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ORIGINAL ARTICLE

Hb VILA REAL [β36(C2)Pro → His] IN ITALY: CHARACTERIZATION OF THE AMINO ACID SUBSTITUTION AND THE DNA MUTATION

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ABSTRACT

A rare high oxygen affinity hemoglobin variant was identified in a 22-year-old male patient from Napoli (Naples, Italy) affected by erythrocytosis. A detailed structural characterization of the variant hemoglobin was carried out, both at the protein and DNA levels essentially by mass spectrometric procedures and allele-specific amplification techniques. The amino acid substitution was determined by liquid chromatography tandem mass spectrometric analysis of the tryptic digest as β36(C2)Pro → His; the corresponding DNA mutation was identified as C → A at the second position of codon 36 of the β chain (C*C*T → C*A*T). These variations identified the presence of Hb Vila Real, described only once before in a Portuguese woman. Haplotype analysis of DNA polymorphisms showed that the β-globin gene of Hb Vila Real was associated with haplotype I.

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INTRODUCTION

Hb Vila Real is an extremely rare high oxygen affinity variant hemoglobin (Hb) carrying the substitution Pro → His at position 36 of the β chain. This variant was described in 2000 in an asymptomatic 15-year-old Portuguese girl, and her mother with a long history of polycythemia (1). The abnormal Hb was undetectable by conventional electrophoresis and isoelectrofocusing (IEF) techniques and its presence was eventually revealed by cation exchange high performance liquid chromatography (HPLC). Finally, reversed phase HPLC analysis showed the presence of a variant β chain. Localization of the DNA mutation in exon 2 of the β chain was accomplished by single strand conformation analysis (SSCA), and DNA sequencing showed the mutation at codon 36 (1).

This paper reports the first observation of Hb Vila Real in Italy, in a patient from Napoli (Southern Italy) affected by erythrocytosis. The variant Hb was structurally characterized at the protein level essentially by mass spectrometric techniques, leading to the identification of the amino acid substitution Pro → His at position 36 of the β chain. The corresponding DNA mutation was confirmed as C*C*T → C*A*T by the amplification refractory mutation system (ARMS). This is only the second known instance of Hb Vila Real.

MATERIALS AND METHODS

Routine Examinations

Blood samples were collected with EDTA as anticoagulant. Hematological data were obtained by standard procedures. Red cell lysates were analyzed by electrophoretic techniques both at alkaline pH on cellulose acetate and at acidic pH on agar citrate. The different Hb components were separated and quantitated by cation exchange HPLC using the VARIANT^{TM} system according to the procedures provided by the manufacturer (Bio-Rad Laboratories, Richmond, CA, USA). Hb heat stability and isopropanol precipitation tests were performed as indicated (2).

Structural Characterization of the Variant Hemoglobin

The hemolysate from the red cells was directly analyzed by "on line" liquid chromatography-mass spectrometry (LC/MS) using an LCQ ion trap instrument (Finnigan Corporation, San Jose, CA, USA) equipped with an HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) as previously described (3,4). Mass spectra were acquired and elaborated by the software provided by the manufacturer. Individual globins were purified by preparative HPLC on an HP 1100 apparatus (Agilent Technologies) as reported before (3). Protein fractions were manually collected and dried down in a Speed Vac centrifuge (Savant, Farmingdale, NY, USA).

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Tryptic hydrolysis of individual globins was performed in 0.4% ammonium bicarbonate (pH 8.5) at 37°C for 4 hours using an enzyme to substrate ratio of 1:50 w/w. An aliquot of the resulting peptide mixture was directly analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS) on a Voyager DE mass spectrometer (Applied BioSystems, Boston, MA, USA), as previously reported (5). The tryptic digest was also analyzed by "on-line" LC/MS techniques using the LCQ ion trap mass spectrometer (Finnegan Corporation) described above. The peptide mixture was fractionated on a narrow bore C_{18} Jupiter reverse phase column (250 × 2.1 mm, 300 Å) (Phenomenex Inc., Torrance, CA, USA) using a two-step gradient from 7 to 17% of acetonitrile in 0.1% trifluoroacetic acid (TFA) over 5 minutes, and to 50% over 45 minutes, at a flow-rate of 0.2 mL/min. The effluent was inserted directly into the ion source of the LCQ instrument (Finnegan Corporation) and both electrospray (ES) and ES tandem mass spectra were acquired throughout the entire analysis by using the software provided by the manufacturer.

DNA Analysis

DNA was obtained from peripheral blood leucocytes by phenol-chloroform extraction and the mutation characterized by polymerase chain reaction (PCR)-ARMS. The β-globin gene was amplified in 30 µL of amplification mixture containing 50 µM dNTPs and 0.5 µM of each primer. The β mutation was detected using allele-specific amplification (ASA) of the Hb Vila Real mutation. The following primers were employed: 5′-CAAAGGACTCAAAGAACCTCTG-GGTCGAAT-3′ and 5′-ACCTCACCCTGTGGAGCCAC-3′, with the former containing the expected base mutation for β36His at the 3′ end, and a further mismatched base located four nucleotides upstream of the 3′ end to enhance specificity according to Old et al. (6). PCR amplification was performed using the thermal gradient profile already described (3).

Restriction Fragment Length Polymorphism (RFLP) Haplotypes

β-Globin gene RFLPs (*Hinc*II/ε; *Hind*III/$^{G}\gamma$ and $^{A}\gamma$; *Hinc*II/$\psi\beta$ and 3′$\psi\beta$; *Ava*II/β; *Bam*HI/3′β) were analyzed in all members of the family, and RFLP haplotypes were defined through family linkage analysis (7).

RESULTS

Case Report

The proband (Fig. 1, II-1) was a 22-year-old male of Campanian origin, who was referred to the Thalassemia Unit of the Cardarelli Hospital in Naples, Italy,

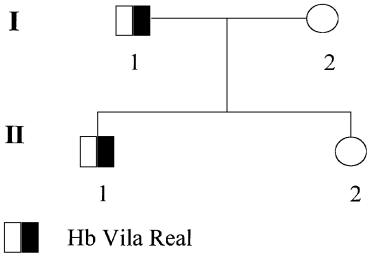

Figure 1. Pedigree of the Italian family with Hb Vila Real.

and was found to be affected by erythrocytosis. Bone marrow biopsy and cytogenetic analysis ruled out the occurrence of myeloproliferative disorders. Hematological findings of the proband and his family are reported in Table 1.

Hemoglobin Analysis

Both cellulose acetate (pH 8.6) and agar citrate (pH 6.0) electrophoreses showed only normal Hb components. The hemolysate was then fractionated by ion exchange chromatography on the VARIANT^{TM} HPLC system (Bio-Rad Laboratories) and the presence of an anomalous peak eluting in the Hb A_2 region was easily detected. A relative abundance of about 47% was estimated for the abnormal Hb by peak area integration. The isopropanol and heat stability tests performed on the whole blood were normal.

Abnormal Hemoglobin Analysis

The red cell lysate from the proband was directly analyzed by on-line LC/MS and the corresponding total ion current profile is shown in Fig. 2A.

Table 1. Hematological Data from the Family Carrying Hb Vila Real

Subject	I-1	I-2	II-1	II-2
Sex-Age	M-51	F-44	M-22	M-18
RBC (10^{12}/L)	6.3	4.6	6.1	4.9
Hb (g/dL)	19.7	14.2	20.3	16.2
PCV (L/L)	0.5	0.4	0.6	0.4
MCV (fL)	87.3	87.0	93.2	94.0
MCH (pg)	31.4	41.1	33.2	32.0
Reticulocytes (%)	1.0	0.8	1.1	0.7
Hb A_2 (%)	2.9	2.6	2.7	2.9
Hb F (%)	0.9	0.8	0.8	0.9
Hb Vila Real (%)	44.0	–	47.0	–
Isopropanol	–	–	–	–
Serum ferritin (ng/mL)	270.0	60.0	46.0	37.0

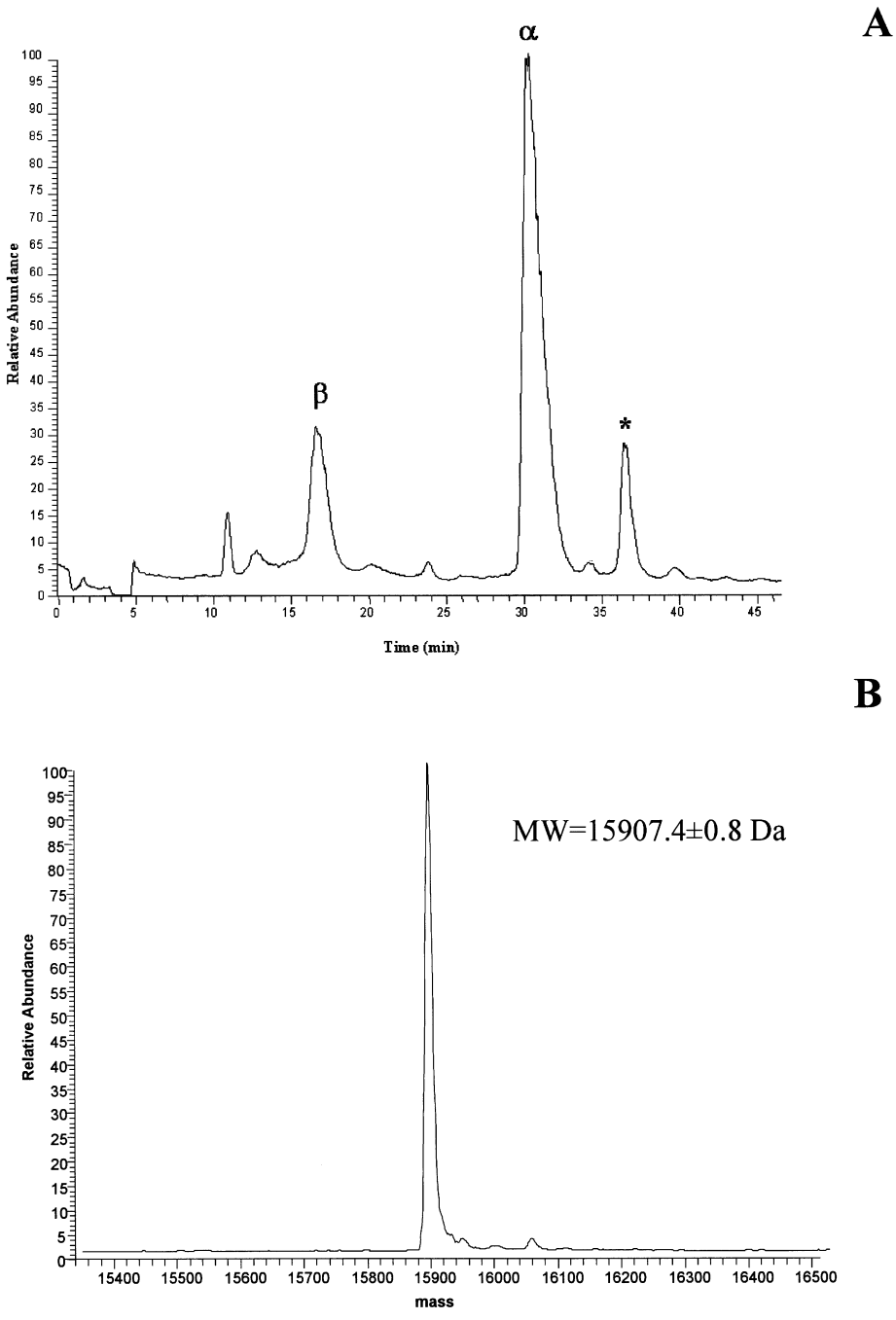

Figure 2. (A) TIC profile of the LC/MS analysis of a hemolysate sample from patient II-1. The abnormal β chain is indicated with an asterisk (*). (B) Transformed ES/MS of the variant globin; the measured molecular mass is indicated.

An abnormal globin chain eluting after the normal α chain was clearly detected and the corresponding ES/MS analysis displayed a molecular mass of 15,907.4 ± 0.8 Da (Fig. 2B). This mass value is about 40 Da higher than that of the normal β chain, indicating the occurrence of a β variant. On the basis of single point mutations, this mass difference could only be associated with a Pro → His substitution (8).

The variant globin was purified by preparative reversed phase HPLC, digested with trypsin, and the resulting peptide mixture directly analyzed by MALDI/MS. The mass spectrum showed the absence of the expected signal at m/z 1,275.5, corresponding to peptide 31–40. If the amino acid substitution had occurred within this peptide, and considering the estimated mass increase of 40 Da, peptide 31–40 would have shown a mass signal at m/z 1,315.5, exactly coincidental with the theoretical value of normal peptide 18–30.

An aliquot of the peptide mixture was then submitted to LC/MS analysis on a LCQ instrument (Finnegan Corporation) equipped with an ion-trap analyzer able to provide MS/MS analysis. Figure 3 shows the total ion current (TIC) profile of

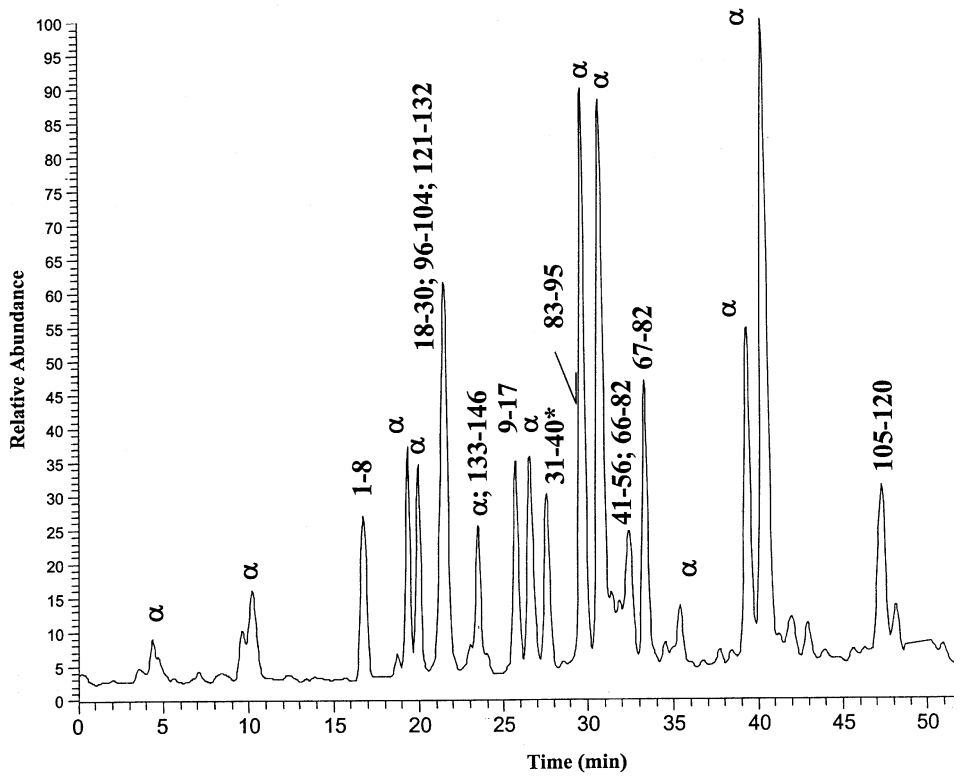

Figure 3. TIC profile of the LC/MS analysis of the tryptic digest of the variant chain. Each peak is associated with a peptide(s) within the β-globin sequence. The symbol α indicates peaks from the α-globin. The mutated peptide is indicated with an asterisk (*).

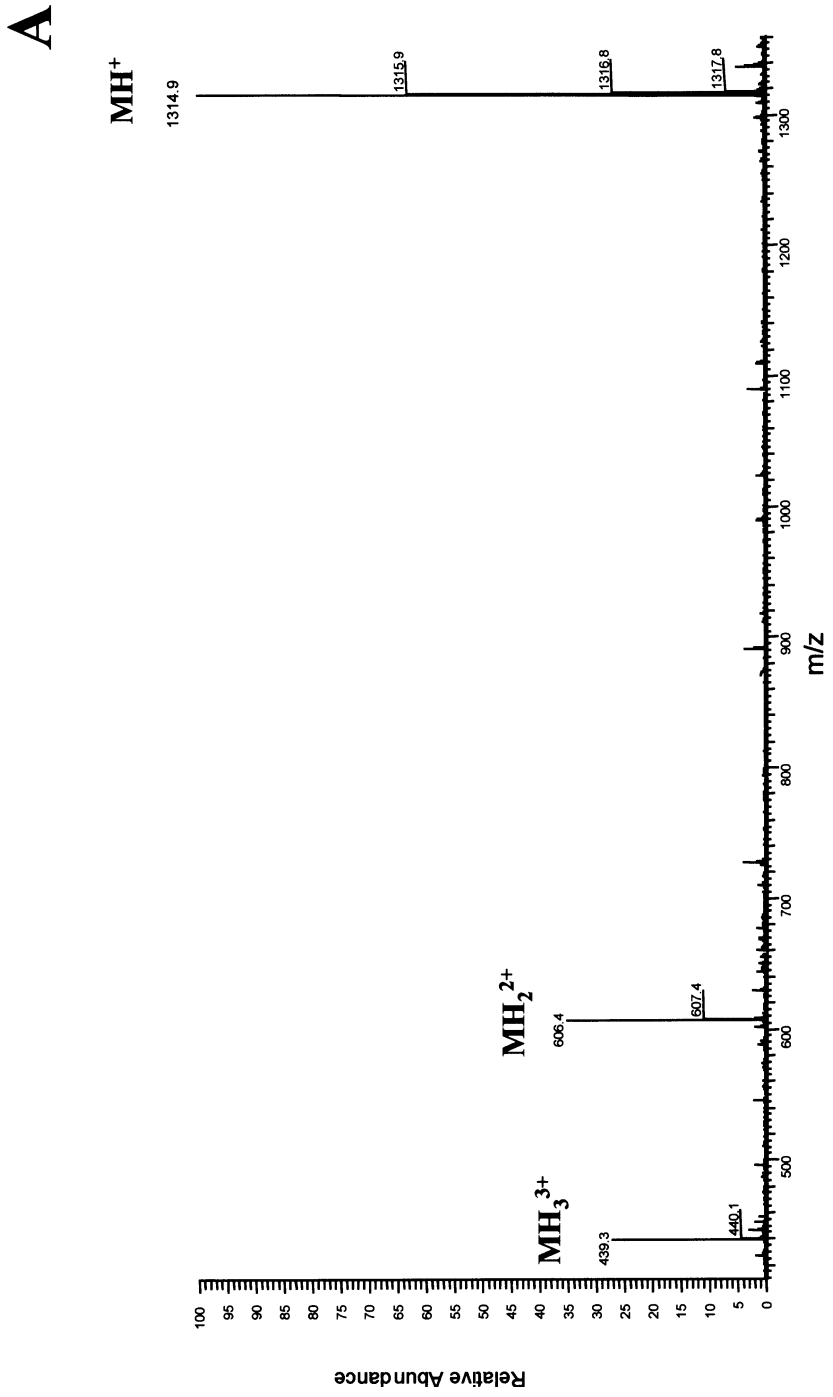

Figure 4. ES/MS (A) and tandem ES/MS (B) of the variant peptides (31–40) from the LC/MS/MS analysis of the tryptic digest of the variant chain. The y4 and y5 signals that identify the loss of the newly inserted His*β*36 are boxed. The letter "c" refers to a type of fragmentation from the C-terminal side.

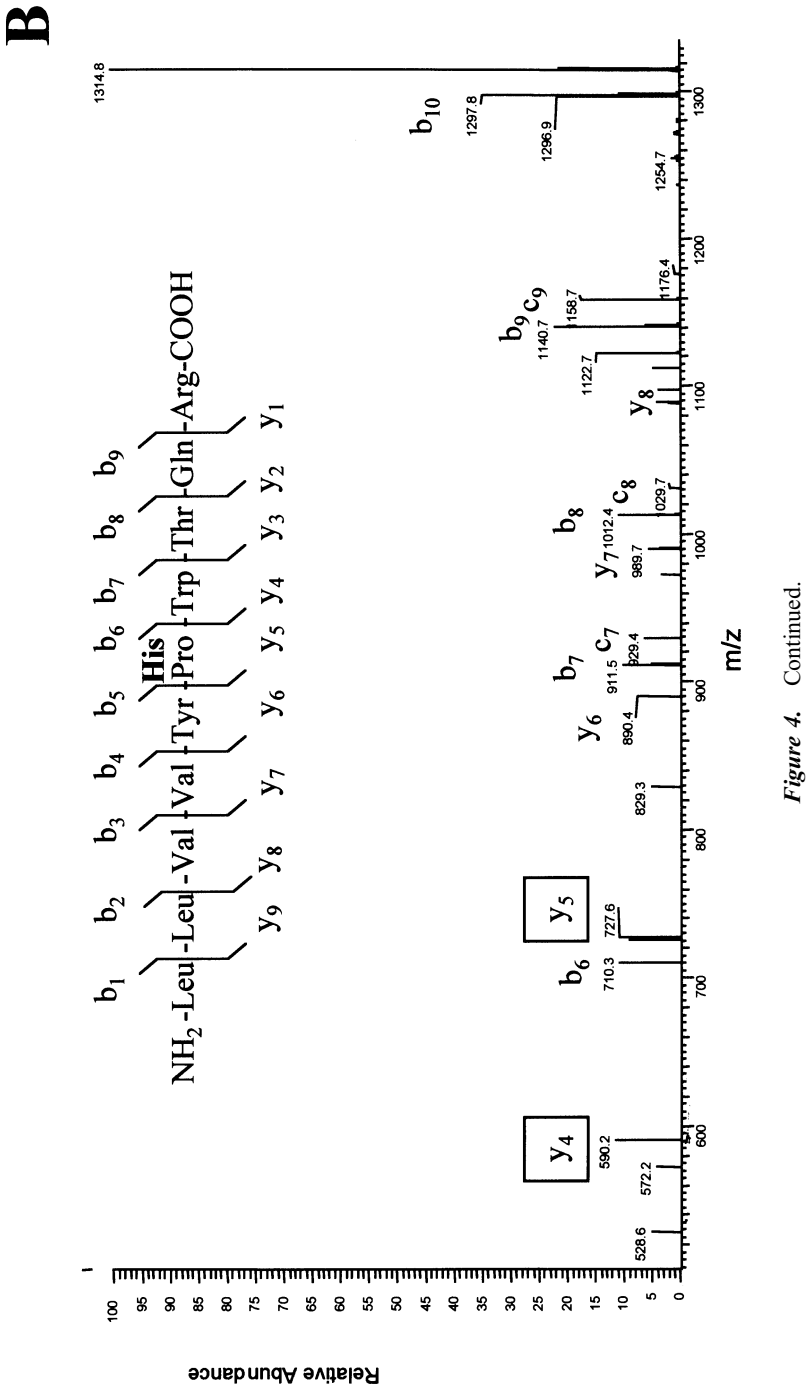

Figure 4. Continued.

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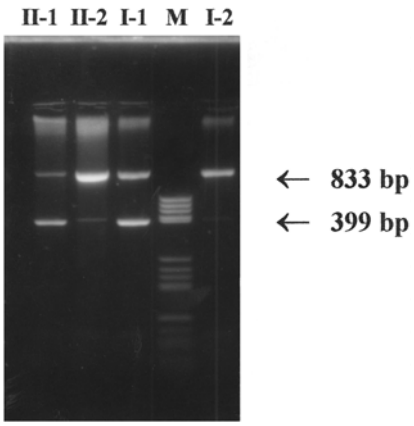

Figure 5. ASA for Hb Vila Real. Lane 1: proband (II-1); lane 2: (II-2); lane 3: (I-1) father; lane 4: markers; lane 5: (I-2) mother. The 399 bp fragment detected in lanes 1 and 3 indicates the presence of Hb Vila Real in the proband and his father. The 833 bp fragment is the amplification control.

the LC/MS analysis displaying the *β*-globin peptides identified by their characteristic mass values. The peak labeled with an asterisk is absent in the chromatogram of the normal *β*-globin digest, and its corresponding ES/MS analysis showed a singly charged ion at m/z 1,314.8 (Fig. 4A). This signal was isolated into the ion trap and fragmented by helium collision yielding the ES-MS/MS shown in Fig. 4B; fragmentation analysis identified the sequence of peptide 31–40. Moreover, the mass difference between fragment ions at m/z 727.6 (y5) and m/z 590.5 (y4) revealed the presence of histidine at position 36 substituting the normally occurring proline residue. This amino acid replacement identified the abnormal variant as Hb Vila Real (1).

DNA Analysis

The DNA mutation of Hb Vila Real at the second position of codon 36, from C*C*T to C*A*T, was confirmed by ASA or ARMS analysis. The DNA from the proband and his family were analyzed by using an oligonucleotide probe, synthesized on the basis of the structural data provided by the MS investigation. Figure 5 shows the resulting electrophoretic analysis; the occurrence of an abnormal 399 bp band peculiar of Hb Vila Real, absent in the control, was detected in the proband (II-1) and his father (I-1).

Haplotype analysis of DNA polymorphisms on the *β*-globin gene cluster was performed by amplification of the seven DNA segments containing the common polymorphic restriction sites occurring within the *β*-globin gene cluster (4), using different sets of oligonucleotide primers. This analysis showed that the *β*-globin gene from Hb Vila Real was associated with haplotype I, according to Orkin et al. (7).

DISCUSSION

The occurrence of an abnormal Hb was detected in a patient affected by erythrocytosis. A detailed structural characterization of the variant Hb, carried out at both the protein and DNA levels, eventually demonstrated the presence of Hb Vila Real or β36(C2)Pro → His. This Hb is a rare, high oxygen affinity variant, described only once before in a Portuguese woman with a long history of polycythemia (1).

The amino acid replacement Pro → His at β36(C2) was assessed by advanced MS methodologies including the LC/MS/MS analysis of the tryptic digest. The corresponding DNA mutation was established as C → A at the second position of codon 36 (C*C*T → C*A*T) by PCR-ASA experiments using a synthetic oligonucleotide probe designed on the basis of the MS results. The Hb Vila Real gene was associated with haplotype I according to Orkin et al. (7) by RFLPs through family linkage studies. This result reflects the frequency of haplotypes observed in β^A and β-thalassemia (thal) chromosomes of Campanian origin. Haplotype frequency analysis, in fact, had shown that haplotypes I and II account for more than 50% of both β^A and β-thal chromosomes (9).

The Pro → His substitution at β36(C2) caused an alteration of the physicochemical properties of the variant Hb that affected its chromatographic behavior. As a consequence, Hb Vila Real was eluted later than Hb A on the VARIANT™ ion exchange HPLC system (Bio-Rad Laboratories), that can then be useful for diagnostic purposes.

Four other mutations at position β36(C2)Pro have been described so far: Hb Linköping (→ Thr) (10), Hb Sunnybrook (→ Arg) (11), Hb North Chicago (→ Ser) (12), and Hb Brie Comte Robert (→ Ala) (13). In all cases, including Hb Vila Real, a bizarre hydrophobicity of the β chains was reported, with the variant globins eluting after the α chains on reversed phase HPLC. This behavior is rather unexpected for substitutions of proline with serine or threonine, and partly unaccountable even for a Pro → His replacement, suggesting the occurrence of conformational changes associated with these mutations. β36(C2)Pro is one of the invariant residues of the β chain in all known mammalian and most vertebrates Hbs. This amino acid is located between the B and C helices, and is involved in the $\alpha1\beta2$ contacts of the Hb molecule; its substitution changes the angle between these helices, altering contacts among several residues of $\alpha1$ and $\beta2$ chains, and originating changes in functional properties (11). As a consequence, all the β36(C2)Pro variants described so far exhibit an increased oxygen affinity.

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