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β^+-thalassaemia in the Po river delta region (northern Italy): genotype and β globin synthesis

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SUMMARY Six β^+-thalassaemic patients from the Po river delta region have been studied. Using synthetic oligonucleotides as specific hybridisation probes, the β^+ IVS I mutation (G→A at position 108) was demonstrated. This lesion and the enzyme polymorphism pattern in the subjects examined are the same as have been described for other Mediterranean β^+-thalassaemias. Antenatal diagnosis through DNA analysis of β^+-thalassaemia is therefore possible. The production of β globin in a β^+ homozygote and in a β^+, β° 39 (nonsense mutation at codon 39) double heterozygote is approximately 20% and 10% respectively of total non-α globin synthesis. Despite some overlapping of the results, similar β globin synthesis levels have been obtained in 43 β^+-thalassaemia patients. This suggests that in the Po river delta region the most common thalassaemic genes are β° 39 and β^+ IVS I.

The frequency of the thalassaemic gene in Italy is very high[1] and in the Po river delta (northern Italy) it can reach 20%.[2] In this region, in addition to a form of β°-thalassaemia which has already been characterised,[3–23] forms of α-thalassaemia have been described.[4 5] The aim of this paper is to report the presence in this region of a form of β^+-thalassaemia and to describe its molecular lesion.

When the population with Cooley's anaemia in the Po river delta region was examined for haemoglobin patterns, in addition to the typical Ferrara β°-thalassaemia subjects, patients with β globin production were found. Since restriction enzyme polymorphisms[6–19] and synthetic oligonucleotides have been used as highly specific techniques for the identification of different mutant β globin genes,[10–11] DNA analysis was performed both with restriction enzymes[6] and with specific synthetic oligonucleotides[6] in six β^+-thalassaemic subjects. The results obtained showed that the subjects examined carry a G to A transition at position 108 of the first intervening sequence of the β globin gene,[7 8] as found in Greek and other Mediterranean populations.[6]

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The level of β globin synthesis of this β^+-thalassaemia gene has been determined in intact erythroid cells and in cell free incubations, both in homozygous and in doubly heterozygous subjects. The amount of β globin synthesis directed by a single mutated β gene is approximately 10% of the total non-α globin synthesis.

Materials and methods

SUBJECTS

Cooley's anaemia patients with some reticulocyte β globin synthesis were examined. They were unrelated and all originated from the Po river delta region.

GLOBIN SYNTHESIS DETERMINATION

Heparinised samples of peripheral blood, the reticulocytes isolated through Percoll gradient centrifugation,[15] and bone marrow erythroid cells were washed with saline and incubated with ^{3}H leucine in Eagle's MEM (Flow Laboratories) for one hour.[16] Globins prepared by acid acetone precipitation were separated on carboxymethyl cellulose column chromatography and the radioactivity was determined as previously described.[17]

RESTRICTION ENZYME ANALYSIS OF GENOMIC DNA

A total of 10 μg DNA from white blood cells was digested with HindIII, BamHI, HincII, and AvaII under the conditions recommended by the manufacturers (Boehringer–Mannheim; Bethesda Research Laboratories). The digested DNA was electrophoresed on 0·8% agarose gel, transferred to a nitrocellulose filter, and hybridised to four ^{32}P labelled nick translated plasmids containing γ, β, $\psi\beta$, and ε globin sequences respectively.[9]

SYNTHETIC OLIGONUCLEOTIDE ANALYSIS OF GENOMIC DNA

A total of 5 to 10 μg BamHI digested DNA was electrophoresed on agarose gel, denatured, and dried as described by Pirastu *et al.*[10]

The two nonadecamers, specific for the thalassaemic lesions β° 39[10] and β^+ IVS I,[11] were synthetised by the solid phase phosphotriester method in an automatic DNA synthetiser (Microsin 1450, SYSTEC). The two probes were labelled with ^{32}P and hybridised to gels according to Pirastu *et al.*[11]

RNA PURIFICATION AND ANALYSIS

Total RNA was obtained by phenol extraction and poly A^+ RNA was prepared by oligo dT cellulose column chromatography.[12] The poly A^+ RNA was translated in vitro using a rabbit reticulocytes translation kit (New England Nuclear Chemicals) under the conditions already described.[13] The amount of β globin mRNA was measured by liquid hybridisation with ^3H β and ^3H α cDNA probes according to the method of Ottolenghi *et al.*[12]

Poly A^+ RNA was electrophoresed on agarose gel and transferred to a nitrocellulose filter as described by Thomas.[14] The nitrocellulose blots were baked for two hours at 80°C, then hybridised to a ^{32}P β cDNA plasmid and autoradiographed, as described by Flavel *et al.*[9]

Results

LEVEL OF β GLOBIN SYNTHESIS IN UNFRACTIONATED PERIPHERAL BLOOD

Globin synthesis in intact reticulocyte incubations was examined in 43 β^+-thalassaemia patients from the Po delta region. The data obtained are shown in fig 1. The values of β synthesis relative to non-α globin ranges from 5 to 30%, the most frequent values being around 10 and 20%.

RESTRICTION ENDONUCLEASE ANALYSIS OF GENOMIC DNA

The DNA from six β^+-thalassaemia patients was analysed by the blotting technique using HincII, HindIII, AvaII, and BamHI restriction enzymes, which have polymorphic sites within the β gene cluster.[6] The combined use of these four enzymes has allowed the identification of restriction patterns typical of several forms of thalassaemia.[6] The hybridisation conditions and the data obtained are shown in fig 2. Two of the six cases were homozygous for haplotype I, described by Orkin *et al*,[6] in association with β^+-thalassaemia presenting a G to A transition at nucleotide 108 of the first intron.[7 8] The other four cases showed polymorphic patterns compatible with heterozygosity for haplotype I and IX, described by Orkin *et al*,[6] the latter in linkage with β°-thalassaemia presenting a nonsense mutation at codon 39.[18]

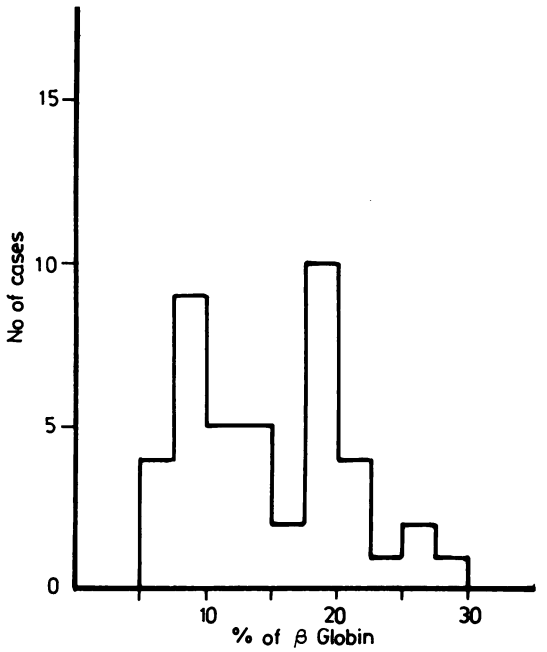
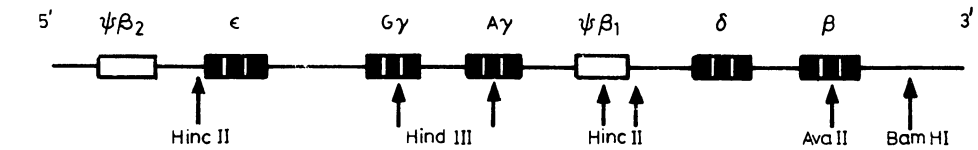


FIG 1 *Percentage of β globin synthesis in 43 β^+ Cooley's anaemia subjects.*

HYBRIDISATION WITH SPECIFIC OLIGONUCLEOTIDES

The presence of the IVS I and β° 39 mutations, suggested by the restriction pattern of fig 2, was further investigated, hybridising the DNA of the same subjects with the synthetic oligonucleotides specific for the two mutations.[10 11] The results obtained in three β^+- (MD, AR, and BF of fig 2) and in one β°-thalassaemia patients are shown in fig 3.

The DNA of the patients was digested with



Subjects							Haplotypes
AR	+/−	−/+	−/−	−/+	−/+	+/+ +/+	I - IX
BF	+/−	−/+	−/−	−/+	−/+	+/+ +/+	I - IX
MD	+/+	−/−	−/−	−/−	−/−	+/+ +/+	I - I
FA	+/−	−/+	−/−	−/+	−/+	+/+ +/+	I - IX
BS	+/+	−/−	−/−	−/−	−/−	+/+ +/+	I - I
GP	+/−	−/+	−/−	−/+	−/+	+/+ +/+	I - IX
Haplotype I (β⁺IVS I)	+	−	−	−	−	+ +	
Haplotype IX (β°39)	−	+	−	+	+	+ +	

FIG 2 *Restriction site pattern in the β globin gene cluster of six β⁺ Cooley's anaemia subjects. + and − indicate the presence or absence of the cleavage site in the DNA regions indicated. Haplotype I and IX described by Orkin et al [6] are shown.*

BamHI restriction enzyme which gives rise to a β globin fragment of 1·8 kb including both codon 39 and the first intervening sequence. The two probes hybridise only if the corresponding mutation is present. Therefore subject 1 is homozygous for the β⁺ IVS I mutation, subject 2 is homozygous for the β° 39 mutation, and subjects 3 and 4 carry the two mutations. The hybridisation patterns obtained in the six subjects studied (two homozygous for the IVS I mutation and four double heterozygotes) are compatible with the restriction patterns of fig 2.

β GLOBIN SYNTHESIS IN $\beta^+\beta^+$ AND $\beta^+\beta^\circ$ PATIENTS

In order to estimate the level of β globin synthesis due to this β⁺-thalassaemia gene in single or double

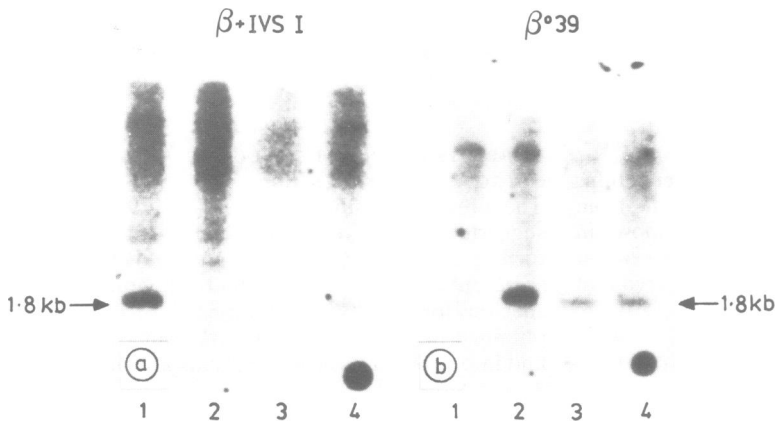


FIG 3 *Autoradiographic pattern of BamHI digested thalassaemic DNA. Gels were hybridised (a) with β⁺ IVS I and (b) with β° 39 oligonucleotides. For experimental details see Methods section.*

TABLE *Globin biosynthesis ratio ($\beta/\beta+\gamma$) in two β^+-thalassaemia subjects.*

Subject	*Erythroid cells*	*Intact cell incubation*	*Cell free incubation*
$\beta^+\beta^+-$	Reticulocytes	0·21	0·20
	Younger reticulocytes	0·20	—
	Nucleated cells	0·23	0·22
$\beta^+\beta^\circ-$	Reticulocytes	0·11	0·12
	Younger reticulocytes	0·09	—
	Nucleated cells	0·11	—

dose, globin synthesis was studied in two β^+-thalassaemia patients, one homozygous for the β^+ IVS I mutation and the other doubly heterozygous for the IVS I and β° 39 mutation (MD and FA of fig 2 and cases 1 and 4 of fig 3). Biosynthetic data were obtained in whole reticulocyte and bone marrow cell incubations and by cell free translation of the poly A^+ mRNA isolated from the two cell types.

The ratio of β to $\beta+\gamma$ globin synthesis both in young and mature erythroid cells was about 0·20 in the β^+ homozygous subject and 0·10 in the doubly heterozygous (table). The two ratios were maintained in cell free conditions. In addition, the same percentages of β globin synthesis were found in cells of different ages in both subjects, thus indicating the stability of β globin mRNA and excluding a decrease of β globin synthesis during cell maturation. The relative amount of β globin mRNA was measured by cDNA hybridisation in the reticulocytes from the homozygous subject. The $\beta/\beta+\gamma$ mRNA ratio was 0·19 and corresponded to the globin synthesis ratio already reported for other β^+-thalassaemic patients.[24–26]

The same RNA sample was analysed by agarose gel electrophoresis and hybridised to a β cDNA probe. The autoradiographic pattern showed the usual faint band approximately 800 nucleotides long preceding the normal 10 S band of mature β globin mRNA (fig 3).

Discussion

The data presented in fig 1 show that in the Po river delta, as in other Mediterranean regions, β^+-thalassaemia is present as well as β°- and α-thalassaemia. These β^+-thalassaemics represent approximately 50% of the total Cooley's anaemia population and are preferentially distributed in the northern part of the river delta (data not presented).

In six β^+-thalassaemics examined, both restriction enzyme polymorphism analysis and oligonucleotide hybridisation show that this form of β-thalassaemia is caused by the already described[7 8] point mutation G→A at position 108 of the IVS I of the β gene. This nucleotide substitution causes a new splicing signal which results in abnormal splicing and instability of the RNA. Only a small amount of RNA is correctly processed and this leads to low levels of β globin synthesis.[20 21]

Supporting this abnormal processing, a β globin RNA fragment, larger than normal, was found in the β^+ homozygous subject examined. Similar data have been reported in β^+-thalassaemia patients of Greek origin.[22]

In addition, as expected, the β globin synthesis levels observed both in erythroid cells at different maturation phases and in a cell free system are constant, thus confirming the stability of the decreased number of β globin mRNA molecules correctly processed.

The ratios of β to $\beta+\gamma$ globin synthesis are approximately 0·20 in the homozygote and 0·10 in the double heterozygote. Correspondingly, in 43 β^+-thalassaemic patients examined, the distribution of β synthesis levels shows two main peaks, one approximately at 10% and the other at 20% (fig 4). However, owing to some overlapping of the values,

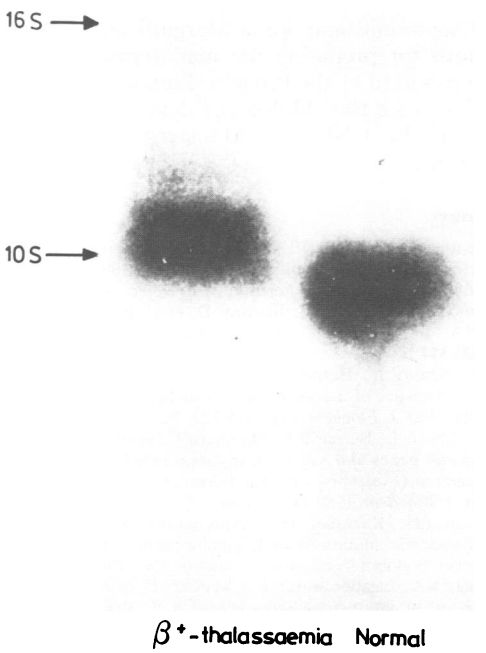


FIG 4 *Autoradiographic pattern of reticulocyte poly A^+ β globin RNA in a β^+-thalassaemia homozygote and in a normal subject. RNA was electrophoresed on agarose gel, transferred to a nitrocellulose filter, hybridised to a β cDNA plasmid, and exposed to x-ray film as described in the Methods section.*

the distinction between the β^+ homozygotes and double heterozygotes based on globin synthesis is not always clear. The distinction between the β^+- and β°-thalassaemia gene is also difficult in heterozygotes. The healthy carriers of the two genes studied so far (data not reported) show only slight differences in the mean cellular volume (β^+ parents (38): MCV 75·1±7·2 SD; β° parents (37): MCV 70·5±4·6 SD).

In conclusion, the form of β^+-thalassaemia in the Po river delta region is caused by the IVS I mutation and presents the same restriction enzyme pattern (haplotype I of Orkin *et al* [6]). It is therefore possible that this thalassaemia gene came from Greece to the Po delta region where Greek influence has been demonstrated.

Since the genotype found in six β^+ and in eight β° Cooley's anaemia patients examined so far (except one)[23] are either β^+-thalassaemia IVS I or β°-thalassaemia 39, the synthetic oligonucleotide technique can be applied to the antenatal diagnosis of forms of β-thalassaemia in the Po river delta.

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References

1 Weatherall DJ, Clegg JB. The thalassaemia syndromes. 3rd ed. Oxford: Blackwell Scientific Publications, 1981.

2 Vullo C. Thalassemia in Ferrara. *Birth Defects* 1982;**18**:199–202.

3 Conconi F, Bernardi F, Buzzoni D, *et al.* β-globin messenger RNA in Ferrara β° thalassemia. *Ann NY Acad Sci* 1980;**344**:120–31.

4 Del Senno L, Bernardi F, Buzzoni D, *et al.* Molecular characteristics of a non deletion α-thalassemia of the Po river delta. *Eur J Biochem* 1981;**116**:127–30.

5 Del Senno L, Bernardi F, Marchetti G, *et al.* Organization of α-globin genes and mRNA translation in subjects carrying Hb Hasharon (^{47} asp-his) from the Ferrara region (northern Italy). *Eur J Biochem* 1980;**111**:125–30.

6 Orkin SH, Kazazian HH, Antonarakis S. Linkage of β-thalassaemia mutations and β-globin gene polymorphisms in human β-globin gene cluster. *Nature* 1982;**296**:627–31.

7 Spritz RA, Jagadeeswaran P, Choudary P, *et al.* Base substitution in an intervening sequence of a β^+-thalassemia human globin gene. *Proc Natl Acad Sci USA* 1981;**78**:2455–9.

8 Westaway D, Williamson R. An intron nucleotide sequence variant in a cloned β^+-thalassemic gene. *Nucleic Acids Res* 1981;**9**:1777–88.

9 Flavell RA, Kooter JM, De Boer E, *et al.* Analysis of the β-δ-globin gene loci in normal and Hb Lepore DNA: direct determination of gene linkage and intergene distance. *Cell* 1978;**15**:25–41.

10 Pirastu M, Kan YW, Cao A, *et al.* Prenatal diagnosis of β-thalassemia. *N Engl J Med* 1983;**309**:284–7.

11 Orkin SH, Markham AF, Kazazian HH. Direct detection of the common Mediterranean β-thalassemia gene with synthetic DNA probes. *J Clin Invest* 1983;**71**:775–9.

12 Ottolenghi S, Comi P, Giglioni B, *et al.* Direct demonstration of β-globin mRNA in homozygous Ferrara β° thalassemia patients. *Nature* 1977;**266**:231–5.

13 Del Senno L, Bernardi F, Marchetti G, *et al.* Organization of α-globin genes and mRNA translation in subjects carrying Hb Hasharon (α^{47} asp-his) from the Ferrara region (northern Italy). *Eur J Biochem* 1980;**111**:125–30.

14 Thomas PS. Hybridization of denaturated RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* 1980;**77**:5201–5.

15 Bernardi F, Marchetti G, Del Senno L, Perrotta C, Conconi F. Decay of β-globin synthesis in heterozygous β° Ferrara thalassaemia. *Haematologica (Pavia)* 1982;**67**:No 5.

16 Bargellesi A, Pontremoli S, Conconi F. Absence of β-globin synthesis and excess of α-globin in homozygous β-thalassemia. *Eur J Biochem* 1967;**1**:13–79.

17 Clegg J, Naughton MA, Weatherall DJ. Abnormal human haemoglobins: separation and characterization of α and β chains by chromatography and the determination of two variants, Hb Chesapeake and Hb J Bankok. *J Mol Biol* 1966;**19**:91–108.

18 Trecartin RF, Liebhaber SA, Chang JC, *et al.* β°-thalassemia in Sardinia is caused by a nonsense mutation. *J Clin Invest* 1981;**68**:1012–7.

19 Antonarakis SE, Boehm CD, Giardina PJV, *et al.* Nonrandom association of polymorphic restriction sites in the β-globin gene cluster. *Proc Natl Acad Sci USA* 1982;**79**:137–41.

20 Busslinger M, Moschonas N, Flavell RA. β-thalassemia: aberrant splicing results from a single point mutation in an intron. *Cell* 1981;**27**:289–98.

21 Fukamaki Y, Ghosh PK, Benz J, *et al.* Abnormally spliced messenger RNA in erythroid cells from patients with β^+ thalassemia and monkey kidney cells expressing a cloned β^+ thalassemic gene. *Cell* 1982;**28**:585–93.

22 Kantor JA, Turner PH, Nienhuis AW. β thalassemia: mutations which affect processing of the β-globin mRNA precursor. *Cell* 1980;**21**:149–57.

23 Pirastu M, del Senno L, Conconi F, Kan YW. Ferrara β° thalassemia is caused by a β 39 nonsense mutation. *Nature* 1984;**307**:76.

24 Benz EJ, Forget BG, Hilman DG, *et al.* Variability in the amount of β globin mRNA in β° thalassaemia. *Cell* 1978;**14**:299–312.

25 Housman D, Forget BG, Skoultchi A, *et al.* Quantitative deficiency of chain-specific globin messenger ribonucleic acids in the thalassaemia syndromes. *Proc Natl Acad Sci USA* 1973;**70**:1809–13.

26 Kacian DL, Gambino R, Daw LW, *et al.* Decreased globin messenger RNA in thalassemia detected by molecular hybridization. *Proc Natl Acad Sci USA* 1973;**70**:1880–90.

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