 22:181–182.

Dodé C, Krishnamoorthy R, Lamb J, Rochette J. 1993. Rapid analysis of -alpha 3.7 thalassaemia and alpha alpha alpha anti 3.7 triplication by enzymatic amplification analysis. Br J Haematol 83:105–111. [Erratum: Br J Haematol 1993 83:684]

Eng B, Patterson M, Walker L, Chui DH, Waye JS. 2001. Detection of severe nondeletional alpha-thalassemia mutations using a single-tube multiplex ARMS assay. Genet Test 5: 327–329.

Fischel-Ghodsian N, Nicholls RD, Higgs DR. 1987. Unusual features of CpG-rich (HTF) islands in the human alpha globin complex: association with non-functional pseudogenes and presence within the 3′ portion of the zeta gene. Nucleic Acids Res 15:9215–9225.

Foglietta E, Bianco I, Maggio A, Giambona A. 2003. Rapid detection of six common Mediterranean and three non-Mediterranean alpha-thalassemia point mutations by reverse dot blot analysis. Am J Hematol 74:191–195.

Ghanem N, Girodon E, Vidaud M, Martin J, Fanen P, Plassa F, Goossens M. 1992. A comprehensive scanning method for rapid detection of beta-globin gene mutations and polymorphisms. Hum Mutat 1:229–239.

Hardison RC, Chui DHK, Riemer C, Giardine B, Lehvaslaiho H, Wajcman H, Miller W. 2001. Databases of human hemoglobin variants and other resources at the globin gene server [Review]. Hemoglobin 25:183–193.

Hardison RC, Chui DHK, Riemer C, Giardine B, Lehvaslaiho H, Wajcman H, Miller W. 2004. HbVar: a database of human hemoglobin variants and thalassemias. http://globin.cse.psu.edu/hbvar/menu.html.

Harteveld KL, Heister AJ, Giordano PC, Losekoot M, Bernini LF. 1996. Rapid detection of point mutations and polymorphisms of the alpha-globin genes by DGGE and SSCA. Hum Mutat 7:114–122.

Higgs DR, Hill AV, Bowden DK, Weatherall DJ, Clegg JB. 1984. Independent recombination events between the duplicated human alpha globin genes; implications for their concerted evolution. Nucleic Acids Res 12:6965–6977.

Higgs DR, Vickers MA, Wilkie AO, Pretorius IM, Jarman AP, Weatherall DJ. 1989. A review of the molecular genetics of the human alpha-globin gene cluster. Blood 73:1081–1104.

Lacerra G, Fioretti G, De Angioletti M, Pagano L, Guarino E, de Bonis C, Viola A, Maglione G, Scarallo A, De Rosa L, Carestia C. 1991. (Alpha)alpha 5.3: a novel alpha(+)-thalassemia deletion with the breakpoints in the alpha 2-globin gene and in close proximity to an Alu family repeat between the psi alpha 2- and psi alpha 1-globin genes. Blood 78:2740–2746.

Lacerra G, De Angioletti M, Di Girolamo R, Sciorio A, Testa R, Schilliró G, Carestia C. 1997. Hb Caserta e Hb Bronte: two novel hemoglobin variants caused by alpha-2 globin gene mutation. In: Proceedings of the 6th International Conference on Thalassaemia and the Haemoglobinopathies. The Thalassemia International Federation, Malta, April 5–10. Abstract no. 51.

Lacerra G, Testa R, De Angioletti, Schiliró G, Carestia C. 2003. Hb Bronte or α93(FG5)Val→Gly: a new unstable variant of the α2-globin gene, associated with a mild α+ -thalassemia phenotype. Hemoglobin 27:149–159.

Lerman LS, Silverstein K. 1987. Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis. Methods Enzymol 155:482–501.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215.

Myers RM, Maniatis T, Lerman LS. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol 155:501–527.

Old JM, Varawalla NY, Weatherall DJ. 1990. Rapid detection and prenatal diagnosis of beta-thalassaemia: studies in Indian and Cypriot populations in the UK. Lancet 336:834–837.

Orkin SH, Goff SC, Hechtman RL. 1981. Mutation in an intervening sequence splice junction in man. Proc Natl Acad Sci USA 78:5041–5045.

Pirastu M, Saglio G, Chang JC, Cao A, Kan YW. 1984. Initiation codon mutation as a cause of alpha thalassemia. J Biol Chem 259:12315–12317.

Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, Davis RW, Cavalli-Sforza LL, Oefner PJ. 1997. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 7: 996–1005.

Weatherall DJ, Clegg JB. 1986. The thalassemia syndromes. Oxford: Blackwell Scientific Publications. p 508–612.

Weatherall DJ. 2001. Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat Rev Genet 2:245–255.