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δ°-Thalassemia in *cis* of $\beta^{Knossos}$-globin gene

Normal structure and normal transient expression of the δ-globin gene

R. Ouazana*, D. Bozon*, F. Baklouti*+, C. Gonnet*, J. Delaunay+ and J. Godet*

**Centre de Génétique Moléculaire et Cellulaire, CNRS UMR 106, 43 boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex* and *+Génétique Moléculaire de la Membrane Erythrocytaire, CNRS URA 1171, Faculté de Médecine Grange-Blanche, 69373 Lyon Cedex 08, France*

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We have previously described the first homozygous cases of Hb Knossos in an Algerian family. The Hb A_2 was completely absent, ascertaining the presence of a δ°-thalassemia determinant in *cis* of the $\beta^{Knossos}$ gene. Here, we investigate the affected δ-globin gene. The complete DNA sequence of the gene and its 5′ and 3′ flanking regions was determined. Only two nucleotide changes were recorded: a C → T substitution at −199 and an AT insertion at −448 upstream from the cap site. To examine the involvement of these changes in gene function, the δ-gene was subcloned in an expression vector and introduced into COS cells. Analysis of RNA derived from these cells, using an S_1 protection assay and dot-blot hybridization, revealed qualitatively and quantitatively normal transcription. The loss of δ-globin gene activity in vivo may be due to the alteration of a tissue-specific control.

Gene, δ-globin; DNA sequence polymorphism; Thalassemia; Transient expression

1. INTRODUCTION

The δ-globin chain is the β-like subunit of the minor adult hemoglobin (Hb), Hb A_2, which is synthesized at about 2.5% in normals [1]. The underproduction of δ-globin chains results, at least in part, from the imperfect constitutive δ-promoter structure [2]. δ-Thalassemia is rather a rare condition which has been identified mainly in Mediterraneans [3–5] and Japanese [6,7] individuals. Like β-chain synthesis in β-thalassemia, both partial and total suppression of δ-globin chain synthesis occur in δ-thalassemia, giving rise to δ^{+} and δ° forms, respectively.

Correspondence address: F. Baklouti, Génétique Moléculaire de la Membrane Erythrocytaire, CNRS URA 1171, Faculté de Médecine Grange-Blanche, 69373 Lyon Cedex 08, France

Abbreviations: Hb, hemoglobin; δ-thal, δ-thalassemia

Increased Hb A_2 is a major criterion for diagnosis of the β-thalassemia trait. Nevertheless, distinct forms of non-deletion β-thalassemia are associated with normal Hb A_2, suggesting the coinheritance of separate β- and δ-thalassemia determinants [4,8–10]. Hb Knossos (β 27 Ala ⟶ Ser) is a structural variant with a mild β-thalassemia phenotype and an almost constant decrease in Hb A_2 percentage in heterozygotes [8]. The presence of a δ°-thalassemia (δ°-thal) determinant in *cis* of the $\beta^{Knossos}$ gene has been demonstrated in a [$\beta^{Knossos}/\delta\beta^{Lepore}$] double heterozygote [11] and, more recently, in homozygous Hb Knossos [12]. Here, we have analysed the δ-globin gene isolated from a previously reported patient with homozygous Hb Knossos [12]. A single base substitution at −199 and an AT insertion at −448 have been identified in 5′ of an otherwise normal δ-gene. Assays of transient expression in COS cells showed the affected gene to be quantitatively and qualitatively normally expressed.

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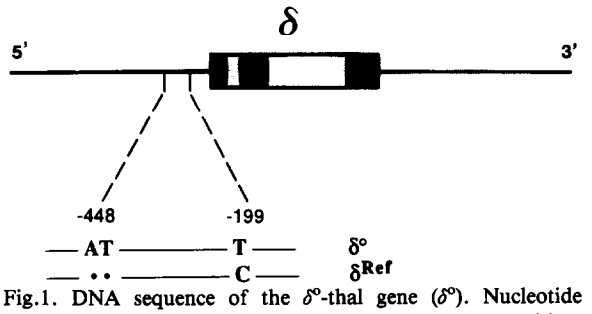

Fig.1. DNA sequence of the δ°-thal gene (δ°). Nucleotide changes are depicted at the bottom, in comparison with a normal δ-globin gene, considered as the reference (δ^{Ref}) [16].

2. MATERIALS AND METHODS

2.1. *Gene cloning and DNA sequencing*

DNA was obtained from peripheral blood leucocytes and an enriched library was constructed in bacteriophage λL47 as described [13]. The δ-globin gene was cloned as a 7–8 kb *Bgl*II fragment by substitution of the central *Bam*HI fragment of the phage. A 2.3 kb *Pst*I fragment containing the entire δ-gene was subcloned in M13 mp18 and M13 mp19 vectors. Its complete nucleotide sequence was determined by the dideoxy method of Sanger et al. [14], using several M13 templates containing *Alu*I, *Hae*III, *Rsa*I or *Sau*3A restriction fragments derived from the 2.3 kb *Pst*I insert.

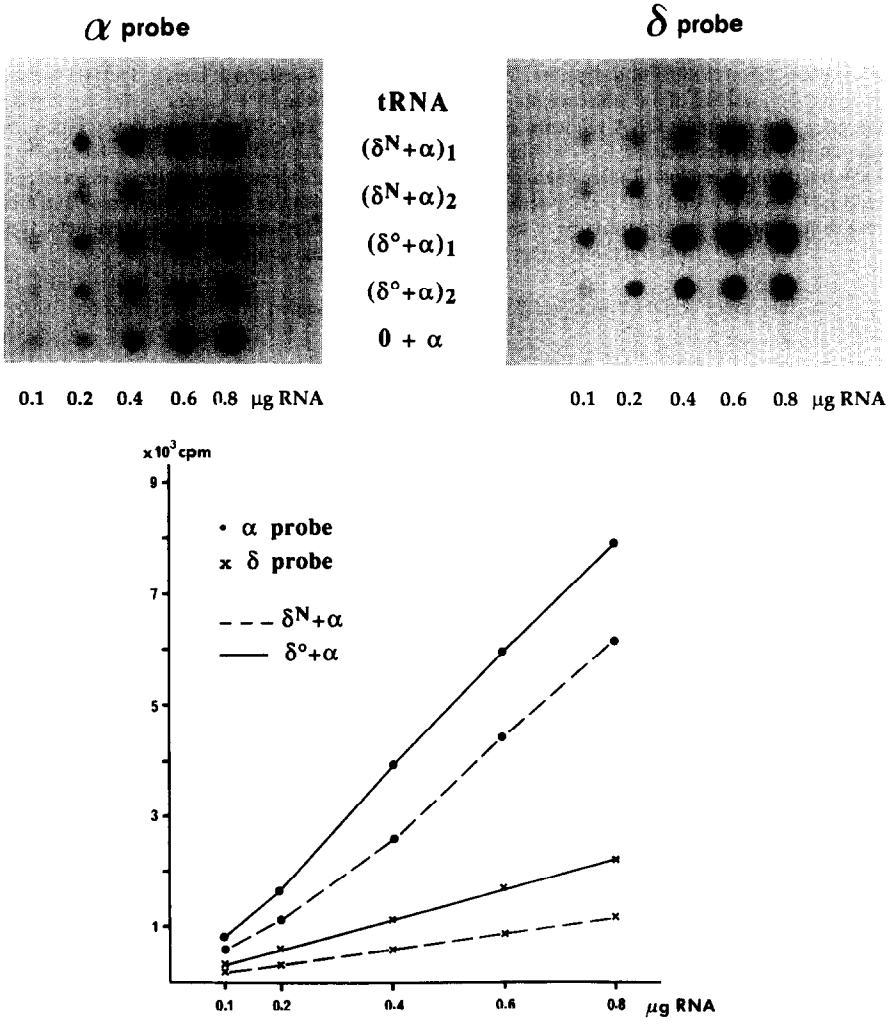

Fig.2. Dot-blot analysis of the δ-mRNA level following transient assay in COS cells. (Top) Autoradiograph of duplicated filters after hybridization with α- and δ-probes. tRNA is a negative control. δ^{N} and δ° refer to normal and thalassemic genes, respectively. ($\delta^{x}+\alpha$) RNA from δ and α co-transfected cells. 1 and 2 indicate RNAs extracted from two different culture dishes. ($0+\alpha$) RNA from cells transfected only by α-recombinant plasmid. (Bottom) Plots of radioactivity count vs increasing quantities of RNA applied to nitrocellulose filters; radioactive spots were excised from filters and determined by liquid scintillation counting.

2.2. *Gene expression in COS cells*

A 2.3 kb *Pst*I fragment containing either the δ^o-thal or normal δ-gene was subcloned in pEMBL 8 SV expression vector containing the SV40 enhancer element [15]. The recombinant plasmids were co-transfected in COS cells with an α-globin gene recombinant plasmid as an internal standard [15].

Dot-blot assay of COS cell RNA was carried out as in [13]. Duplicate nitrocellulose filters were hybridized with anti-mRNA probes encompassing either δ- or α-globin exon 1. These probes were prepared using T_7 RNA polymerase and Bluscribe recombinant DNA templates.

Analysis of COS cell RNA was performed by S_1 nuclease mapping according to [13,15]. Two uniformly ^{32}P-labeled δ-globin gene probes were prepared from single-strand M13 clones containing a normal δ-globin 2.3 kb *Pst*I fragment: (i) a 950 bp *Bam*HI/*Hin*dIII fragment encompassing exon 1 and the 5′-end of exon 2; and (ii) a 550 bp *Hpa*I fragment containing exon 3.

3. RESULTS

3.1. *Sequence analysis*

The nucleotide sequence of the δ-thal gene was examined from 458 bp 5′ to the cap site to 207 bp 3′ to the polyadenylation signal. Comparison with a reference sequence [16] led to the detection of two variations: C → T substitution at −199 and AT insertion at −448 upstream from the cap site (fig.1). No abnormal sequence was found to explain the dysfunction of this gene in vivo.

3.2. *Transient expression in heterologous cells*

The δ-thal gene and its flanking regions including the two mentioned variations were introduced together as recombinant plasmid in cultured COS cells. Transient expression of the affected gene and a normal δ-gene was examined in the presence of an equal amount of a co-transfected α-globin gene as an internal control.

Dot-blot assay was performed on transfected COS cell RNA to evaluate quantitative expression of the δ-thal gene. RNA analysis with both α- and δ-globin riboprobes would allow monitoring of variability due to differences in transfection efficiency or RNA recovery. As shown in fig.2, there was no difference in the quantity of δ-globin mRNA transcribed from each δ-globin.

Alternatively, RNAs derived from COS cells were analysed with respect to the functional integrity of the δ-thal gene by S_1 nuclease mapping, using two δ single-strand DNA probes: the first probe is protected by δ-globin mRNA to produce a 143-nucleotide fragment encompassing the

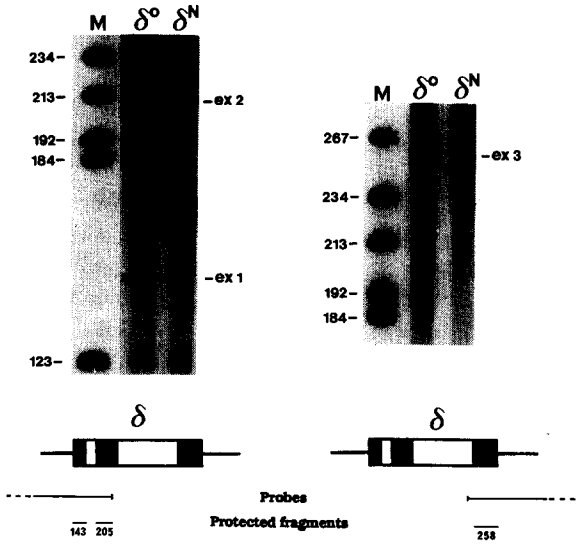

Fig.3. S_1 mapping of δ-globin gene transcripts derived from transfected COS cells. Uniformly labeled probes and protected fragments (with their expected lengths) are depicted at the bottom of schematic diagrams of the δ-globin gene. The broken line signifies that the end of the probe is within the M13 polylinker. The low intensity of exon 1 protected bands (▷) results from the low specific activity of the probe in this region, due to the primer extension reaction. δ^o and δ^N, see legend to fig.2. M, ^{32}P-labeled *Hae*III pBR322 restriction fragments used as size markers. All sizes expressed in base pairs.

5′-untranslated region and all of exon 1, and a 205-nucleotide fragment containing exon 2 to the *Bam*HI site. The second probe is protected by normally spliced δ-globin mRNA to produce a 258-nucleotide fragment encompassing all of exon 3. As demonstrated by the presence of the expected bands and their equal intensities, the δ-thal gene initiates transcription normally and produces a transcript from which the two introns are spliced correctly.

4. DISCUSSION

In $\delta\beta$-thalassemia and some HPFH conditions, the δ-globin gene is usually deleted, and therefore completely unexpressed. In contrast, no large deletion affecting this gene was observed in δ-thal conditions [5,7,17–19]. Only two mutations are known to be responsible for the δ-thal phenotype [5].

In the present study, we tried to define the

molecular defect of the δ^0-thal gene present in *cis* of the $\beta^{Knossos}$ gene. The restriction haplotype suggested the absence of an extended deletion around the δ-gene [12]. The DNA sequence was normal and the gene was expressed normally when introduced into COS cells.

Both the two nucleotide changes observed at -199 and -448 upstream from the cap site have been encountered in a Japanese individual in whom δ-globin gene was expressed normally in vivo. They have also been found to be associated with a T → C substitution at -77 of a δ^0-thal gene [7]. There is no evidence that the -77 base substitution is involved in the δ^0-thal phenotype. Nevertheless, the present δ^0-thal gene is flanked by a T at -77 in the 5′-side, and a $\beta^{Knossos}$ mutation in the 3′-side, and is associated with a different β-globin gene cluster haplotype [12]. It should therefore differ from the δ^0-thal gene described in Japan.

The finding that an intact δ-globin gene is inactive in vivo on a chromosome bearing no large deletion at its proximity rules out a mechanism identical to that involved in some $\gamma\delta\beta$-thalassemia (review [1]); which inactivates a structurally normal β-globin gene. Nevertheless, in the Japanese and the δ^0-thal described here, the molecular lesion appears to be associated with erythroid-specific control of the δ-gene, since either of the two genes was expressed normally in heterologous cells.

Recently, the DNA sequence at -200, and with lower efficiency that at -120, upstream from the human β-globin gene, have been shown to bind a specific nuclear erythroid factor, NF-E1 [20]. These sites contain a consensus sequence, T/A ATC A/T Py (with a mismatch at -119) also found in the 3′ flanking β-globin enhancer [20,21]. On the other hand, the 5′-flanking sequence of the δ-globin gene contains only one copy of the consensus T/A ATC A/T Py sequence at position -150. Therefore, the -199 C → T substitution probably occurs outside of an NF-E1 binding site.

Although the -199 substitution has been considered as a polymorphism, it should be pointed out that it occurs in an upstream region containing, at least for β- and γ-globin genes, a selective (or tissue-specific) promoter [22,23]. This region is characterized by the presence in globin expressed cells of 'minor' DNase I-hypersensitive sites [24,25]. In order to elucidate the pattern of these sites upstream from the δ-gene, collection of bone marrow cells from the patient is necessary.

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