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DETECTION OF α-GLOBIN GENE DISORDERS BY A SIMPLE PCR METHODOLOGY

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ABSTRACT

Background. **α thalassemias are very common in all thalassemic areas; however, complete knowledge of the phenotypic, genotypic and epidemiological features of these thalassemias has not yet been achieved for a number of reasons: the frequent absence of a thalassemic hematologic picture, the lack of a specific characteristic comparable to the Hb A_2 increase for β thalassemias, and the almost complete homology between the two α genes.**

Methods and Results. **A new set of PCR techniques, each based on primer(s) specific for a particular type of α globin gene disorder, has been devised in our laboratory. The procedures are simple, and non-radioactive. They lead to the identification of all α globin disorders common in the Mediterranean area [$-\alpha^{3.7}$, $-\alpha^{4.2}$, α^{HphI}, α^{NcoI}, – –MED, $-(\alpha)^{20.5}$, $\alpha\alpha\alpha^{anti\ 3.7}$]. The electrophoretic patterns specific for the main α globin alterations as observed with this set of techniques, are presented.**

Conclusions. **Owing to their advantageous properties, these techniques are suitable for precise molecular characterization of the numerous subjects selected through mass population screenings.**

Key words: α thalassemias, PCR methodology

α thalassemias are a group of inherited hemoglobin disorders based on alterations of the α_2 and/or the α_1 globin gene that cause a reduction or even a complete suppression of α globin chain synthesis. α_2 and α_1 globin genes are located close to the tip of the short arm of chromosome 16 (16p13-pter) in a region consisting of three duplicated segments (X, Y, Z) separated by non-homologous regions (Figure 1). The α genes are embedded in the homologous sub-regions Z_2 and Z_1; their coding regions are identical and these genes differ (Figure 2) only in the second intron (two single nucleotide substitutions and a 7-nucleotide insertion in the α_1 gene) and in the 3' untranslated region (18 single nucleotide substitutions and one single base deletion in the α_1 gene).

The most commonly adopted molecular subdivision of α globin gene alterations distinguishes *deletions* of one or both the α genes (large-scale variations due to displaced recombinational events) and *point mutations*.

Deletions

Among the single α globin gene deletions the most common is $-\alpha^{3.7}$, produced by a displaced crossing-over which occurred somewhere in the Z_2 and Z_1 homologous regions (rightward crossing-over, Figure 3). The two products of this displaced crossing-over are a *single* α gene and a *triplicated* $\alpha\alpha\alpha^{anti\ 3.7}$ gene cluster. In Italian subjects the $-\alpha^{3.7}$ deletion can be subdivided into two types according to the location of the crossing over site with respect to a marker site of the Z regions: type I, if the crossing-over site is to the left of the ApaI restriction site (thus the hybrid α gene has the ApaI site), and type II, if the crossing-over site is to the right of the ApaI

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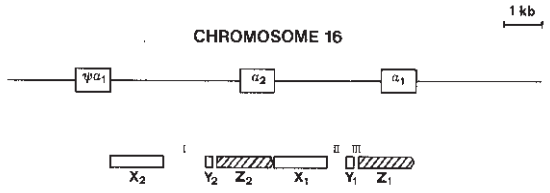

Figure 1. Organization of the α globin gene; of the homologous X, Y and Z regions and of the non-homologous regions.

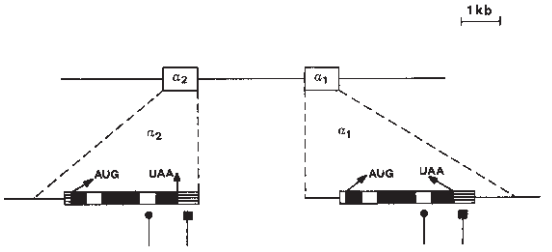

Figure 2. The α_2 and the α_1 globin genes.
□ = introns; ■ = coding sequences; ▤ = untranslated exonic sequences.
$\overline{Y}$ and $\overline{y}$ indicate the sequences (the 2nd intron and the 3' untranslated section of the 3rd exon) where the differences between α_1 and α_2 genes are located.

site (and thus the hybrid α gene has no ApaI site). A second, relatively common deletion of a single α gene is $-\alpha^{4.2}$, in which the homologous regions involved in the displaced crossing-over are X_2 and X_1 (leftward crossing-over, Figure 3). In this case the region deleted in the $-\alpha^{4.2}$ α-thalassemic cluster or added in the $\alpha\alpha\alpha^{4.2}$ triplicated α cluster is 4.2 Kb long and includes the whole α_2-globin gene. Other, rarer deletions (Figure 4) involving only the α_1 gene ($-\alpha^{3.5}$) or only the α_2 gene [$(\alpha)\alpha^{5.3}$] have been reported.[1,2] The $-\alpha^{3.7}$ deletion is common to all the thalassemic areas of the world, including Italy. The $-\alpha^{4.2}$ is found mainly in Southeast Asia, but is also present in our country.

Among the deletions of both α genes the ones most common[3,4] in the Mediterranean area are – –MED and $-(\alpha)^{20.5}$, whereas in Southeast Asia the most frequent is – –SEA.[1]

Point mutations

The most common α gene point mutations in the Mediterranean regions are: (a) the deletion of the TGAGG pentanucleotide located in the 5' region of the 1st intron of the α_2 gene that removes an HphI restriction site, and (b) a single nucleotide pair substitution (SNS) at the ini-

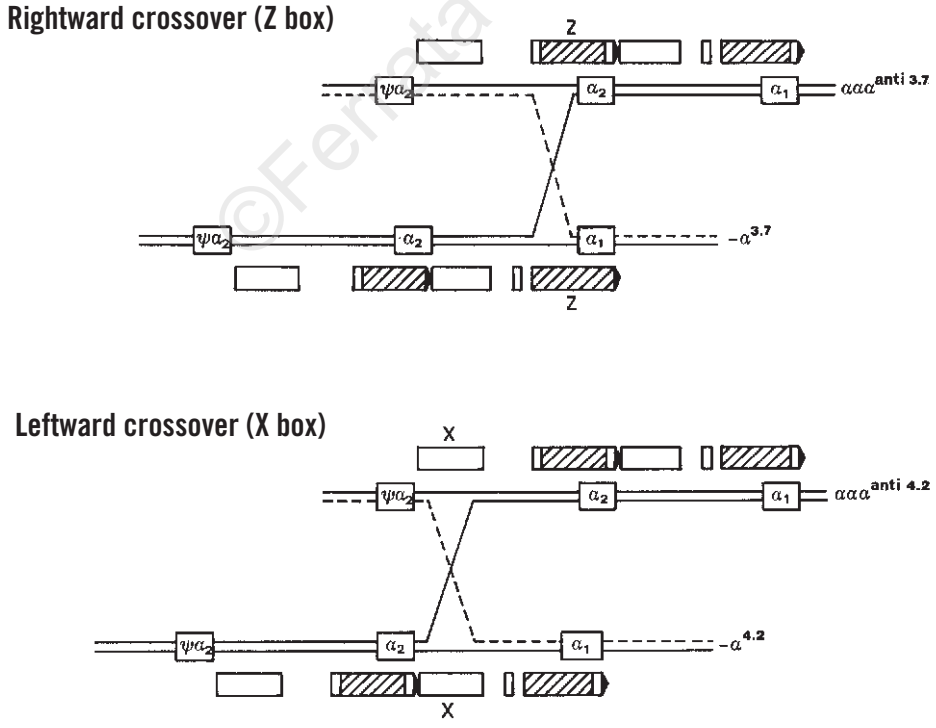

Figure 3. Displaced, but homologous, crossing-overs which produce the $-\alpha^{3.7}$ (Z boxes) and the $-\alpha^{4.2}$ (X boxes)[1].

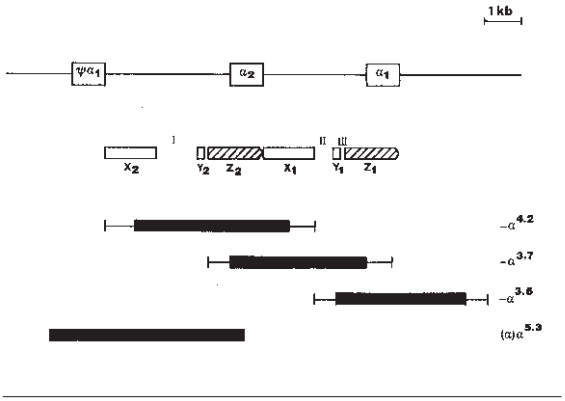

Figure 4. Site and extension (m) of the deletions within the α globin gene cluster in various types of α^+ thalassemias[1].

tiation codon (ATG→ACG) of the α_2 or (ATG→GTG) of the α_1 globin gene, which in either case removes an NcoI restriction site. In Saudi Arabia an SNS in the polyadenylation site (AATAAA→AATAAG) of the α_2 globin gene is rather common.

α-thalassemias can be divided into two classes according to the phenotype of the heterozygotes: α^+ thalassemias (or α-thal 2), caused by a defect in a single α globin gene and characterized by a mild reduction of α-globin chain synthesis (α/non-α globin chain synthesis ratio ≃0.80) and a minor or absent hematologic thalassemic phenotype; α° thalassemias (or α-thal 1), caused by complete suppression of α-globin chain synthesis by the affected cluster, with a clear-cut reduction of the α/non-α globin synthetic ratio (~0.60) and an evident hematologic thalassemic phenotype.

Homozygosity for α^+-thalassemias (of the same or of different types) may cause phenotypes that are indistinguishable from those associated with heterozygosity for α°-thalassemias.

Until a few years ago Southern blotting was the main technical approach for identifying in a single experiment a number of known and unknown varieties of α-thalassemias. However, this procedure is very time-consuming, cumbersome and involves radioactive materials, and is therefore not realistically feasible for studying many individuals.

In the last few years our laboratory has devised a series of simple, rapid, reliable, non-radioactive PCR techniques. These lead to unambiguous identification of the molecular lesion in almost all types of α-thalassemias and anti$^{3.7}$ α-gene triplication. Furthermore, they can be utilized not only for pure research purposes but also as the final, conclusive step in mass screening programs. They are in fact routinely utilized in our laboratory, where some 55,000 subjects are examined yearly (~15,000 in the laboratory and ~40,000 in schools during scholastic screenings)[5], and approximately 14.3% turn out to be carriers of an α-globin gene defect.[6]

In the present paper these techniques and their results are described.

Materials and Methods

DNA was purified from blood leukocytes by the salting out procedure of Miller *et al.*[7]

Amplification was carried out with a *DNA thermal cycler-480 Perkin Elmer* apparatus. It was checked on 1.5% agarose-TBE and the specific bands were visualized on a UV transilluminator after ethidium bromide staining. The DNA marker used in each experiment was DNA molecular weight marker VI cat. No 106250 Boehringer Mannheim. A blank test on the amplification buffer was carried out to detect possible contamination.

The following amplification buffer and thermal-cycling conditions were adopted for all PCR protocols ($\alpha^{4.2}$, α^{NcoI}, α^{HphI}, −−MED, $-(\alpha)^{20.5}$, $\alpha\alpha\alpha^{anti3.7}$ triplication), except for $-\alpha^{3.7}$.

One γ of genomic DNA was used for each PCR reaction (25 μL). The amplification buffer consisted of 2.5 mM $MgCl_2$, 50 mM Tris-HCl pH 8,9, 200 μM dNTPs, 13% glycerol, and contained 1.25 U native Taq polymerase (Perkin Elmer). The primer concentrations are shown in Table 2. A total of 30 cycles were performed: 5 cycles at 98°C for 1', 55°C 30", 72°C 45", and 25 cycles at 96°C for 30", 55°C 30", 72°C 45"; an additional 72°C 10' extension step was included.

For the $-\alpha^{3.7}$ deletion the amplification buffer was: 1.7 mM $MgCl_2$, 50 mM Tris-HCl pH 8.9, 200 μM dNTPs (G+C), 100 μM dNTPs (A+T), 13% glycerol, and contained 1.25 U native Taq (Perkin Elmer). A total of 35 cycles were per-

formed: 5 cycles at 98°C for 30", 66°C 90", 72°C 180", and 30 cycles at 96°C for 30", 66°C 30", 72°C 120", with an additional 15' extension at 72°C.

Subtyping of the $-\alpha^{3.7}$ genotype into type I and II was obtained by digesting the amplified PCR product with ApaI, followed by electrophoresis on 4% NU-SIEVE agarose.

Preliminary testing of these protocols was performed on a selected sample of 120 α thalassemia blood specimens previously characterized by Southern blotting.

The procedures adopted for the specific identification of the various α gene disorders and the documentation of the results are reported below. The sequences and the quantities of the primers are listed in Tables 1 and 2.

Table 1. Sequences and locations of the primers used in each PCR reaction.

Primer	Sequence
A (5' Z box α_2)	5' - CTGTCCTTTCCCTACCCAGAGCCA - 3'
B (3' α_1)	5' - CCATGCCTGGCACGCTTTGCTGAG - 3'
C (5' Y_1)	5' - GCTGAAGGGTGCTGACCTGATGCA - 3'
D (3' α_2)	5' - AACACCTCCATTGTTGGCACATTCC - 3
E (α_1/α_2) *	5' - CCCTGGGTGTCCAGGAGCAAGCC - 3'
F (3' $\psi\alpha_1$)*	5' - GGCACATTCCGGGACAGAGAGAA - 3'
G ($\psi\alpha_1$ exon 3)*	5' - CCGGTTTACCCATGTGGTGCCTC - 3'
L (5' α)	5' - TCCCCACAGACTCAGAGAGAACC - 3'
5' MED (upstream $\psi\zeta_1$)	5' - TCCCTGAAGCTGAAACATACAGGT - 3'
3' MED (downstream α_1)	5' - CCCCCGTCTTAGGTGTTCTGTAG - 3'
5' 20.5 (downstream ζ_2)	5' - GAGGCCAAGGTGGGATGGT - 3'
T37 (5' Z box α_1)	5' - GCTCCAGCCGGTTCCAGCTATTGC - 3'

*published in Baysal and Huisman.[5]

Results

Deletions

The $a^{3.7}$ deletion (Figure 5)

A mixture of three primers (A, B and C) allows one to unambiguously distinguish among homozygosity for the absence of this defect ($\alpha\alpha/\alpha\alpha$), $-\alpha^{3.7}/-\alpha^{3.7}$ homozygosity and $\alpha\alpha/-\alpha^{3.7}$ heterozygosity (Figure 5b). In fact, the αα normal cluster produces a CB amplified 2,300 bp fragment, but not an AB fragment (because A and B are too far from each other), whereas the $-\alpha^{3.7}$ cluster produces an AB fragment of 1,911 bp (the distance between A and B when 3.7 Kb have been deleted between them), but not a CB fragment (because the sequence complementary to C is not present in the $-\alpha^{3.7}$ cluster).

The existence of a marker distinguishing α_2 from α_1 [an ApaI restriction site in position 1,594 of the AB fragment (see Figure 6a) is present only in α_1] allows one to ascertain whether the crossing-over which produced $-\alpha^{3.7}$ occurred to the left or to the right of that site. In the first case ($-\alpha^{3.7}$ I type), the ApaI restriction site is present in the α gene so that the 1,911 bp fragment is split by ApaI into a fragment of 317 bp and a fragment of 1,594 bp). In the second case ($-\alpha^{3.7}$ II type), the 1,911 bp fragment does not contain the ApaI site so that it remains unsplit after incubation with ApaI (Figure 6b).

$-\alpha^{4.2}$, --MED and $-(\alpha)^{20.5}$ deletions

The specific primers utilized to look for these deletions are based on the same principles devised for the $-\alpha^{3.7}$ deletion: for each of these three deletions a mixture of oligonucleotides allows one to obtain one specific fragment from the normal αα cluster and one specific fragment from the α cluster with the deletion (Figures 5,

PCR product	Specific primers				Internal control primers
	primer 1		primer 2		
α_2	L	18 pmoles	D	18 pmoles	
α_1	L	18 pmoles	B	18 pmoles	
−−MED	5' MED	20 pmoles	3' MED	21 pmoles	L 23 pmoles + D 23 pmoles
$-(\alpha)^{20.5}$	5' 20.5	5 pmoles	B	23 pmoles	L 23 pmoles + D 23 pmoles
$-\alpha^{4.2}$	F	5 pmoles	G	14 pmoles	E 14 pmoles
$\alpha\alpha\alpha^{anti\ 3.7}$	T37	30 pmoles	D	30 pmoles	G 9 pmoles + F 9 pmoles
$-\alpha^{3.7}$	A	35 pmoles	B	28 pmoles	C 18 pmoles

Table 2. Quantities of the specific primers in each PCR reaction.

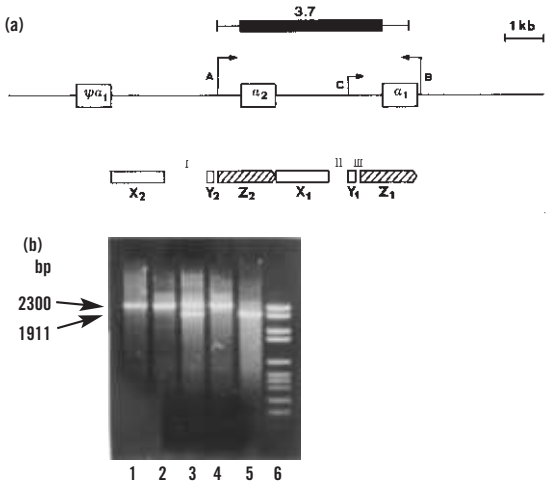

Figure 5. The -$\alpha^{3.7}$ cluster. A schematic representation of the normal α cluster and of the $\alpha^{3.7}$ cluster. Identification of the -$\alpha^{3.7}$ deletion by PCR and electrophoresis.
(a) The normal α cluster, the 3.7 Kb deletion and the sites of the sequences complementary to the primers utilized (A, B and C, see Table 1).
(b) Electrophoretic patterns of fragments derived from DNA of αα/αα, αα/-$\alpha^{3.7}$ and -$\alpha^{3.7}$/-$\alpha^{3.7}$ subjects amplified with specific primers. In αα/αα normal individuals (lanes 1, 2 and 4) one obtains a fragment of 2,300 bp that represent the distance between the sequences C and B (the site of the A sequence is too far from B to allow the production of an AB amplified fragment); in αα/-$\alpha^{3.7}$ subjects (lane 3) one obtains both the 1,911 bp and 2,300 bp fragments; in -$\alpha^{3.7}$/-$\alpha^{3.7}$ homozygous individuals (lane 5) one only obtains a 1,911 bp long fragment, which corresponds to the distance between A and B after the deletion of 3.7 Kb (the C site is absent because it was lost with the deletion). Lane 6: DNA marker.

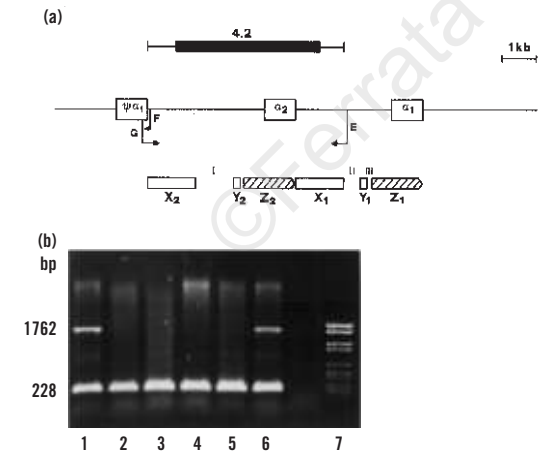

Figure 7. Detection of -$\alpha^{4.2}$ thalassemia; (a) the deletion and the positions of the sequences complementary to the specific primers E, F and G (see Table 1); (b) electrophoretic bands visible after amplification: a 228 bp long fragment (G and F primers*, internal control) and a 1,762 bp long fragment (G and E primers*) in subjects carrying the $-\alpha^{4.2}$ deletion. In this case these two primers can promote the amplification because, owing to the deletion, they are only 1,762 bp far from on another.
Lanes 1 and 6: individuals with the $-\alpha^{4.2}$ deletion. Lanes 2, 3, 4 and 5: Individuals with no $-\alpha^{4.2}$ deletion. Lane 7: DNA marker.
**Ref. #5*

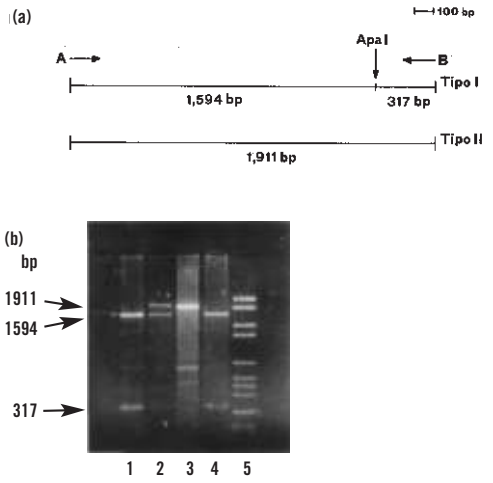

Figure 6. Identification of the two types (I and II) of the $-\alpha^{3.7}$ deletion by ApaI digestion of the PCR amplified $-\alpha^{3.7}$ cluster.
(a) Position of the ApaI site in the fragment obtained by PCR amplification of the $-\alpha^{3.7}$ cluster [(see (b) of Figure 5)].
(b) Electrophoretic patterns after ApaI digestion of the 1,911 bp amplified sequence.
Lanes 1 and 4: heterozygotes for the $-\alpha^{3.7}$ deletion type I;
Lane 2: a heterozygote for both $-\alpha^{3.7}$ type I and $-\alpha^{3.7}$ type II;
Lane 3: a heterozygote for the $-\alpha^{3.7}$ type II;
Lane 5: DNA marker.

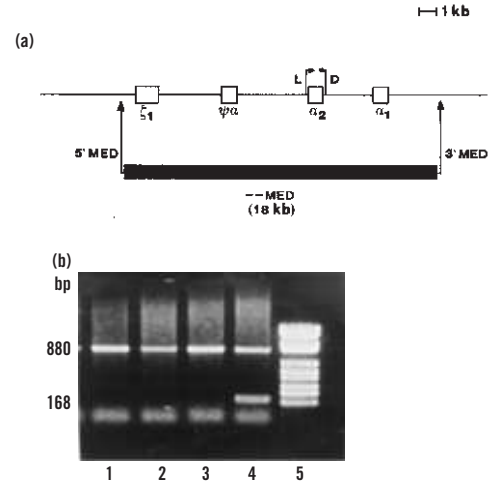

Figure 8. Detection of - -MED thalassemia. (a) The 18 Kb deletion and the sites of the sequences recognized by the specific primers. These primers are: 5' Med (upstream of $\psi\alpha_1$); 3' Med (downstream of α_1); L and D sites flanking the α_2 gene (see Table 1); (b) electrophoretic patterns after PCR amplifications of DNA from four individuals. Lanes 1, 2 and 3: Individuals with no - -Med deletion. Only an 880 bp band derived by PCR amplification of the LD region (present in the α cluster with no - -Med deletion) is visible; Lane 4: a heterozygote for the - -Med deletion and a normal αα cluster. The αα cluster gives rise to the 880 bp fragment whereas the - -Med cluster produces a 168 bp fragment derived from amplification of the region still left between the 5'Med and the 3'Med sequences after the - -Med deletion; Lane 5: DNA marker.

7, 8 and 9).

Point mutations

A preliminary step common to any point mutation consists of two amplifications, one of the α_2 and one of the α_1 gene, that must be carried out separately.

α^{NcoI}

This is a single nucleotide pair substitution in the initiation codon of either the α_2 (ATG→ACG) or the α_1 gene (ATG→GTG) that abolishes an NcoI restriction site in both cases. α_2 is amplified with primers A and D, and α_1 with primers T37 and B (Table 1). The positions of the sequences complementary to the primers are shown in Figure 10a. The 1,801 bp α_2 and α_1 fragments are then examined separately with NcoI. The initiation codon mutation is diagnosed in the α_2 or in the α_1 gene depending on which of these two gene fragments fails to be split into the 948 and 853 bp complementary fragments (Figure 10b).

α^{HphI}

This is a 5-nucleotide deletion (GAGGTGAG-GCTCC→GAGGCTCC) in the 5' end of the 1st intron of the α_2 gene that abolishes an HphI restriction site. It can be identified by incubating with HphI an 880 bp fragment obtained by a specific amplification of the α_2 gene (Table 1).

One of the fragments derived from this process, which is 456 bp long, is further subdivided into two visible complementary fragments (of 134 and 322 bp) if it is derived from the normal α_2 gene, whereas it remains unsplit if it is derived from the α^{HphI} allele of that gene and exhibits a length of 451 bp. Obviously, fragments of 250, 11 and 163 bp are produced by the digestion of DNA from both normal and mutated α genes (Figure 11).

$\alpha\alpha\alpha^{anti\ 3.7}$ *triplication*

As shown in Figure 3, the two complementary recombinant products of the displaced crossing-over in the Z region (Z_2 and Z_1) are the $\alpha^{3.7}$ deletion (see above) and the $\alpha\alpha\alpha^{anti3.7}$ triplication. The $\alpha\alpha\alpha^{anti3.7}$ cluster consists of three α genes: α_2, α_1-α_2; α_1 (Figure 12a).

This triplication is detected by two specific primers: T37 and D (Table 1). If a cluster contains an α_1-α_2 hybrid gene – and this can only occur in an ααα cluster – amplification with these two primers produces a 1,799 bp fragment which includes that gene (Figure 12b).

Discussion

α thalassemias are present throughout vast areas of the world and have such high prevalences in many parts of these areas that they make up a large share of all thalassemias. Phenotypically, however, they are much less identifiable than β thalassemias both at the hematologic and at the protein level: at the hematologic level because in many cases α thalassemias cause an extremely mild, if not altogether silent, phenotype which mimics sideropenia; at the protein level because they are not associated with a diagnostically precious feature such as the Hb A_2 increase in β thalassemias.

Thus, in contrast to β thalassemias, α thalassemias require DNA studies for mere detection and not only for full characterization. Until recently, Southern blotting was in practice the only approach available for DNA studies of globin genes. This technique, however, is cumbersome, time-consuming, expensive and requires technical skill, so that it can be realistically employed to study only a few individuals.

In the last few years a completely new approach, based on PCR, has been proposed. It was initially adopted for β thalassemias but was later successfully extended to α thal defects too.[2,9-12]

The set of reliable as well simple as PCR techniques devised in our laboratory allows one to identify all the α globin disorders common in the Mediterranean area [$-\alpha^{3.7}$, $-\alpha^{4.2}$, α^{NcoI}, α^{HphI}, $\alpha\alpha\alpha^{anti3.7}$, – –Med and $-(\alpha)^{20.5}$]. Furthermore, these techniques allow one to detect α globin gene disorders not only in a few sporadic subjects but also in great numbers of individuals identified by *mass* screening as neither normal nor β thalassemic (~18%, namely classes A, B and C, Figure 13). At present only 2% of these numerous subjects (≃ 0.4% of the total sample) remain incompletely diagnosed at the molecular level.

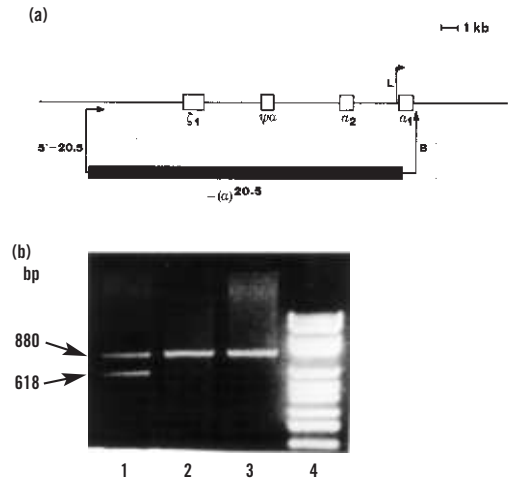

Figure 9. Detection of $-(\alpha)^{20.5}$ thalassemia.
(a) The $-(\alpha)^{20.5}$ deletion and the sites of the sequences complementary to the specific primers. These primers are: 5'-20.5 downstream from ζ_2; L and B (see Table 1). The sum of the 5' and 3' segments left after the -$(\alpha)^{20.5}$ Kb deletion is 618 bp;
(b) Electrophoretic patterns after PCR amplifications of DNA from three individuals.
Lane 1: a subject heterozygous for the $-(\alpha)^{20.5}$ deletion. Besides the 880 bp long fragment (L and B primers) derived from the normal αα cluster, which acts as an internal control, a specific 618 bp long fragment (5'-20.5 and B primers) is visible. 5'-20.5 and B work as primers because, following the deletion, they are only 618 bp apart;
Lanes 2 and 3: αα/αα normal individuals; Lane 4: DNA marker.

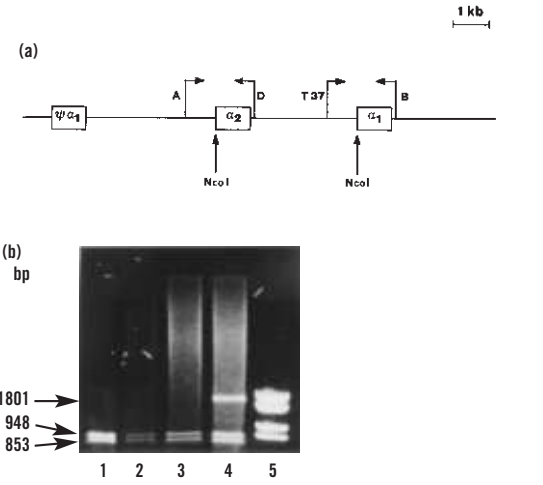

Figure 10. Detection of α^{NcoI} thalassemia.
(a) Positions of the sequences complementary to A, D (α_2 gene), T37 and B (α_1 gene) primers (see Table 1) and of the NcoI restriction site, which, following digestion with this enzyme of the AD or the T37 B amplified normal α gene, produces 948 and 853 bp long fragments from both these genes. The α^{NcoI} thal genes (α_2 or α_1) do not contain this site. Therefore the amplified AD or T37 B fragment is not split by NcoI and exhibits a length of 1,801 bp.
(b) Electrophoretic bands derived from NcoI digestion of normal or α^{NcoI} thalassemic genes.
Lanes 1, 2 and 3: αα/αα normal individuals;
Lane 4: αα/αα^{NcoI} subject;
Lane 5: DNA marker.

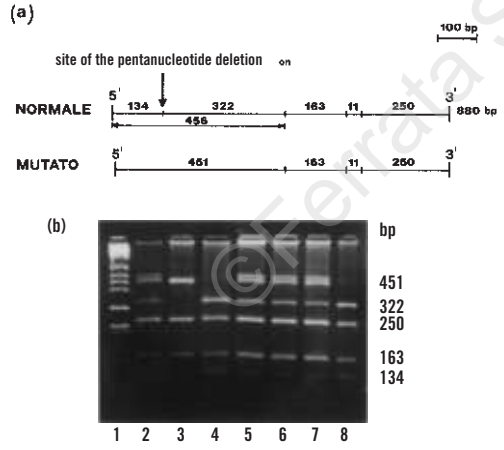

Figure 11. Detection of $\alpha^{HphI}\alpha$ thalassemia.
(a) Fragments derived from HphI digestion of a normal α_2 gene amplified with the L and D primers (see Table 1). The α^{HphI} gene has lost 5 nucleotides and this results in the loss of the HphI site of position 134, so that a single fragment of 451 bp [that is (134+322)-5] is formed in place of the 134 and 322 normal fragments;
(b) electrophoretic bands observed after LD amplification followed by HphI digestion of DNA from selected subjects.
Lane 1: DNA marker; Lane 3: $\alpha^{HphI}\alpha/\alpha^{HphI}\alpha$ homozygous subject; Lanes 4 and 8: αα/αα normal subjects; Lanes 2, 5, 6 and 7: αα/$\alpha^{HphI}\alpha$ heterozygous subjects. In these cases a heteroduplex complex appears close to the 451 bp fragment.

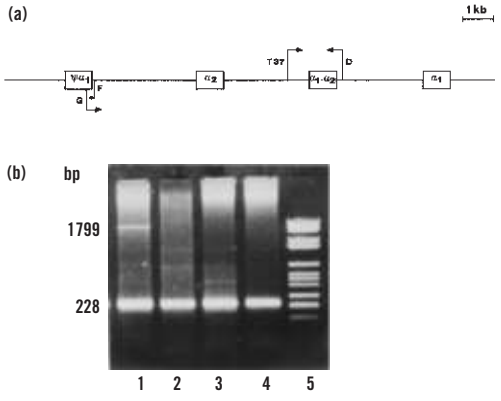

Figure 12. Detection of $\alpha\alpha\alpha^{anti3.7}$.
(a) A schematic representation of the $\alpha\alpha\alpha^{anti3.7}$ cluster and of the sites complementary to the specific primers (G, F, T37 and D, see Table 1). In the normal αα cluster only G and F primers can promote amplification, with the production of a 228 bp long fragment (internal control). In an $\alpha\alpha\alpha^{anti3.7}$ cluster the T37 and D primers recognize two sequences flanking the α_1-α_2 hybrid gene which are 1,799 bp far apart; (b) electrophoretic patterns of fragments derived from DNA of the $\alpha\alpha\alpha^{anti3.7}$ triplication.
Lane 1: an individual with the 228 bp fragment as internal control and the 1,799 bp fragment derived from the $\alpha\alpha\alpha^{anti3.7}$ cluster; Lanes 2, 3 and 4: αα/αα normal subjects; Lane 5: DNA marker.

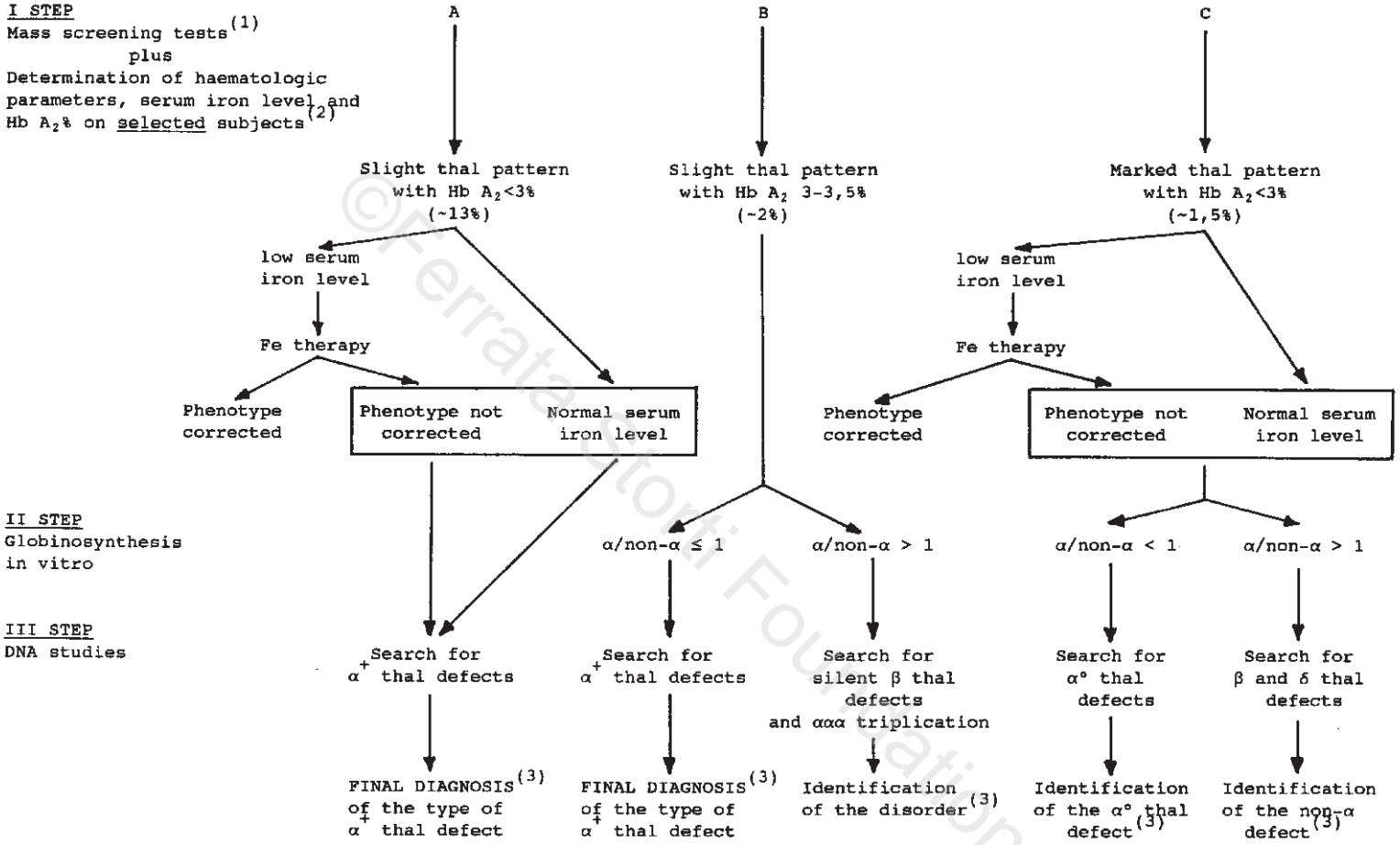

Figure 13. Chart flow of diagnostic tests which has been adopted to elucidate the etiopathogenesis of the phenotypic alterations found in subjects neither normal nor clearly thalassemic.
(1) red cell osmotlc fragility + microscopic examination of a fresh unstained thin blood smear;
(2) approximately 20% of the total sample of screened individuals. In order not to miss any thalassemic subjects, the criteria adopted to include an individual in this subsample were extremely unselective; even the faintest suspicion of abnormality was sufficient to process to the next analyses; (3) some 2% of these cases remain undiagnosed.

The diagnostic protocol routinely adopted in our laboratory is presented in Figure 13.

I step consisted of a red cell osmotic fragility test and of red blood cell morphology study for all individuals. For approximately 20% of selected individuals it consisted of sideremia studies and of the Hb electrophoretic pattern (Hb A_2 dosage). This step led to the identification of perfectly normal subjects and of typical β thalassemics (~80% of the total number, N, of subjects examined, who were excluded from the present study), and of three other classes of subjects, namely: class A, which includes individuals with a slight thal pattern, but with a normal percentage of Hb A_2 and normal or low sideremia; class B, which consists of a small group with a slight thal pattern and a moderate increase of Hb A_2 (3-3.5%); class C, which encompasses subjects with a hematologically marked thal phenotype, but a normal percentage of Hb A_2 and normal or low sideremia.

Class A is the largest group in our region (~13% of N); class B contains ~2% and class C ~1.5% of the selected subjects. All individuals with low sideremia were reexamined after adequate iron therapy and were not studied further if their phenotype was found to be normal.

II step. In vitro globin synthesis was performed on all subjects in classes A, B and C with normal sideremia and abnormal phenotype not corrected by iron therapy.

III step consisted of the DNA studies. The choice of the cluster (α or β) and of the type of DNA studies to be performed (search for α globin gene deletion or triplication, etc.) depended on the findings of steps I and II (Figure 13).

All subjects in class A selected for further studies went on to the II (globin synthesis) and the III step (DNA studies to search for α^+ thal defects). The decision to carry out DNA studies irrespectively of the globin synthesis findings was based on the well-known notion that some mild α thalassemias exhibit a normal hematologic and globin synthesis picture (adequate population studies estimating the prevalence of these *silent* α thalassemias have not yet been reported).

Class B: individuals were all studied for globin synthesis. Those with an α/non-α ratio ≤1 were examined for α^+ thal defects; those with an α/non-α ratio >1, for silent β thal defects (-101 C→T, IVS II 844 C→G, etc.) and for ααα triplication.

Class C: for these subjects the globin synthesis findings were especially relevant to the decision of which DNA investigations to undertake; if the α/non-α synthesis ratio was clearly greater than 1, the DNA studies were directed toward the search for β and δ globin defects because combined β and δ thalassemia is *a priori* a reasonable explanation for the whole phenotype. If, instead, the α/non-α ratio was considerably lower than 1, the DNA studies investigated defects (deletions or mutations) that involve two α genes in cis or in trans. In these cases one must also take into account that even a simple heterozygosity for one of the above mentioned α thal defects (for instance $-\alpha^{3.7\ II}$) may sometimes appear as the only basis for a striking α thalassemic phenotype.

In conclusion, the large-scale utilization of this ensemble of techniques enabled us to identify the precise molecular alteration in almost all the α thalassemias studied. It may thus be confidently predicted that in the very near future the exact incidence of the various types of thalassemias, α and non-α, will be clarified, at least for the best studied populations. The cases which remain unsolved or only partially diagnosed because of the incompleteness of studies at the DNA level will progressively decrease or even disappear altogether. Finally, the relative incidence of the various types of thalassemias (silent, mild and marked), at present all undetermined, will eventually be known. This would in turn contribute to clarifying the pathogenesis of thalassemia intermedia.[12]

With the techniques illustrated here ~600 α thalassemia carriers have already been identified in our laboratory. A new long-term research project aimed at solving the above listed problems on a precise statistical basis has just been programmed in the Latium population.

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