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A molecular study of a family with Greek hereditary persistence of fetal hemoglobin and β-thalassemia

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A family was studied in which two inherited defects of the non-α-globin cluster segregate: Greek hereditary peristence of fetal hemoglobin (HPFH) and β-thalassemia. Fragments of the non-α-globin cluster from two patients were cloned in cosmid and phage lambda vectors, and assigned to either the HPFH or β-thalassemic chromosome on the basis of the demonstration of a polymorphic *Bgl*II site in the HPFH γ-globin cluster. The thalassemic β-globin gene carries a mutation at nucleotide 1 of the intervening sequence I, known to cause β°-thalassemia; the β-globin gene from the HPFH chromosome is entirely normal, both in the intron-exon sequence and in 5′ flanking regions required for transcription. As the compound HPFH/β-thalassemia heterozygote synthesizes HbA, these data prove that the HPFH β-globin gene is functional, although at a decreased rate; its lower activity is likely to be due to a distant mutation. The HPFH $^{A}\gamma$-globin gene shows only two mutations: a T→C substitution in the large intervening sequence (responsible for the *Bgl*II polymorphic site) and a C→T substitution 196 nucleotides 5′ to the cap site; the 5′ flanking sequence is normal up to −1350 nucleotides upstream from the gene. Circumstantial evidence suggests that the mutation at −196 may be responsible for the abnormally high expression of the $^{A}\gamma$-globin gene.

Key words: $^{A}\gamma$ globin gene/β-thalassemia/polymorphism/gene regulation/point mutation

Introduction

Inherited disorders of human globin synthesis characterized by persistent expression of either or both fetal $^{G}\gamma$- and $^{A}\gamma$-globin genes during adult age and defective δ- and β-globin production are known as δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH), the former being in general associated with more unbalanced α-/non-α-globin synthetic ratios than the latter (Weatherall and Clegg, 1981). Many of these syndromes are due to large deletions in the non-α-globin cluster (summarized in Ottolenghi and Giglioni, 1982; Tuan *et al.*, 1983); in other cases no alterations can be detected by restriction enzyme analysis, suggesting that small deletions or even point mutations might be responsible for these defects. Leaving aside heterocellular HPFH, which may be due to genes linked to the non-α-globin cluster, but mapping outside it (Old *et al.*, 1982) or even to unlinked genes (Gianni *et al.*, 1983), three different syndromes have been analyzed recently by restriction enzymes: $^{G}\gamma$-β^{+} HPFH (Basley *et al.*, 1982; Jones *et al.*, 1982), Greek HPFH (Bernards and Flavell, 1980; Tuan *et al.*, 1980; Jones *et al.*, 1982) and Sardinian δβ°-thalassemia (Ottolenghi *et al.*, 1982; Guida *et al.*, 1984; Pirastu *et al.*, 1984). Here, we describe cloned genes from patients from the Southern Italian region of Puglia, with hematological characteristics similar to those of previously described individuals with Greek HPFH.

Results

The main hematological data of the family studied are reported in Table I. In this family, an HPFH gene appears to segregate with a β-thalassemia gene; two compound heterozygotes for these conditions (II.1 and II.2) can be identified. Heterozygous HPFH patients have a high level of HbF, mostly of the α_2-$^{A}\gamma_2$ type, and essentially normal red cell indices and biosynthetic ratios. These data suggest that the HPFH chromosome might contribute a relatively high level of hemoglobin (HbF and perhaps HbA) possibly similar to that of a normal chromosome; indeed, in the compound HPFH-β-thalassemic heterozygotes, red cell indices and biosynthetic ratios are almost superimposable to those of the β-thalassemic parent, although the higher HbF, $^{G}\gamma$ proportion and γ/α synthetic ratio suggest that the biosynthetic balance may be achieved, in these patients, at least in part, by increased activity of γ-globin genes in *cis* and in *trans* to the HPFH locus.

To investigate the nature of the genetic defect, we decided to clone the non-α-globin cluster from both the HPFH and β-thalassemic chromosomes. Restriction enzyme analysis with several probes derived from the globin cluster (ε-, γ-, ψβ-, RIH- and β-globin probes) fails to demonstrate any rearrangement in the DNA of these patients (not shown). Moreover, the compound heterozygotes appear to be haplotype V homozygotes when analyzed with restriction enzymes according to Orkin *et al.* (1982). In order to discriminate the HPFH from the β-thalassemic chromosome, we relied on a newly discovered *Bgl*II polymorphic site, that appears to cut the usual 13-kb *Bgl*II γ-globin band into a 8-kb band and a very faint 5-kb band, not easily seen with γ cDNA probes (Figure 1). This polymorphism is present in the HPFH patients, but not in the β-thalassemic carrier, indicating that it is on the HPFH chromosome. Figure 2 shows two different cosmid clones from the non-α-globin cluster of the compound heterozygote II.1, and a phage lambda clone containing a 18-kb *Bcl*I γ-globin fragment from the HPFH chromosome of patient I.1. *Hind*III digestion of the latter clone generates (Figure 3) a 3.3-kb fragment (the size expected for the $^{A}\gamma$-globin gene) which can be demonstrated by hybridization (not shown) to contain γ-globin sequences; this fragment is further cut by *Bgl*II to yield 2.5-kb and 0.8-kb fragments. These data locate the *Bgl*II site in the $^{A}\gamma$-globin gene and are the basis for identifying cosmid 2, whose $^{A}\gamma$-globin gene does not contain this site, as deriving from the β-thalassemic chromosome. Unfortunately cosmid 1 does not include the $^{A}\gamma$-globin gene; to determine its origin, we first showed by sequencing (Figure 4a) that the β-globin gene from cosmid 2

Table I. Main hematological and globin data of the family under study

Patient	Condition	%HbF	%HbA_2	MCH (pg)	MC HbA (pg)	MCV (fl)	Synthetic ratios		
							β/α	γ/α	$^{G}\gamma/^{G}\gamma+^{A}\gamma$
I.1	HPFH	16	1.9	31	25.4	90	0.76	0.18	<0.05
I.2	β-thalassemia	1.2	4.8	21.5	20.2	67.9	0.51	–	–
II.1	HPFH/β-thalassemia	40	4	23.4	13.1	67.3	0.31	0.25	0.20
II.2	HPFH/β-thalassemia	38	4.2	21.7	12.8	69.6	0.35	0.22	–
II.3	HPFH	15	2.1	29.4	24.4	86	0.78	0.13	<0.05
II.4	HPFH	12	2.7	29.5	25.2	90	0.84	0.15	<0.05

Patients I.1 and I.2 are the father and the mother respectively of patients II.1–4. The data are compiled in part from Camaschella *et al.* (1978). MCH, mean cellular hemoglobin; MC HbA, mean cellular HbA; MCV, mean cellular volume; $^{G}\gamma/^{G}\gamma+^{A}\gamma$ globin ratios are based on isoelectric focusing of globin chains (Comi *et al.*, 1979, and unpublished data).

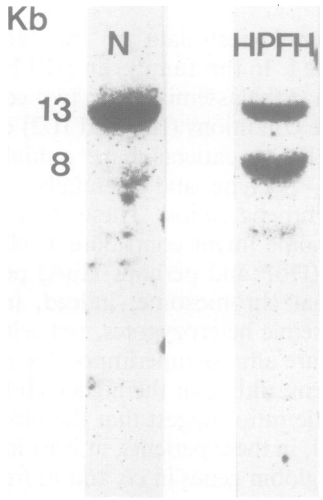

Fig. 1. Restriction enzyme analysis with *Bgl*II of DNA from patient II.1 and a normal control. The probe was a γ-cDNA plasmid from Little *et al.* (1978). Kb, kilobase.

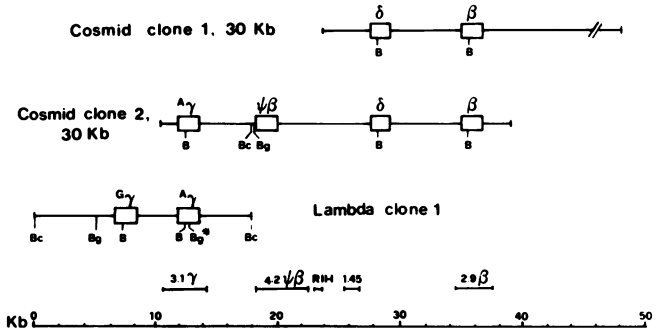

Fig. 2. Schematic representation of the inserts in two cosmid clones (from patient II.1) and in an EMBL-4 phage (patient I.1). Some relevant restriction enzyme sites are shown. Bc, *Bcl*I; Bg, *Bgl*II; B, *Bam*HI. An asterisk indicates the polymorphic *Bgl*II site. Probes used for detailed analysis of the clones are shown; these probes were excised from plasmid or phage clones with the enzymes listed. γ, a 3.3-kb *Hind*III fragment from the $^{A}\gamma$-globin gene; ψβ; a 4.2-kb *Bgl*II fragment; RIH, an *Eco*RI 0.5-kb fragment from plasmid RIH (Fritsch *et al.*, 1979); 1.45, a *Bgl*II-*Eco*RI 1.45-kb fragment; β, a 2.9-kb *Hpa*I-*Pst*I fragment.

contains the IVS 1 splice mutation that is commonly associated with haplotype V (Orkin *et al.*, 1982), then we demonstrated that the β-globin gene from cosmid 1 is normal in this region. These results assign cosmid 1 to the HPFH

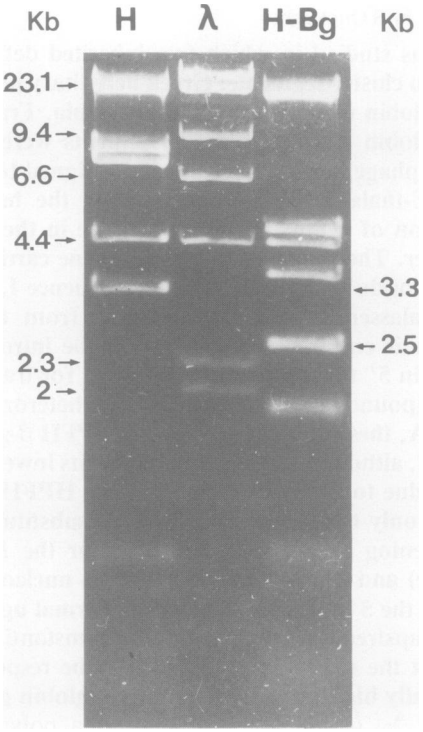

Fig. 3. Restriction enzyme analysis of the HPFH γ-globin clone in phage EMBL-4; H, *Hind*III; Bg, *Bgl*II; λ, phage lambda DNA digested with *Hind*III.

chromosome; to ascertain if the β-globin gene from this chromosome might itself be a thalassemic one, we completely sequenced it (Figure 4b) from nucleotide −280 (relative to the cap site) in the 5′ flanking sequence to nucleotide +200 in the 3′ flanking sequence, and demonstrated that its sequence is completely normal, corresponding to that of the β-globin gene (type 2) usually associated with haplotype V (Orkin *et al.*, 1982). These data indicate that the defect causing the decreased activity of the HPFH β-globin gene must lie at least at some distance from this gene. In the hope of finding an explanation for the abnormal expression of the HPFH $^{A}\gamma$-globin gene, we then sequenced the clone from nucleotide −1348 5′ to the cap site to nucleotide 380 beyond the 3′ end of the structural gene sequence. Two different $^{A}\gamma$-globin alleles have been sequenced so far, and differ considerably due to a variety of insertions, deletions and single nucleotide substitutions (Slightom *et al.*, 1980; Shen *et al.*, 1981; Slightom *et al.*, personal communication). The HPFH $^{A}\gamma$-

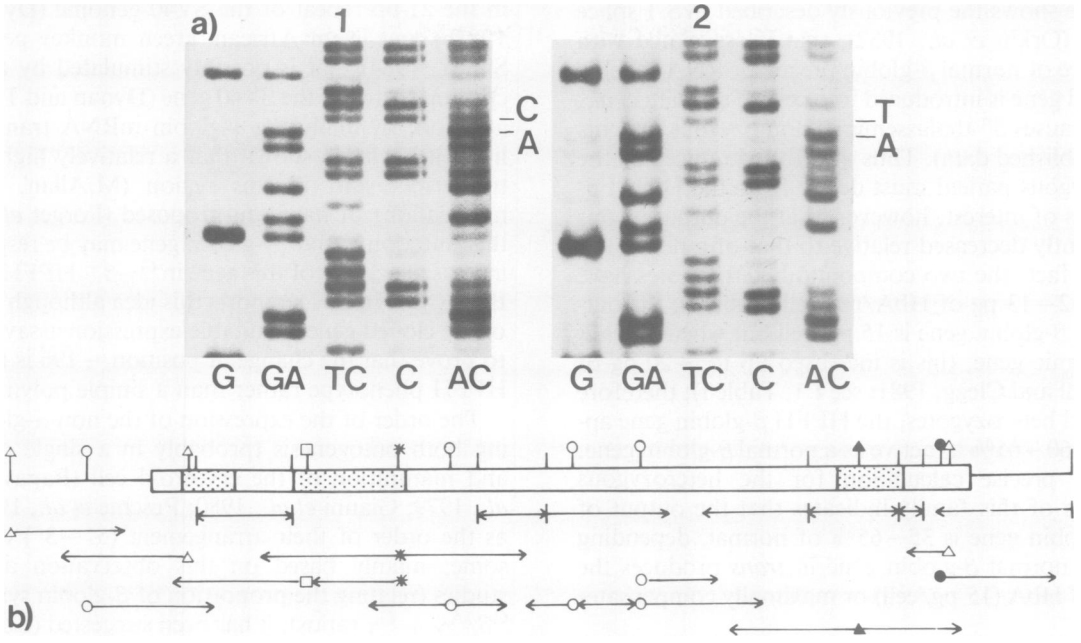

Fig. 4. (a) Sequence from cosmid I.2 of nucleotides adjacent to the donor site of the intervening sequence I of the β-globin gene. The sequence was read in the antisense strand; therefore CA = GT, normal splice signal; TA = AT, mutated splice signal. (b) Sequencing strategy for the HPFH β-globin gene. Arrows pointing to the right indicate that sequence was determined on the sense strand; arrows pointing to the left indicate that it was determined on the antisense strand. Enzymes used to generate the fragments to be labelled for analysis are as follows: △, *Hinf*I; ○, *Rsa*I; |, *Dde*I; □, *Ava*II; *, *Bam*HI; ●, *Hae*III; ▲, *Eco*RI.

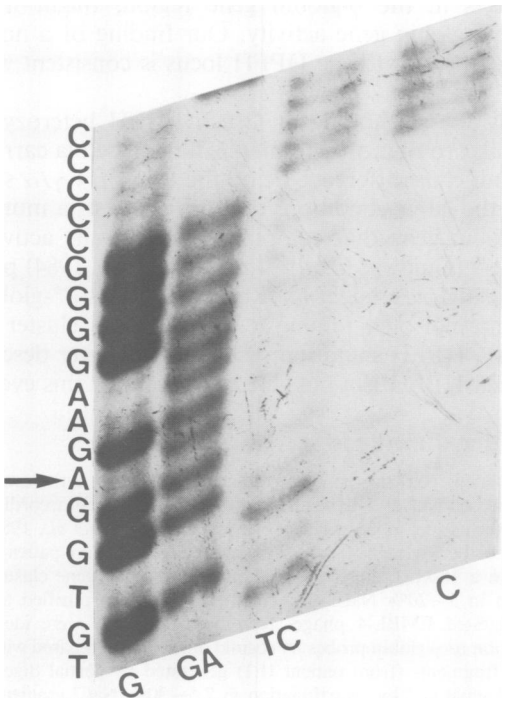

Fig. 5. Mutation in the 5′ flanking sequence of the HPFH γ-globin gene. The sequence was read on the strand opposite to that shown in Figure 6, starting from an *Ava*II site downstream from it, and confirmed by reading the opposite strand. An arrow shows the mutation.

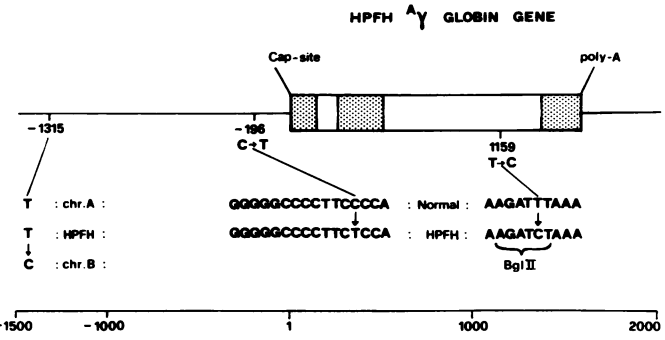

Fig. 6. A scheme summarizing the mutations detected in HPFH $^{A}\gamma$-globin gene.

globin sequence closely corresponds to that of the $^{A}\gamma$-gene from chromosome B, with three differences (Figures 5,6) (Slightom *et al.*, 1980; Slightom *et al.*, personal communication); of these changes, the mutation (relative to chromosome B) found in our gene at position −1315 is unlikely to be of any significance, as the $^{A}\gamma$-globin gene from chromosome A is identical to the HPFH $^{A}\gamma$-gene at this position. We are therefore left with only two real differences: a T→C substitution at position 1159 of the $^{A}\gamma$-globin gene, and a C→T substitution at position −196 of the 5′ flanking region (Figure 6).

Discussion

The hematological parameters of the patients with HPFH reported in this paper closely resemble those of individuals with the Greek type of HPFH (Fessas and Stamatoyannopoulos, 1964; Sofroniadou *et al.*, 1975; Clegg *et al.*, 1979; Weatherall and Clegg, 1981), although our patients are the first ones whose Greek ancestry is not obvious, they live in an area at the tip of the Italian peninsula, where Greek-speaking populations are still reported. For these reasons, in accordance with Weatherall and Clegg (1981), we will refer to these patients as carriers of Greek type HPFH.

The analysis of the β-thalassemic gene in *trans* to the HPFH chromosome solves the important question as to whether the HPFH β-globin gene is active. In fact, the β-

thalassemic gene shows the previously described IVS 1 splice junction defect (Orkin *et al.*, 1982), that is associated with complete absence of normal β-globin precursor RNA splicing when this cloned gene is introduced into cells (Treisman *et al.*, 1983) and that causes β°-thalassemia in homozygous patients (G.Saglio, unpublished data). Thus the HbA produced by the doubly heterozygous patient must derive from the HPFH β-globin gene. It is of interest, however, that the output of this gene is significantly decreased relative to that of a normal β-globin gene. In fact, the two compound heterozygotes have approximately 12–13 pg of HbA/red cell (Table I); the output of a normal β-globin gene is 15 pg/cell but when in *trans* to a β°-thalassemic gene, this is increased up to ~20 pg of HbA (Weatherall and Clegg, 1981; see I.1, Table I); therefore in the compound heterozygotes, the HPFH β-globin gene appears to be only 60–65% as active as a normal β-globin gene. A similar, less precise calculation for the heterozygous HPFH members of this family indicates that the output of the HPFH β-globin gene is 35–65% of normal, depending on whether the normal β-globin gene in *trans* produces the usual amount of HbA (15 pg/cell) or maximally compensates (20 pg/cell).

These calculations are only approximate, as it is known (Sofroniadou *et al.*, 1975; Clegg *et al.*, 1979) that in these patients (particularly in the compound HPFH/β-thalassemia heterozygotes) cells containing more HbF survive preferentially; however, the data indicate that the activity of the HPFH β-globin gene is significantly decreased relative to normal. Overall, the combined output of the γ- and β-globin genes on the HPFH chromosome may be slightly lower (80%) than normal. The determination of the nucleotide sequence of the HPFH β-globin gene shows that the structure of this gene is completely normal, including the putative upstream regulatory regions that are required for correct transcription of cloned β-globin genes *in vitro* and upon reintroduction in cells (Treisman *et al.*, 1983). This is the first reported functionally defective β-globin gene, that is nonetheless structurally normal and within a (grossly) intact non-α-globin cluster; a structurally normal β-globin gene was reported (Van der Ploeg *et al.*, 1980; Kioussis *et al.*, 1983) in a case of γ-δ-β-thalassemia, but in this instance a large deletion almost completely removes the non-α-globin cluster up to ~2 kb 5′ to the β-globin gene. It follows that a distant mutation elsewhere in the non-α-globin cluster must be responsible directly or indirectly, for the observed defect in the Greek HPFH β-globin gene. As the $^{A}\gamma$-globin gene is expressed at a very high level in this condition, we hypothesized that such a mutation might lie in some proximity to it. Only two mutations occur in this gene: in the large intron a T→C substitution causing the appearance of the abnormal *Bgl*II site is unlikely to affect to any degree the function of this gene; the remainder of the intron-exon sequence is normal; in the 5′ flanking region a single nucleotide substitution is found (at position −196 relative to the cap site) within the first 1350 nucleotides (Figures 5,6). The region where this mutation lies is completely conserved in several different $^{G}\gamma$ and $^{A}\gamma$-globin genes sequenced so far (Slightom *et al.*, 1980; Shen *et al.*, 1981; Slightom *et al.*, personal communication); moreover (Forget *et al.*, 1984) the only nucleotide change found in an over-expressed $^{G}\gamma$-globin gene from a patient with $^{G}\gamma$-β^{+} HPFH, is very close (position −201) to our mutation in a G-C rich region that is similar to regulatory sequences in the herpes virus thymidine kinase gene (McKnight *et al.*, 1984), in the 21-bp repeat of the SV40 genome (Dynan and Tjian, 1983b) and in an African green monkey gene (Saffer and Singer, 1984) that is possibly stimulated by the same transcription factor as the SV40 gene (Dynan and Tjian, 1983a). In addition, by analyzing γ-globin mRNA transcribed in fetal liver, it has been shown that a relatively high proportion of transcripts start in this region (M.Allan, personal communication). It has been proposed (Forget *et al.*, 1984) that the mutation in the $^{G}\gamma$-globin gene may be responsible for the increased activity of this gene in $^{G}\gamma$-β^{+} HPFH: our finding in the $^{A}\gamma$-β^{+} HPFH supports this idea although functional tests of the cloned gene in suitable expression assays will be needed to prove that the change at position −196 is the cause of the HPFH phenotype rather than a simple polymorphism.

The order of the expression of the non-α-globin genes during both ontogenesis (probably in a single cell population) and maturation of the erythroid cell (Papayannopoulou *et al.*, 1979; Gianni *et al.*, 1980; Peschle *et al.*, 1984) is the same as the order of their arrangement (5′→3′) on the chromosome; mainly based on this observation and on cellular studies (relating the proportion of β-globin synthesized to the $^{G}\beta/^{A}\gamma + {}^{G}\gamma$ ratios), it has been suggested (Comi *et al.*, 1980; Wood and Jones, 1981) that the normal expression of the β-globin gene is the end-result of the sequential propagation of changes in chromosome structure, beginning 5′ from the ε-$^{G}\gamma$-globin gene region and moving 3′ towards the β-globin gene during erythroid maturation. This model predicts (Jones *et al.*, 1982) that a delay or inhibition of this progression, due to changes in the γ-globin gene region, might indirectly decrease β-globin gene activity. Our finding of a normal β-globin gene in *cis* to the HPFH locus is consistent with this idea.

Finally the phenotype of Greek HPFH heterozygotes is very similar to that of Sardinian δβ°-thalassemia carriers, the only significant difference being the lower β+γ/α synthetic ratio in the latter condition, that results from a mutation in the β-globin gene that completely prevents its activity. We and others (Guida *et al.*, 1984; Pirastu *et al.*, 1984) proposed that crossing over might have exchanged this β°-globin gene with a normal one, joining it to a γ-globin cluster already carrying a HPFH mutation; the type of cluster described in this Greek HPFH is a possible candidate for this event.

Materials and methods

DNA obtained from the patients studied was digested with restriction enzymes, fractionated and hybridized with cloned probes according to the Southern technique, as described previously (Ottolenghi *et al.*, 1982).

To clone the γ-globin genes, *Bcl*I-digested DNA from patients I.1 was enriched in a 18-kb fragment containing the γ-globin gene cluster by centrifugation in 5–20% NaCl gradients, and ligated to purified arms from *Bam*HI-digested EMBL-4 phage. Recombinant clones were identified by hybridization to γ-globin probes. A cosmid library was prepared with 30–40-kb DNA fragments (from patient II.1) generated by partial digestion with *Mbo*I and separated by centrifugation in 7.5–30% NaCl gradients, before ligation into phosphatase-treated arms of the pTM cosmid vector (from F.Grosveld). The ligated DNA was packaged *in vitro* and transfected into ED8767 cells and clones containing the β-globin gene identified by hybridization to a purified β-globin fragment excised from a genomic clone.

For further characterization, digests from EMBL-4 and cosmid clones were analyzed by hybridization to probes excised from genomic clones.

After subcloning in pBR322, DNA sequencing was carried out according to Maxam and Gilbert (1980).

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