Molecular characterization of a novel form of ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemia deletion with a 3' breakpoint close to those of HPFH-3 and HPFH-4: insights for a common regulatory mechanism

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ABSTRACT

We have molecularly characterized a novel ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemia associated with increased synthesis of HbF in three members of a German family. The levels of HbF in the peripheral blood red cells of the heterozygotes ranged between 9.9% and 12.5% with a heterocellular distribution in the red cells, as detected by immunofluorescence. The mutation resulted from a deletion starting about 1.5 to 1.9 kb from the 3' end of the G_{γ} -gene and ending 27 ± 0.5 kb 3' to the β -globin gene. Thus, the total deletion is 52 ± 0.5 kb. Its 5' breakpoint is similar to that of the previously described ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemias, while the location of the 3' breakpoint is placed very close to the 3' breakpoints of HPFH-4 and HPFH-3 deletions. The proximity of the 3' breakpoint of the German ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemia to those of HPFH-3 and HPFH-4 deletions raises the possibility that a common mechanism, such as the juxtaposition of an enhancer, might underlie the activation of the γ -globin genes in these three mutants.

INTRODUCTION

The $\delta\beta$ -thalassemias and hereditary persistence of fetal hemoglobin (HPFH) represent two heterogeneous groups of mutations, characterized by the continuous expression of fetal hemoglobin in adult life, as a result of either variable length deletions or point mutations in the β -globin gene cluster (1). Distinct features between the two groups exist, in that heterozygotes for the $\delta\beta$ -thalassemias exhibit moderate levels of HbF (10-15%) with a heterocellular distribution in the red cells, while in HPFH mutations, there is a much higher level of HbF (15-25%) with a pancellular distribution. In a subset of the $\delta\beta$ -thalassemias, the deletion involves the A_{γ} -gene as well, resulting in the so-called ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemia. Heterozygotes for this condition exhibit 10-15% HbF, being mostly of the G_{γ} -type (2). Molecular analysis of several patients with ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemia has so far revealed the existence of seven different types of deletions (3-11). The molecular mechanisms involved in the generation of the above phenotypes remain largely unknown. Current models attempting to explain the continuous expression of the γ -globin genes in adults carrying these mutations, include the deletion of negative regulatory sequences, the juxtaposition of enhancer-like sequences in the vicinity of the γ -globin genes, and modifications of the chromatin structure within the β -globin cluster as a result of the deletion (2).

In this study, we molecularly characterized a novel type of deletion resulting in ($A_{\gamma\delta\beta}$) $^{\circ}$ -thalassemia in three individuals of a German family. We also have analyzed the cellular and biochemical phenotypes of these heterozygotes and tested the response of their erythroid progenitors (BFUe) to the fetal sheep "switching factor" *in vitro*. The deletion removes the

A_{γ} -, ψ -, δ - and β -globin genes and its total length is 52 ± 0.5 kb. The data regarding its 5' and 3' breakpoints are consistent with the other $(A_{\gamma}\delta\beta)^{\circ}$ deletions where they characteristically exhibit similarities of the locations of the 5' breakpoints and heterogeneity in the location of their 3' breakpoints. The biochemical phenotype of the deletion was found also to be consistent with those of the other $(A_{\gamma}\delta\beta)^{\circ}$ -thalassemias. Finally, the cultures of the erythroid progenitor cells (BFUe) of the heterozygotes responded to the "switching activity" of the fetal sheep serum, by decreasing the synthesis of HbF in vitro.

MATERIALS AND METHODS

Hematological studies

Routine hematological parameters were measured with standard methodology. Distribution of HbF in erythrocytes (F-cells) was assayed with a monoclonal anti- γ -globin antibody (12). Labeling of the erythrocytes was performed using methanol-fixed preparations and stained with anti- γ antibody conjugated to rabbit anti-mouse F(ab')₂-fluorescein isothiocyanate (FITC) (12). Globin biosynthetic ratios in reticulocytes were measured by ³H-leucine labeling and isoelectric focusing, as previously described (12).

Preparation of DNA and Southern blot analysis

High molecular weight DNA from peripheral blood was extracted, as previously described (13). Following digestion with restriction endonucleases, separation of the DNA fragments was performed in 1.0% agarose in vertical slab gels. Transfer of DNA to nitrocellulose filters, hybridization and washing of the filters were performed, as previously described (13). Genomic DNA fragments derived from the β -like globin gene cluster were used as probes (as specified in the legends of Figures 1 and 2) after labeling with ³²P by nick-translation, as previously described (13).

Anti-globin monoclonal antibodies

Two previously well-characterized human anti-globin monoclonal antibodies were utilized for immunofluorescence-labeling of the hybrids: 51.7 specific for γ -globin chains, and 37 specific for β -globin chains (12).

Culture of erythroid progenitors

Peripheral blood mononuclear cells were isolated using Hypaque-Ficoll centrifugation and were further proceeded and cultured as previously described in detail (14). BFUe were collected after 12-16 days of culture and were incubated with ³H-leucine for globin biosynthetic studies as described previously (12). Control cultures contained fetal calf serum (FCS) while, in experimental cultures, FCS was substituted with a preparation of fetal sheep serum (FSS) which has been shown previously to induce HbF to HbA switching in cultures of BFUe from neonates, normal adults and patients with continuous expression of fetal hemoglobin in adult life (15-18).

RESULTS

Hematological Data

Three members of the German family (father, daughter, and granddaughter) exhibited increased amounts of HbF in peripheral blood. All three individuals were asymptomatic and their hematological parameters are shown in Table 1. Their erythrocyte morphology was characterized by moderate microcytosis and hypochromia. HbF ranged from 9.9% to 12.5%. The $G_{\gamma}/A_{\gamma}+G_{\gamma}$ ratio was 0.8, indicating that the majority of HbF produced was of the G_{γ} type. The distribution of HbF in the red blood cells was heterocellular. The F-cells ranged from 53.9% to 76.6%. These results were compatible with

Table 1. Hematological data of the three ($A_{\gamma}\delta\beta$)^o-thalassemia heterozygotes in the German family.

Individual	Hb	PCV	RBC	MCV	MCH	MCHC	HbF	F-cells	HbA ₂	Biosynthetic ratios	
	g/dl	l/l	x10 ¹² /l	fl	pg	g/dl	%	%	%	$\gamma/\gamma+\beta$	$G_{\gamma}/G_{\gamma}+A_{\gamma}$
Father	14.0	0.44	5.8	76.1	24.1	31.6	ND	67.4	2.5		
Daughter	12.0	0.36	5.3	69.0	22.5	32.7	9.9	53.9	2.4	0.13	0.84
Granddaughter	13.3	0.41	5.9	68.8	22.2	32.2	12.5	76.6	2.6		

($A_{\gamma}\delta\beta$)^o-thalassemia in the family. This interpretation was further documented by molecular analysis of the DNA of the three individuals.

Molecular analysis

To test the possibility of a deletion underlying the $\delta\beta$ -thalassemia phenotype, DNA of the proband was digested with several enzymes and hybridized with a $\psi\beta$ probe. The expected normal fragments were documented (data not shown); however the intensity of the bands was decreased to one-half of that of the normal control, as expected if only one copy of the $A_{\gamma}\delta\beta$ region was present. Since the intensity of the $\psi\beta$ -specific fragments was one-half the intensity of normal, these data suggested that a deletion removing the A_{γ} -, $\psi\beta$ -, δ -, and β -globin genes was underlying the ($\delta\beta$)^o-thalassemia phenotype of this family. The ($\delta\beta$)^o-thalassemia was preliminarily designated as the German variation of ($A_{\gamma}\delta\beta$)^o-thalassemia.

Mapping the 5' breakpoint. To probe the status of the region 5' of the $\psi\beta$ -globin gene further, including the A_{γ} -gene region, DNA from these individuals was digested with Bgl II and hybridized with the 0.9 kb Bam HI-Eco RI fragment from the IVS-II of the G_{γ} -gene (which is highly homologous to that of the A_{γ} -gene). This study revealed the normal 13.0 kb fragment and an abnormal 9.3 kb fragment (Figure 1), indicating that the single Bgl II site 3' of the A_{γ} -gene had been deleted, as shown in diagram of Figure 1. Further mapping using the enzymes Sac I, Eco RI, and Hind III with the G_{γ} IVS-II probe confirmed complete deletion of the A_{γ} -gene on one chromosome (Figure 1); no abnormal fragments were found in the patient's lanes, but the intensity of the signal from fragments containing the A_{γ} -gene (3.8 kb Sac I, 2.7 kb Eco RI, and 2.7 kb Hind III) was half of that of the G_{γ} -gene containing fragments (Figure 1).

To further localize the exact 5' breakpoint, a 1.6 kb Eco RI fragment probe containing the 3' of the G_{γ} -gene was used (Figure 1). The patient's DNA digested with Bgl I, BstE II, Hpa I, Nco I, or Xba I revealed abnormal fragments, while digestion with EcoRI, Acc I and Sac I showed no abnormal fragments (Figure 1). These data suggest that the EcoRI site situated 1.5 kb 3' of the G_{γ} -gene is intact, while the Nco I site located 440 bp downstream from the EcoRI site is deleted. In addition, hybridization with the 0.7 probe (Figure 1) revealed no abnormal fragments with EcoRI, Acc I or Nco I (data not shown) suggesting that this 0.7 kb region is mostly deleted in this patient. Therefore, the 5' breakpoint of the German ($A_{\gamma}\delta\beta$)^o-thalassemia deletion can be localized within this 440 bp region, which lies 1.5 to 1.9 kb downstream from the 3' end of the G_{γ} -globin gene (Figure 1).

Figure 1
Mapping of the 5' breakpoint of the German ($A\gamma\delta\beta$) $^\circ$ -thalassemia deletion. Three DNA fragments derived from the region of the γ -globin genes were used as probes and are indicated by brackets over the map: IVS-II, Eco RI 1.6, and 0.7 Eco RI. The deletion is shown as a heavy black line under the map. The region of the uncertainty of the 5' breakpoint is designated by broken lines.

Mapping the 3' breakpoint. To map the 3' breakpoint of the deletion three probes were used. Probe pRK29 was initially used since it represents(19) the closest available nonrepetitive DNA region 3' of the β -globin gene (Figure 2), probe 3'IH and probe 3D. Analysis with the pRK29 probe which lies 18 kb 3' from the β globin gene (Figure 2), revealed no abnormal fragments in the patient's lanes, but the intensity was one-half of that of the normal control (Figure 2, left panel) suggesting that the deletion extended beyond this region. To exclude whether the breakpoints of the deletion are extending near the HPFH-1 and HPFH-2 deletions, we used a probe derived from the cloned 3' region of HPFH-1 deletion (20). We documented that the region included within the 22.0 kb Hpa I fragment (Figure 2, right panel) is intact on the mutant chromosome. Therefore, the 3' breakpoint appeared necessarily to lie in a region upstream from the Hpa I site 5' to the 3D probe and downstream from the Hpa I site located 5' to the

Figure 2

Mapping of the 3' breakpoint of the German ($A\gamma\delta\beta$) $^\circ$ -thalassemia deletion. Three fragments from the 3' region of the β -globin gene cluster were used as probes, as indicated by the brackets over the map: RK 29, 3' IH, and 3D. The total length of the deletion and the region of the 3' breakpoint are shown as a heavy black line. The region of uncertainty is indicated by broken lines. Vertical arrows indicate the 3' breakpoints of the four types of HPFH. Bg = Bgl II, X = Xba I, E = Eco RI, S = Sac I, Nc = Nco I, H = Hind III, Hp = Hpa I. Note that the restriction map shown in the diagram was derived from mapping genomic DNA using the 3' IH probe (22).

pRK29 probe. This was confirmed by detailed mapping using the third probe (3' IH) a Hinf I-Bgl II 0.7 kb fragment (kindly provided by P. Henthorn and D. Mager) from the cloned bridging fragment of the HPFH-3 (Indian) deletion (21,22); digestion with Bgl II, Hpa I, and Xba I generated 9.3, 11.5, and 11.5 kb abnormal fragments, respectively (Figure 2). These represent the bridging fragments of the deletion, since fragments of identical size were detected with the 1.6 Eco RI probe (Figure 1). Furthermore, no abnormal band was present in Eco RI digested DNA, suggesting that the 3' breakpoint of the deletion lies between the Xba I and the Eco RI sites (Figure 2). These data allowed the localization of the 3' breakpoint 27 ± 0.5 kb downstream from the 3' end of the β -globin gene and defined the total length of the deletion at 52.5 ± 0.5 kb. The 3' breakpoint, as it is shown in Figure 2, lies about 1.5 kb and 3.5 kb from the HPFH-4 (23) and HPFH-3 (21,22) breakpoints, respectively.

Studies in BFUe cultures. Previous studies have shown that HbF expression in cultures of erythroid progenitors (BFUe) colonies can be

Figure 3

Effect of the "switching activity" of the fetal sheep serum on the biosynthetic ratio of γ -chains in cultures of the erythroid progenitors (BFUe) from a heterozygote with German ($A_{\gamma\delta\beta}$)^o-thalassemia. The two pairs of columns represent two different experiments. FC = fetal calf serum; FS = fetal sheep serum.

modulated by the culture environment (15-18); thus, culture in fetal sheep (instead of fetal calf serum) drastically decreases HbF expression in HPFH or $\delta\beta$ -thalassemia BFUe. To test whether "switching activity" of the fetal sheep serum can affect HbF expression in the German ($A_{\gamma\delta\beta}$)^o-thalassemia, we cultured erythroid progenitors from two heterozygous individuals. $\gamma/\gamma+\beta$ ratio in erythroid progenitors cultured in FCS was 0.2 to 0.3. This ratio was reduced to 0.01 or 0.02 in BFUe cultures done in FSS (Figure 3). To test the effect of culture conditions on HbF productions by individual cells of the clones, individual colonies were lifted from the plates and were labeled with anti- γ and anti- β antibodies. Over 80% of the cells of the FCS-grown clones exhibited HbF synthesis. In contrast, less than 5% of the FSS-grown cells displayed γ -globin expression (data not shown).

DISCUSSION

We have molecularly characterized a novel type of ($A_{\gamma\delta\beta}$)^o-thalassemia deletion associated with increased expression of γ -globin genes in adult life. The deletion of the German ($A_{\gamma\delta\beta}$)^o-thalassemia starts 1.5 to 1.9 kb from the 3' end of the G_{γ} -gene and ends 27 ± 0.5 kb from the 3' end of the β -globin gene, removing 52 ± 0.5 kb of DNA.

The 5' end of the new deletion is similar in location to the seven previously described ($A_{\gamma\delta\beta}$)^o-thalassemia deletions (Figure 4) that remove part or all of the A_{γ} -gene but leave the G_{γ} -gene intact (3-11). In contrast, the 3' ends of the eight deletions exhibit considerable heterogeneity

Figure 4

Comparison of the various locations of the breakpoints and of the variable sizes of deletions in the eight types of $(A\gamma\delta\beta)^{\circ}$ -thalassemias. All of the mutants exhibit very close 5' ends, while their 3' ends differ considerably. The arrowheads show the location and orientation of the Alu repetitive sequences. The slashed boxes downstream of ϵ - and β -genes show the location of Kpn repeats. The 3' endpoint of the Chinese $(A\gamma\delta\beta)^{\circ}$ -thalassemia is close to a region that contains a sequence with enhancer properties (24-26), a hypersensitive site (26), and a retroviral element (30).

regarding their locations in the cluster as shown in Figure 4. The 3' end of the Chinese $(A\gamma\delta\beta)^{\circ}$ -thalassemia (8) lies more than 40 kb from the 3' ends of the Indian, Turkish, U.S. Black, and German $(A\gamma\delta\beta)^{\circ}$ -thalassemias (Figure 4). Nevertheless, the observed phenotypes of the heterozygotes with the above types of deletions are strikingly similar; the levels of HbF range between 10-15%, with heterocellular distribution and slight hypochromia, and microcytosis (7).

It is apparent that the molecular heterogeneity of these deletions albeit their phenotypic similarities, precludes any attempt to explain the molecular basis of increased γ -expression by a single unifying mechanism; it rather suggests the presence of several regulatory sequences which are distributed along the β -globin gene cluster and that the resulting phenotypes of the various deletions of $\delta\beta$ - or $(A\gamma\delta\beta)^{\circ}$ -thalassemias (i.e., lower HbF output/heterocellular distribution) and the HPFH syndromes (higher HbF output/pancellular distribution) reflects either their deletion or rearrangement.

The increased expression of γ -genes from the deleted $(A\gamma\delta\beta)^{\circ}$ -thalassemia chromosome can be explained with three hypotheses.

The first involves the juxtaposition of an enhancer, as a result of the deletion, to the proximity of the intact $G\gamma$ -globin gene. It is unlikely that this hypothesis can account for all the mutants; this presupposes the existence of many types of enhancer sequences, dispersed along the 3' region of the β -cluster. On the other hand, the hypothesis can explain the induced $A\gamma$ -gene expression in certain mutants such as the German $(A\gamma\delta\beta)^{\circ}$ -thalassemia. The 3' breakpoint of the German $(A\gamma\delta\beta)^{\circ}$ -thalassemia is very close (1.5 to 3.5 kb) to those of HPFH-4 and HPFH-3 deletions (see Figure 2). Therefore, it is conceivable that all three deletion mutations might juxtapose to the vicinity of the fetal γ -globin genes an enhancer element, normally lying more than 30 kb from the β -globin gene. In support of this hypothesis, is recent experimental evidence which suggests the presence of an enhancer-like element immediately 3' from the breakpoint of HPFH-1 and HPFH-2 (24-26). This

hypothesis could be tested with in vivo bioassays such as the transgenic mice system.

A second possibility is that the enhanced G_γ production reflects simply the deletion of an inhibitor which is also deleted in deletion HPFH syndromes (1,2). The difference in the phenotype between the $(A_\gamma\delta\beta)^\circ$ -thalassemias and HPFH could be explained by the fact that the putative enhancer located 3' to the A_γ -gene (27) is preserved in all the HPFH deletions while is deleted in all the $(A_\gamma\delta\beta)^\circ$ -thalassemias. Furthermore, these phenotypic differences between the $(A_\gamma\delta\beta)^\circ$ -thalassemias and HPFH can not be explained by a possible gene dosage effect: i.e. two intact γ genes in HPFH versus one γ gene in $(A_\gamma\delta\beta)^\circ$ -thalassemia, cis to the deletion chromosome. The estimated output of the remaining A_γ gene in the latter case expressed as HbF/cell is quite efficient and equal to the HbF/cell levels of the $(\delta\beta)^\circ$ -thalassemia heterozygotes where both G_γ and A_γ genes are intact (28).

A third possibility is that the locus contains a repressed enhancer 5' to the breakpoint of the $(A_\gamma\delta\beta)^\circ$ deletions. Removal, by the deletions, of sequences that normally suppress the enhancer, results in activation of this enhancer in a mechanism similar to that of the β -interferon enhancer (29). Although this latter hypothesis can explain the relatively abundant expression of the G_γ -genes in the $(A_\gamma\delta\beta)^\circ$ -thalassemia deletions, it is not compatible with recent experimental data. A detailed survey (27) for enhancer activity of a 22 kb DNA sequence covering the ϵ -, G_γ - and A_γ -globin region, failed to identify any enhancer element situated upstream (i.e., either 5' to G_γ or between the G_γ and A_γ region) from the 5' breakpoints of the $(A_\gamma\delta\beta)^\circ$ deletions.

All of the above hypotheses are not mutually exclusive, however, and each of the $(A_\gamma\delta\beta)^\circ$ -thalassemia deletions may be operating by a different mechanism.

Previous work has shown that normal adult BFUe displays induced HbF expression while cultured in FCS but lose this expression when they are cultured in FSS (15). Other work also suggests that FSS may contain factors which inhibit γ -globin expression in adult and neonatal but not fetal BFUe (16). Paradoxically, culture in FSS can completely inhibit γ -gene expression in cultures of deletion or nondeletion HPFH and $\delta\beta$ -thalassemia BFUe (17,18). The phenomena are observed in the studies of BFUe from two German $(A_\gamma\delta\beta)^\circ$ -thalassemia heterozygotes. While culture in FCS was associated with abundant production of HbF, culture in FSS resulted in almost complete inhibition of γ -gene expression. The cellular and molecular basis of inhibition of γ -gene expression in BFUe culture remains unclear. The results, however, indicate that the mutant γ -genes can be modulated by the in vitro environment as normal γ -genes are.

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