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Genetics

Molecular comparison of δβ-thalassemia and hereditary persistence of fetal hemoglobin DNAs: Evidence of a regulatory area?

(deletion/repetitive sequences/γ-globin genes/polymorphism)

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ABSTRACT **The hematological phenotypes of several Mediterranean patients with δβ-thalassemia and hereditary persistence of fetal hemoglobin have been characterized. Although clinical and hematological characteristics are essentially superimposable in all heterozygous δβ-thalassemics, these patients show typical $^{G}\gamma/^{A}\gamma$ ratios in their Hb F, ranging from ≈0.07 in Sardinian to ≈0.15 in Sicilian and ≈0.35 in Spanish patients. $^{A}\gamma^{\text{Sardinian}}$-(isoleucine$^{-75}\rightarrow$ threonine) is found in Spanish patients and accounts for all of the $^{A}\gamma$ production in heterozygotes, indicating that persistent production of γ chains occurs *cis* to the δβ-thalassemia gene. The molecular heterogeneity of these conditions is demonstrated by restriction enzyme mapping of DNA; Sicilian and Calabrian patients show a deletion starting from the δ-globin intron and extending several kilobases 3′ to the β-globin gene; in Spanish patients the deletion starts ≈2–3 kilobases 5′ to the δ-globin gene and extends well beyond the β-globin gene. Comparison of these deletions with previously described ones in Negro and in a new Southern Italian case of hereditary persistence of fetal hemoglobin suggests that the deletion of a region centered at a cluster of repetitive sequences ≈3.5 kilobases 5′ to the δ-globin gene may be critical for the persistent expression of high levels of γ-globin in hereditary persistence of fetal hemoglobin compared to δβ-thalassemia. The concept that the deletion or mutation of specific areas (rather than nonspecific changes brought about by large deletions in the globin cluster) is important in determining the persistent expression of γ-globin genes is supported by the finding of a nondeletion type of δβ-thalassemia in Sardinians.**

Hereditary persistence of fetal hemoglobin (HPFH) and $\delta^0\beta^0$-thalassemia are inherited conditions characterized by persistent expression of fetal ($^{A}\gamma$ and $^{G}\gamma$) globin chains into adult age, usually in association with decreased or abolished δ- and β-globin synthesis from the affected chromosome (1). In the absence of functional assays for transcriptional regulation, these conditions have attracted considerable interest as molecular models for investigating the role of genomic changes in the control of globin gene expression (2). Following the initial suggestion that deletion may be responsible for these conditions (3), liquid hybridization (reviewed in ref. 1) and gene mapping studies (4–13) defined a number of different deletions which provided the basis for more detailed hypothetical models (7, 12, 13). However, deletions so far demonstrated are very large, and it is not possible to determine whether the observed functional changes represent the result of the removal of specific regulatory areas or of gross DNA rearrangements affecting chromatin configuration and transcription; in addition, although increased γ-globin levels are constant in these conditions, the relative expression of the nonallelic $^{G}\gamma$ and $^{A}\gamma$ genes is very variable.

For these reasons, we have characterized the hematological and biosynthetic phenotypes of several Mediterranean $\delta^0\beta^0$-thalassemic and HPFH patients, and we have studied their globin genes by restriction enzyme mapping. We report a set of different overlapping deletions defining a possible regulatory area 5′ to the δ-globin gene.

MATERIALS AND METHODS

Plasmids. pHγG1 and pHβG1 were obtained from P. Little and R. Williamson (14); plasmids containing the RIH and δ-*Pst* I fragments (7) were from T. Maniatis. Amplification and extraction of these plasmids were as described (7, 14) and were conducted under P2 containment conditions in compliance with National Institutes of Health guidelines for recombinant DNA research. DNA mapping studies with restriction enzymes were carried out according to standard techniques (6, 15).

Globin Analysis. $^{G}\gamma/(^{G}\gamma + {}^{A}\gamma)$ ratios were determined by isoelectric focusing (IEF), in the presence of Nonidet P-40, of globin chains (16) obtained from Hb F partially purified by preparative IEF (17). $^{A}\gamma^{\text{Sardinia}}$ percentage was evaluated by IEF (18) of hemoglobin in the presence of β-alanine (unpublished data).

Patients. Hematological studies of a group of Sardinian and Spanish $\delta^0\beta^0$-thalassemia patients have been described (19, 20). An additional Sardinian family and other $\delta^0\beta^0$-thalassemia and HPFH patients will be reported elsewhere.

RESULTS

Hematological Findings. Table 1 lists the main hematological values found in the different families studied. With the exception of heterozygotes from family 1 (Southern Italian "Negro" type HPFH) who showed a very high level of Hb F and essentially normal hematological values (consistent with a diagnosis of HPFH), heterozygotes from all other families demonstrated uniform and typical $\delta^0\beta^0$-thalassemia characteristics [relatively low, heterogeneously distributed Hb F and low mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV)]; however, IEF examination of the relative proportion of $^{G}\gamma$- and $^{A}\gamma$-globin chains in their Hb F clearly demonstrated consistently different ratios which appear to be at least partly under

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Abbreviations: HPFH, hereditary persistence of fetal hemoglobin; IEF, isoelectric focusing; MCH, mean corpuscular hemoglobin; MCV, mean cellular volume; kb, kilobase(s).

Table 1. Main hematological and globin data for $\delta\beta$-thalassemia and HPFH heterozygotes

Patients	Hb, g/dl	MCH, pg	MCV, fl	Hb F, %	Hb A_2, %	β/α synthesis ratio	γ/α synthesis ratio	$\frac{^{G}\gamma}{^{G}\gamma + ^{A}\gamma}$	$\frac{^{A}\gamma^{Sardinia}}{^{A}\gamma}$
Southern Italian HPFH	14 (12–16)	29 (27–31)	85 (74–91)	30 (29–32)	1.9 (1.7–2.0)	0.66	0.27	0.18 (0.15–0.20)	*
Sardinian $\delta\beta^0$-thalassemia†	13.7 (13–15)	26 (21–29)	78 (70–84)	15 (10–25)	2.4 (1.4–3.2)	0.61 (0.37–0.81)	0.14 (0.08–0.22)	0.07 (0.05–0.1)	*
Sicilian and Calabrian $\delta^0\beta^0$-thalassemia	12.4 (9–14)	23 (21–26)	71 (67–79)	9 (5–13)	2.1 (1.7–2.7)	0.65 (0.60–0.71)	0.08 (0.06–0.09)	0.15 (0.1–0.2)	*
Spanish $\delta^0\beta^0$-thalassemia	13.1 (12–15)	23 (20–26)	69 (63–77)	10 (5–13)	2.3 (1.3–2.7)	—	—	0.35 (0.30–0.45)	1.04 (0.8–1.25)

Data from refs. 19 and 20 and unpublished results. The results are shown as mean and range.
* $^{A}\gamma^{Sardinia}$ was not detectable.
† Double heterozygous $\delta\beta^0$-/β^0-thalassemia patients have no Hb A (20).

hereditary control (Table 1; Fig. 1). Interestingly, homozygotes (Sicilian $\delta^0\beta^0$-thalassemia families 1 and 2 and Spanish $\delta^0\beta^0$-thalassemia) always exhibited higher $^{G}\gamma/(^{G}\gamma + ^{A}\gamma)$ ratios ($\approx$0.45) than their heterozygous relatives. If it is assumed that γ-globin genes in the normal chromosome contribute only to a minor extent to total Hb F production in these heterozygotes (see below), this observation suggests that the abnormal γ-globin synthesis occurring in these patients is not constitutive but is still subjected to modulation, possibly in response to erythropoietic stress. An additional distinctive feature of Spanish $\delta^0\beta^0$-thalassemia is the occurrence of an isoleucine/threonine substitution at position 75 of the $^{A}\gamma$ chain [the known $^{A}\gamma^{Sardinia}$ or $^{T}\gamma$ polymorphism (21)]; in all the heterozygotes studied, from different branches of the family, the level of $^{A}\gamma^{Sardinia}$ is coincident with the $^{A}\gamma$ level, suggesting that abnormal production of γ-globin chains mainly occurs *cis* to the $\delta^0\beta^0$-thalassemia defect, as previously inferred for γ-globin chain synthesis in Hb Kenya carriers (3).

Gene Mapping Studies. *Southern Italian HPFH.* Fig. 2 summarizes our mapping data for DNA from Southern Italian HPFH. Analysis of restriction enzymes fragments from this DNA yielded normal bands only (consistently of lower intensity than corresponding bands in control DNA) when RIH or β probes were used (not shown). Because only heterozygotes are available from this family, these data are consistent with either complete deletion of the $\delta\beta$-globin genes and surrounding DNA or with normality; the latter interpretation, however, is ruled out by the demonstration of abnormal bands with *Kpn* I and *Bam*HI, which hybridize with γ (Fig. 3) but not β and RIH (not shown) probes. From the normality of *Bgl* II, *Pst* I, and *Eco*RI γ fragments (not shown), the 5′ border of the deletion can be approximately defined; the 3′ end remains more uncertain. This case of HPFH with typical "Negro type" features is of interest because, as in previously described cases, HPFH is associated with a complete deletion of the δ- and β-globin genes. The absence of DNA corresponding to the RIH fragment rules out the similarity with the cases studied by Fritsch *et al.* (7) and Tuan *et al.* (9); on the other hand, the relatively small size of the abnormal *Kpn* I fragment suggests an independent origin of this deletion from that observed in the previously studied Ghanian HPFH (10, 12). It should be pointed out, however, that the different *Kpn* I fragment might be due either to a different 3′ break-point or simply to a polymorphic *Kpn* I site.

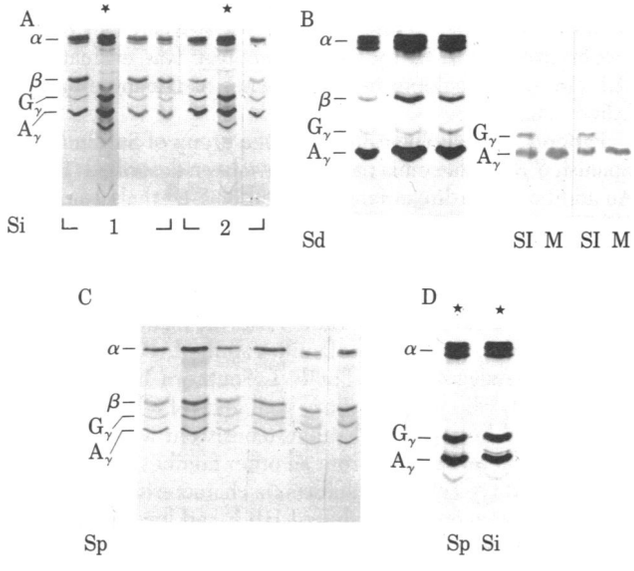

FIG. 1. IEF of $^{G}\gamma/^{A}\gamma$ ratios in partially purified Hb F or γ-globin chains. Si 1 and 2: Sicilian $\delta^0\beta^0$-thalassemia heterozygous and homozygous patients from two different families; Sd, Sardinian $\delta^0\beta^0$-thalassemia heterozygotes from three independent families; SI, Southern Italian HPFH heterozygotes; M, marker $^{A}\gamma$-globin chains; Sp, Spanish $\delta^0\beta^0$-thalassemia heterozygous and homozygous patients. ⋆, Homozygous patients.

Sardinian $\delta^0\beta^0$-thalassemia. Only heterozygotes are known with this type of $\delta^0\beta^0$-thalassemia; no abnormal bands could be found from these DNAs with *Kpn* I and *Bam*HI (Fig. 3), *Hin*dIII (see Fig. 5), *Eco*RI, *Bgl* II, *Pst* I, *Hpa* I and *Xba* I, with γ, β, δ, and RIH probes; the intensity of the bands was in general comparable to that from control DNA (Fig. 4) (also, some data not shown). Because some of the enzymes used yielded large fragments bridging DNA areas that can be analyzed with different probes, it is unlikely that deletions in the $\delta\beta$ cluster would not cause the appearance of abnormal bands with RIH and γ probes, or vice versa; thus, the evidence is compatible with the absence of any gross rearrangement in the $^{G}\gamma$–$^{A}\gamma$–δ–β cluster, although small deletions cannot be ruled out. Similar conclusions have been reached with DNA from Greek HPFH heterozygotes (10, 12).

$\delta^0\beta^0$-Thalassemia from Calabria and Sicily. A deletion map for $\delta^0\beta^0$-thalassemia from Calabria was initially deduced from studies of independent heterozygotes from several families. It showed (Fig. 2) a large deletion extending from the δ-globin gene intron to several kilobases downstream from the β-globin gene. To our surprise, comparison with published data for $\delta^0\beta^0$-thalassemia homozygotes from Sicily (see legend to Fig. 2 and refs. 6–8) demonstrated essentially identical fragment sizes for all enzymes tested, with the exception of *Hin*dIII which gave an $\approx$34-kilobase (kb) RIH (Fig. 5) or δ (not shown) fragments instead of the $\approx$13.5-kb band expected on the basis of a *Pst* I/*Hin*dIII double digestion (8). This prompted reexamination of

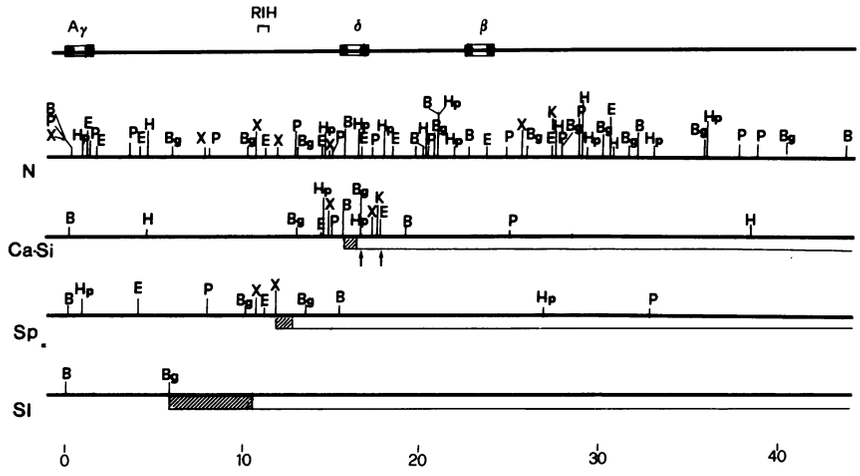

FIG. 2. Restriction enzyme map of the globin gene cluster in normal (N), Calabrian (Ca), Sicilian (Si), and Spanish (Sp) $\delta^0\beta^0$-thalassemia DNAs and Southern Italian (SI) HPFH DNA. The normal map is derived mainly from ref 7. Some additional sites 3′ to the β-globin gene are from ref. 22. The *Hin*dIII and *Kpn* I sites are from refs. 11 and 12; the *Bam*HI site at the extreme 3′ end of the map is from ref. 23. Fragment lengths for the new DNAs studied were calculated by reference to normal globin fragments of appropriate sizes run in parallel, adopting the lengths given in ref. 7. The map and fragment sizes of Calabrian and Sicilian $\delta^0\beta^0$-thalassemia patients are identical and essentially in agreement with those published previously (6–8, 13). Arrows point to the region in Sicilian $\delta^0\beta^0$-thalassemia DNA that is apparently homologous to the normal region 3′ to the β-globin gene. Regions of the globin cluster deleted in $\delta^0\beta^0$-thalassemia and HPFH are indicated by boxes. The precise locations of the end points of the deletion are within the regions defined by the hatched boxes. Scale at the bottom is in kilobases.

mapping data for the previously studied homozygote (6), which demonstrated the abnormal ≈34-kb *Hin*dIII δ (Fig. 5) and RIH (not shown) fragment in both the Sicilian homozygote and the Calabrian $\delta^0\beta^0$-thalassemia group of heterozygotes. In addition the size of the previously unmapped (6, 8) abnormal *Bam*HI fragment containing part of the δ-globin intron was defined; the relative positions of all other restriction sites tested (not shown) are consistent with previous data (6–8).

It had been pointed out (8) that the relative distances of several sites (*Bgl* II, *Eco*RI, *Taq* I, *Kpn* I, and *Hin*dIII) 3′ to the δ-globin gene fragment in Sicilian $\delta^0\beta^0$-thalassemia are similar to those observed in normal DNA 1–4 kb 3′ to the β-globin gene; however, the position of *Hpa* I (7, 8), *Pst* I (6, 7), *Bam*HI, and, in at least some patients, *Hin*dIII sites now appear inconsistent with those found in normal cloned DNA (22) in the ≈20-kb region 3′ to the β-globin gene. It is unlikely that all of these discrepancies are due to polymorphism in the $\delta^0\beta^0$-thalassemia DNA [as suggested for the *Hpa* I site (8)] or in the cloned sequence (22); rather, the data indicate a large extension of the deletion 3′ to the β-globin gene, although a precise definition of the break point will require additional mapping studies with unique-sequence cloned probes from this region.

On the other hand, a problem arises as to whether similarities of several sites 3′ to the deletion with those 3′ to the β-globin gene represent a chance coincidence or bear a special relationship to the deletion. Some of the sequences located 3′ to the β-globin gene appear to be highly repeated in the globin gene cluster and elsewhere in the genome (24–27); in particular, a long (6.4 kb) sequence without internal repetitivity, which is represented 3000–4800 times in the genome, is found ≈3 kb downstream from the β-globin gene (27). If these sequences participate in a recombinational mechanism with distant similar

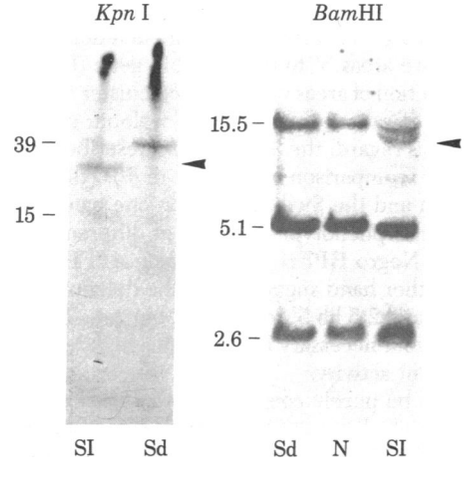

FIG. 3. γ-Globin DNA restriction fragments. N, normal; SI, Southern Italian HPFH heterozygote; Sd, Sardinian $\delta^0\beta^0$-thalassemia heterozygote. Arrows point to abnormal bands; sizes are shown in kilobases.

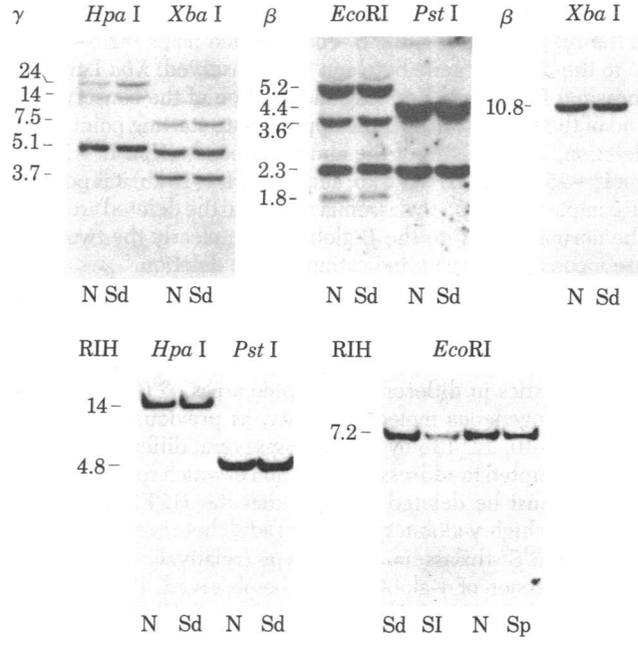

FIG. 4. Analysis of γ, β, and RIH DNA restriction fragments. Sp, Spanish homozygous $\delta^0\beta^0$-thalassemia. For other details, see Fig. 3.

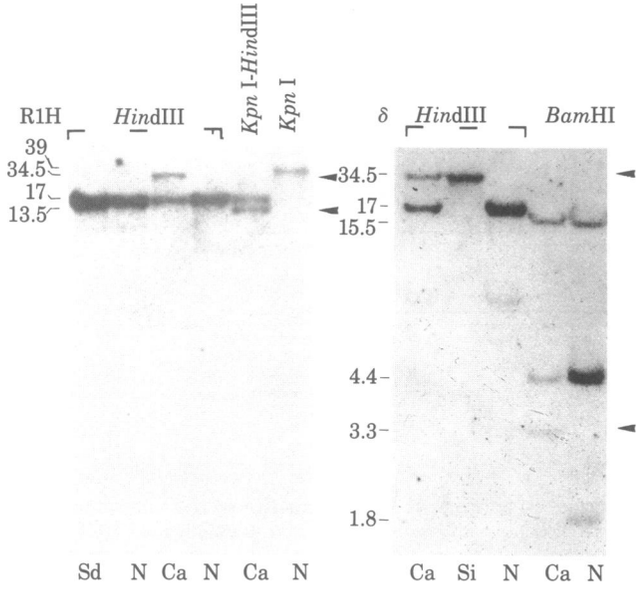

FIG. 5. Analysis of RIH and δ DNA restriction fragments. Ca, Calabrian $\delta^0\beta^0$-thalassemia heterozygote; Si, Sicilian $\delta^0\beta^0$-thalassemia homozygote. Also see Fig. 3.

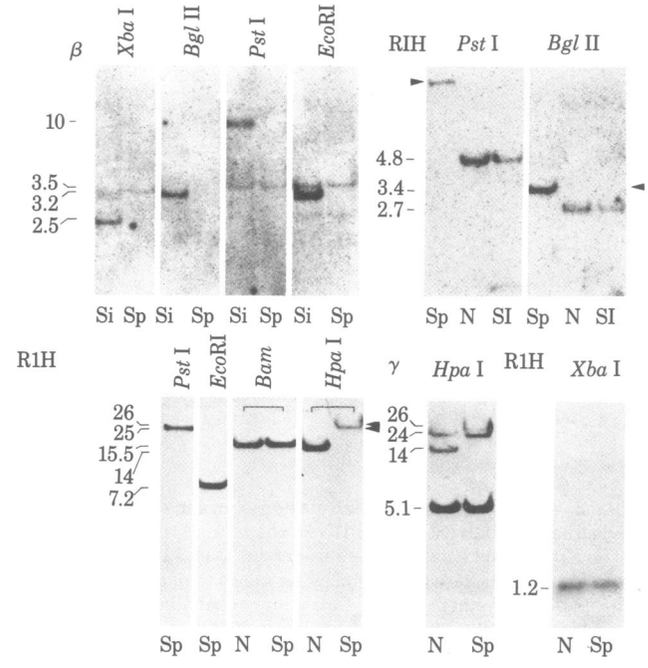

FIG. 6. Analysis of γ, β, and RIH DNA restriction fragments. Also see Fig. 3.

sequences generating the deletion, the observed similarities could be explained.

Based on this type of explanation, the discrepancies in the *Hin*dIII sites might reflect the joining of the fragment of δ-globin gene to similar, but not identical, repetitive regions in different patients; alternatively, however, a mutation might have occurred in the DNA of individuals with this rather widespread type of $\delta^0\beta^0$-thalassemia to generate a polymorphic *Hin*dIII site [the possibility that such a mutation occurred during the *in vitro* propagation of lymphoid cells used for the study (8) should also be considered].

Spanish $\delta^0\beta^0$-thalassemia. No hybridization to the β probe could be detected by using DNA from the homozygous patient (Fig. 6); on the other hand, RIH demonstrated abnormal bands with *Hpa* I, *Pst* I, and *Bgl* II although not with *Eco*RI (Fig. 4) and *Xba* I (Fig. 6). These data demonstrate a complete deletion of the δβ gene cluster, the 5′ end of which maps ≈2.5–3.5 kb 5′ to the δ-globin gene between the (conserved) *Xba* I and the (missing) *Pst* I sites. Knowing the location of the conserved 5′ end of the bands, which is the approximate starting point of the deletion, and the size of the abnormal bands (*Hpa* I, ≈26 kb; *Pst* I, ≈25 kb; *Bgl* II, ≈3.4 kb; and *Bam*HI, ≈15 kb) it is possible to compare the $\delta^0\beta^0$-thalassemia map 3′ to the deleted area with the normal map 3′ to the β-globin gene; clearly the two maps are inconsistent, again indicating a large deletion.

DISCUSSION

This paper demonstrates that, in spite of similar phenotypic characteristics in different geographic areas, $\delta^0\beta^0$-thalassemia is a heterogeneous molecular entity, as previously shown for HPFH (7–10, 12, 13); by comparing several different cases we have attempted to address the question of which specific regions (if any) must be deleted in order that the HPFH phenotype (relatively high γ-globin synthesis in adult heterozygotes) rather than the $\delta^0\beta^0$-thalassemia phenotype (relatively minor persistent expression of γ-globin genes) be observed. Previous comparisons (6–8, 13) of $\delta^0\beta^0$-thalassemia and Hb Lepore DNA maps suggested that either deletions in a small (1.8 kb) area 3′ to the β-globin gene (8) or the loss of complete "δ-globin-like" (6, 13) sequences might be responsible for the higher expression of γ-globin genes in the former condition. Much larger deletions were found on both 5′ and 3′ sides of the δβ cluster in HPFH (7, 9, 10, 12, 22).

The recent publication of a detailed restriction enzyme map for ≈20 kb of cloned normal DNA 3′ to the β-globin gene (22) allows comparison of the mapping data of $\delta^0\beta^0$-thalassemia patients of Sicilian (6–8, 12), Calabrian, and Spanish origin with those of normal DNA downstream from the β-globin gene. Fig. 1 shows that a large deletion 3′ to the β-globin gene occurs not only in Negro HPFH, as previously suggested (7, 9, 10, 12, 22), but also in $\delta^0\beta^0$-thalassemia (Sicilian and Spanish types) and in the Italian HPFH. These data are consistent with the possibility that the deletions removing a large piece of DNA close to the globin cluster affect γ-globin function nonspecifically by altering chromatin conformation and transcription; however, it is clear that even large deletions, as observed in $\delta^0\beta^0$-thalassemia, are not sufficient *per se* to cause the same degree of persistent γ-globin gene expression as in HPFH.

On the other hand, it has been noted (3, 7, 28) that HPFH phenotypic changes are associated with a deletion (in Hb Kenya) that must spare areas 3′ to the β-globin gene (Fig. 1); accordingly, the deletion of areas within the δβ cluster or 5′ to it might be sufficient to cause a high level of γ-globin gene persistent activity. In this regard, the Spanish case described in this paper is informative; comparison of this type of $\delta^0\beta^0$-thalassemia with the Calabrian and the Sicilian cases on one hand (showing essentially similar phenotypes in spite of different 5′ deletions) and with the Negro HPFH [in particular HPFH 1 and 2 (7, 9, 10)] on the other hand suggests that the deletion of sequences between 4.1 and 2.5 kb 5′ to the δ-globin gene (Fig. 7) may be the critical event necessary to establish a high level of γ-globin gene persistent activity.

It may not be purely coincidental that a set of repeated sequences (24) which appear to be transcribed both *in vivo* (24, 25) and *in vitro* (26) lies just in this putative regulatory area. It is possible that these sequences are simply related to the mechanism generating the deletion, but a key functional role is entirely consistent with our data (the potential importance of the $^A\gamma$–δ region and of repetitive sequences in it for regulation of

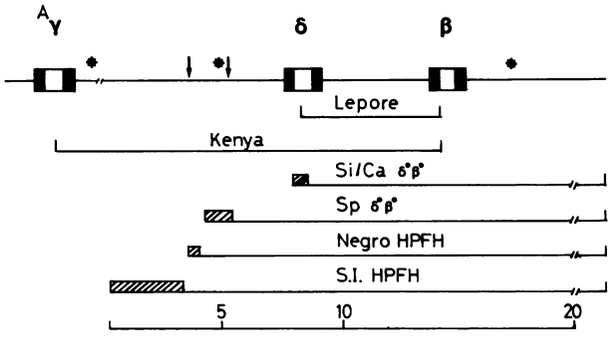

FIG. 7. Schematic representation of DNA deletions in Hb Lepore, Hb Kenya, and $\delta^0\beta^0$-thalassemia and HPFH patients. Negro HPFH is from ref. 7. The putative DNA regulatory area defined by the comparison between $\delta^0\beta^0$-thalassemia and HPFH is within the sites indicated by arrows. Asterisks mark some repeated sequences (ref. 24).

globin gene expression is also discussed in refs. 7, 9, 12, 13, 29).

In conclusion, our results on $\delta^0\beta^0$-thalassemia suggest that functional inherited changes in γ-globin gene activity may be related, at least in part, to deletions or defects of specific DNA sequences rather than to nonspecific changes brought about by large deletions. This idea is strongly supported by the apparent normality of the globin gene cluster in Greek type HPFH (10, 12) [in which $^A\gamma$- and, to some extent, $^G\gamma$-globin genes are expressed in adults (30, 31)] and, in particular, in Sardinian $\delta^0\beta^0$-thalassemia, in which the abnormal expression of γ-globin genes is associated with complete suppression of β-globin (ref. 20; Table 1) and perhaps δ-globin gene activities. In view of our present data, it will be of interest to examine, by cloning, the DNA sequences corresponding to the putative regulatory regions defined in our studies in these nondeletion types of HPFH and $\delta^0\beta^0$-thalassemia. In this respect it is interesting to note that, in at least one different biological model (the histone gene cluster), small distant DNA sequences have been implicated in the regulation of the level of activity of the structural genes (32).

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