

## ORIGINAL ARTICLE

# A Functional Element Necessary for Fetal Hemoglobin Silencing

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## ABSTRACT

**BACKGROUND**

An improved understanding of the regulation of the fetal hemoglobin genes holds promise for the development of targeted therapeutic approaches for fetal hemoglobin induction in the  $\beta$ -hemoglobinopathies. Although recent studies have uncovered *trans*-acting factors necessary for this regulation, limited insight has been gained into the *cis*-regulatory elements involved.

**METHODS**

We identified three families with unusual patterns of hemoglobin expression, suggestive of deletions in the locus of the  $\beta$ -globin gene ( $\beta$ -globin locus). We performed array comparative genomic hybridization to map these deletions and confirmed breakpoints by means of polymerase-chain-reaction assays and DNA sequencing. We compared these deletions, along with previously mapped deletions, and studied the *trans*-acting factors binding to these sites in the  $\beta$ -globin locus by using chromatin immunoprecipitation.

**RESULTS**

We found a new ( $\delta\beta$ )<sup>0</sup>-thalassemia deletion and a rare hereditary persistence of fetal hemoglobin deletion with identical downstream breakpoints. Comparison of the two deletions resulted in the identification of a small intergenic region required for  $\gamma$ -globin (fetal hemoglobin) gene silencing. We mapped a Kurdish  $\beta^0$ -thalassemia deletion, which retains the required intergenic region, deletes other surrounding sequences, and maintains fetal hemoglobin silencing. By comparing these deletions and other previously mapped deletions, we elucidated a 3.5-kb intergenic region near the 5' end of the  $\delta$ -globin gene that is necessary for  $\gamma$ -globin silencing. We found that a critical fetal hemoglobin silencing factor, BCL11A, and its partners bind within this region in the chromatin of adult erythroid cells.

**CONCLUSIONS**

By studying three families with unusual deletions in the  $\beta$ -globin locus, we identified an intergenic region near the  $\delta$ -globin gene that is necessary for fetal hemoglobin silencing. (Funded by the National Institutes of Health and others.)

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**R**EGULATION OF THE SWITCH FROM FETAL hemoglobin ( $\gamma$ -globin) to adult hemoglobin in humans has been of long-standing interest, given the potential for the understanding of this process to inform the development of therapeutic approaches to fetal hemoglobin induction in the  $\beta$ -hemoglobinopathies.<sup>1-3</sup> Furthermore, the process is a paradigm for developmental gene expression and regulation. Recent insight into hemoglobin switching has come from the results of genome-wide association studies of the genetic basis for normal variation in fetal hemoglobin levels.<sup>4-6</sup> This has led to the identification of common genetic polymorphisms in the locus of the  $\beta$ -globin gene (*HBB*) (hereafter referred to as the  $\beta$ -globin locus), a region between the *HBS1*-like gene *HBS1* and the oncogene *MYB*, as well as within the gene *BCL11A* (encoding the transcription factor B-cell lymphoma/leukemia 11A).<sup>7,8</sup> In addition, family studies have revealed that a few known patients with elevated levels of fetal hemoglobin have mutations in the gene encoding the critical erythroid transcription factor Krüppel-like factor 1 (*KLF1*).<sup>9,10</sup>

Although these studies have advanced the identification of critical *trans*-acting factors in the regulation of fetal hemoglobin, limited insight has been gained regarding the *cis*-regulatory sequences of the  $\beta$ -globin locus required for the silencing or activation of fetal hemoglobin. Extensive work over a period of three decades has implicated these sequences in the regulation of this locus. Large deletions from within the locus through regions far upstream have been found to result in underexpressed or silent thalassemic alleles, leading to the identification of an upstream enhancer, the locus control region. In contrast, deletions downstream of the  $\beta$ -globin locus result either in  $(\delta\beta)^0$ -thalassemia or hereditary persistence of fetal hemoglobin (HPFH).<sup>2</sup> HPFH is distinguished from  $(\delta\beta)^0$ -thalassemia by higher fetal hemoglobin levels, balanced synthesis of  $\alpha$ -like and  $\beta$ -like globin chains, and normocytic red-cell indexes in heterozygotes (persons with one normal and one mutant  $\beta$ -globin gene). The interpretation of such mutations has been hampered by the inability to precisely map deletion end points, the rarity and heterogeneity of the mutations,<sup>2</sup> and the failure to recapitulate in animal models the phenotypes seen in humans with the mutations.<sup>11,12</sup>

Three models have been proposed to explain how the  $(\delta\beta)^0$ -thalassemia and HPFH deletions result in elevated fetal hemoglobin levels.<sup>2,13,14</sup> The first model proposes that deletion of the adult

$\delta$ - and  $\beta$ -globin genes leads to unimpeded interaction of the locus control region with the fetal hemoglobin genes, without competition from the adult genes.<sup>14</sup> Although this model could account for the elevated levels of fetal hemoglobin associated with these downstream deletions, it does not account for two differences between  $(\delta\beta)^0$ -thalassemia and HPFH caused by overlapping deletions: the relative amount of fetal hemoglobin produced and the balance among all types of globin chains. A second model proposes that enhancer sequences at the downstream end of the deletions may determine the outcome — when strong enhancer elements are juxtaposed closer to the  $\gamma$ -globin genes, the output of fetal hemoglobin is increased.<sup>14</sup> A third model posits that specific regulatory sequences in the region between the  $\gamma$  and  $\delta$ -globin genes play a role as silencing elements for the  $\gamma$ -globin genes, depending on whether the sequences remain intact.<sup>1,15</sup> Distinguishing between the second and third models has not been possible, given the heterogeneity of the breakpoints in the intergenic and downstream regions.

## METHODS

### STUDY FAMILIES

In the course of widespread screening aimed at assessing the distribution and frequency of thalassemia mutations in the Sri Lankan population,<sup>16,17</sup> unusual elevations in the expression of fetal hemoglobin were found in several persons. We identified two probands with elevated fetal hemoglobin levels and conducted genetic studies of the probands and their families. The third proband and family were identified during a hematologic evaluation for anemia in Seattle, and a similar set of studies was performed. All three probands and their family members provided appropriate informed consent in accordance with the Declaration of Helsinki.

### GENETIC ANALYSES

Since deletions were suspected in all three probands, we used a high-resolution custom microarray to perform array comparative genomic hybridization (CGH). The microarray had a median probe density of 60 bp through the  $\beta$ -globin locus, allowing us to map all three deletions with high resolution and confirm breakpoints by means of polymerase-chain-reaction assays and sequencing (see Fig. 1 and 2 in the Supplementary Appendix,

available with the full text of this article at NEJM.org). Map “coordinates” are given in base pairs according to the March 2006 human reference sequence (hg18) of the National Center for Biotechnology Information (NCBI, build 36.1).

## RESULTS

### CHARACTERISTICS OF THE PROBANDS AND THEIR FAMILIES

The proband in Family A (Patient II-2) was noted to have an elevated fetal hemoglobin level of 13.7%, along with microcytic red-cell indexes (Table 1). Hemoglobin analysis performed in other members of Family A revealed an elevated fetal hemoglobin level (15.3%) in the proband’s mother (Patient I-2) and children (Patients III-1 and III-2) (Fig. 1). The proband’s children also had microcytic red-cell indexes, indicating that they carried a thalassemia allele. Overall, the findings in Family A were consistent with the presence of a  $(\delta\beta)^0$ -thalassemia mutation.

A child in Sri Lanka (Patient II-2 in Family B) with a mild anemia who was otherwise clinically asymptomatic was found to have a fetal hemoglobin level of 97.2%. The patient’s father (Patient I-1) had a fetal hemoglobin level of 40.8% (Table 1) but with normocytic red-cell indexes and without signs of anemia. An elder sibling of the proband (Patient II-1) also had an elevated fetal hemoglobin level (39.3%) without signs of anemia or microcytosis. The mother in Family B (Patient I-2) had a mild anemia and microcytosis with an elevated hemoglobin A<sub>2</sub> level, findings that are consistent with a  $\beta^0$ -thalassemia mutation. The proband had anemia and microcytosis nearly equivalent to that of his mother (Table 1). The presentation was most consistent with the presence of an HPFH mutation in this family (Fig. 1). The proband appeared to have coinherited a  $\beta^0$ -thalassemia mutation from his mother, along with an HPFH mutation from his father.

A child of Kurdish origin (Patient III-4 in Family C) was evaluated in Seattle for pallor and was found to have an anemia with a markedly elevated hemoglobin A<sub>2</sub> level, at 8.7%, along with a fetal hemoglobin level of 41.4%. Given the suspicion of thalassemia on the basis of these clinical features, DNA samples were obtained from the proband and the *HBB* gene was sequenced. This analysis revealed that the child had the 1587A→G  $\beta^+$ -thalassemia mutation and loss of heterozygosity at several single-nucleotide polymorphisms in

the  $\beta$ -globin gene, suggesting a deletion of the other  $\beta$ -globin allele (Fig. 1). Evaluation of the other family members revealed that the mother (Patient II-3), maternal aunt (Patient II-2), and maternal cousin (Patient III-2) all had microcytic red-cell indexes with unusually elevated hemoglobin A<sub>2</sub> levels (Table 1), findings that are consistent with the clinical features described in deletional  $\beta^0$ -thalassemia mutations.<sup>2</sup>

### ANALYSIS OF DELETIONS

Array CGH analysis revealed that Family A had a new form of  $(\delta\beta)^0$ -thalassemia, hereafter called Sri Lankan  $(\delta\beta)^0$ -thalassemia, in which a 38.2-kb deletion extends from the point 200 bp downstream of the  $\delta$ -globin gene into the downstream olfactory receptor gene cluster (hg18 coordinates, chr11:5172267–5210492) (Fig. 1B). This deletion most likely disrupts expression of the  $\delta$ -globin gene by disturbing downstream elements necessary for its transcription, resulting in the  $(\delta\beta)^0$ -thalassemia phenotype. Family B had a 49.8-kb deletion that removes the  $\delta$ -globin gene and extends downstream to the same region as the Sri Lankan  $(\delta\beta)^0$ -thalassemia breakpoint but results in an HPFH phenotype, hereafter called Sri Lankan HPFH (hg18 coordinates, chr11: 5172263–5222029) (Fig. 1B). This deletion is similar to the previously described HPFH-3 deletion.<sup>18,19</sup> The proband in Family B (Patient II-2) also coinherited the IVSI-130G→C  $\beta^0$ -thalassemia allele from his mother (Patient I-2). Family C had a unique pattern in which two separate deletions occur in *cis* (hg18 coordinates, chr11: 5201767–5209375 and 5216566–5224122): a deletion of the  $\beta$ -globin gene and a deletion of the upstream  $\text{Ay}-\delta$ -globin intergenic region, hereafter referred to as the Kurdish  $\beta^0$ -thalassemia deletion (Fig. 1C). This mutant allele may be similar to a previously described set of deletions, though precise mapping was not performed.<sup>18,20</sup>

The Sri Lankan  $(\delta\beta)^0$ -thalassemia and HPFH deletions have downstream breakpoints that are nearly identical, mapping within 4 bp of one another. No other pairs of these types of deletions have had such similar downstream breakpoints, and hence, an assessment of these differences has not previously been possible.<sup>13,14</sup> Our analysis strongly suggests that the deletion of a silencer of the  $\gamma$ -globin genes upstream of the  $\delta$ -globin gene, rather than juxtaposition of a downstream enhancer, is responsible for the dramatic differences between the otherwise similar Sri Lankan

**Table 1.** Clinical Data and Molecular Mutations in Selected Study Patients.\*

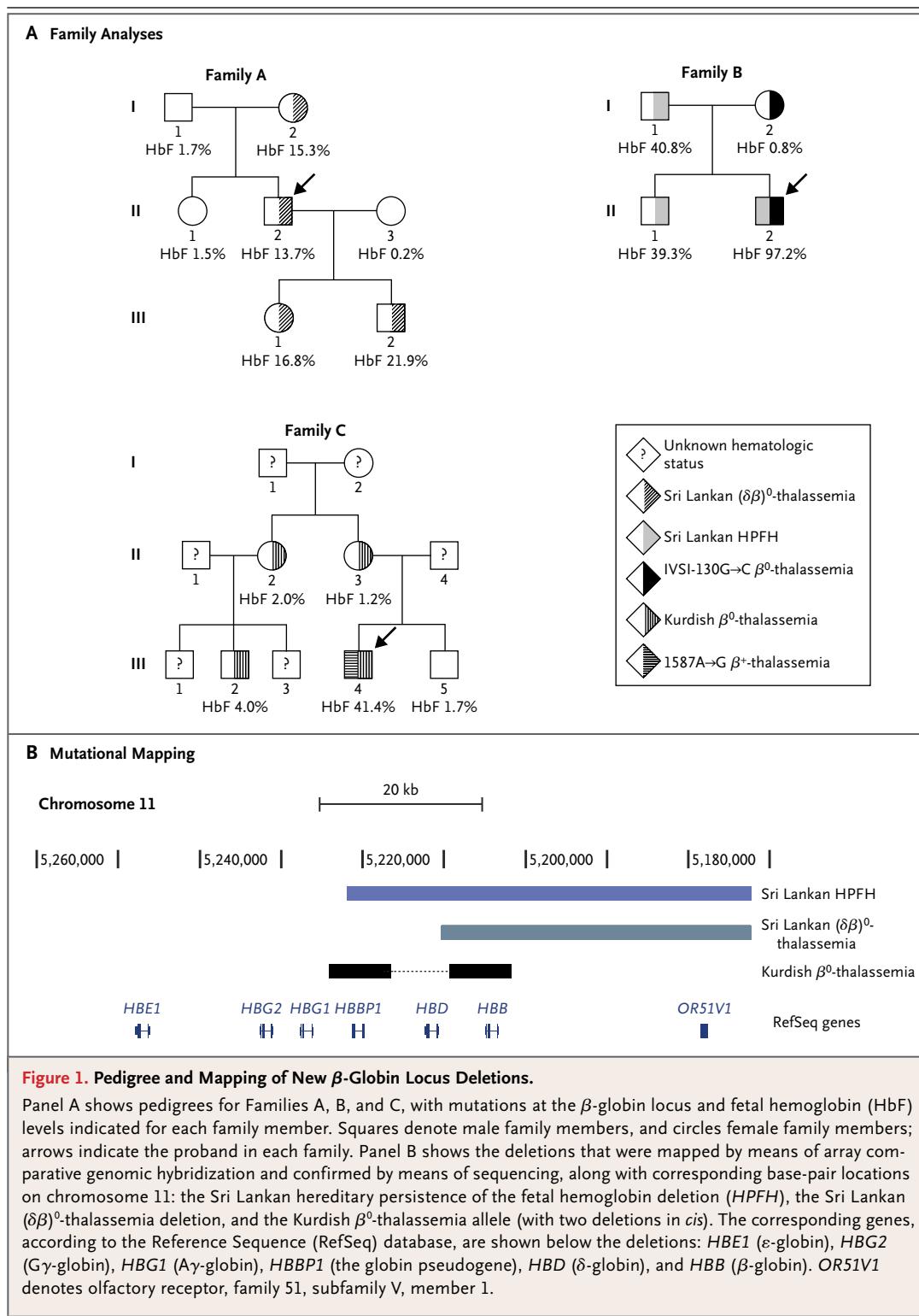
Patient No.	Fetal Hemoglobin	Hemoglobin A <sub>2</sub>	Hematocrit	Mean Corpuscular Volume	Mean Corpuscular Hemoglobin	Mean Corpuscular Hemoglobin Concentration	Genotype at $\beta$ -Globin Loci
	percent			fL	pg	g/dL	
<b>Family A</b>							
II-2	13.7	2.6	46.4	72.7	23.0	31.7	Sri Lankan ( $\delta\beta$ ) <sup>0</sup> -thalassemia/+
III-1	16.8	2.4	36.8	69.1	22.5	32.6	Sri Lankan ( $\delta\beta$ ) <sup>0</sup> -thalassemia/+
III-2	21.9	2.8	34.6	70.1	22.6	32.2	Sri Lankan ( $\delta\beta$ ) <sup>0</sup> -thalassemia/+
<b>Family B</b>							
I-1	40.8	1.9	50.6	83.0	27.1	32.7	Sri Lankan HPFH/+
I-2	0.8	5.0	30.1	59.1	18.1	30.7	IVSI-130G→C $\beta^0$ -thalassemia/+
II-1	39.3	2.2	40.9	76.6	25.4	33.1	Sri Lankan HPFH/+
II-2	97.2	2.8	25.6	58.2	18.5	31.8	Sri Lankan HPFH/IVSI-130G→C $\beta^0$ -thalassemia
<b>Family C</b>							
II-2	2.0	7.3	35.0	65.0	20.7	32.1	Kurdish $\beta^0$ -thalassemia/+
II-3	1.2	8.2	34.0	65.0	20.8	31.9	Kurdish $\beta^0$ -thalassemia/+
III-2	4.0	7.1	30.1	61.4	19.2	31.2	Kurdish $\beta^0$ -thalassemia/+
III-4	41.4	8.7	24.0	60.0	18.5	30.9	Kurdish $\beta^0$ -thalassemia/1587A→G $\beta^+$ -thalassemia

\* HPFH denotes hereditary persistence of fetal hemoglobin.

( $\delta\beta$ )<sup>0</sup>-thalassemia and HPFH deletions (Table 1). We hypothesize that the Kurdish deletion removes an upstream part of the  $\alpha\gamma$ - $\delta$ -globin intergenic region but maintains this putative “silencer” region. Our observation that the Kurdish deletion also results in low levels of fetal hemoglobin expression, similar to those seen with other isolated  $\beta^0$ -thalassemia deletions,<sup>21</sup> is consistent with this hypothesis. Although deletion of this silencer region is essential to obtain the high fetal hemoglobin levels and normal red-cell indexes observed in patients who are heterozygous for HPFH deletions, our data cannot exclude the possibility of contributions from potential regulatory sequences located downstream and juxtaposed to the globin genes by deletion. The shared breakpoint observed in Families A and B brings the genes in proximity to a proposed enhancer (the HPFH-3 region), hypothesized to activate  $\gamma$ -globin, but not  $\delta$ -globin or  $\beta$ -globin, gene expression.<sup>13</sup> This may explain the moderately elevated fetal hemoglobin levels associated with Sri Lankan ( $\delta\beta$ )<sup>0</sup>-thalassemia.

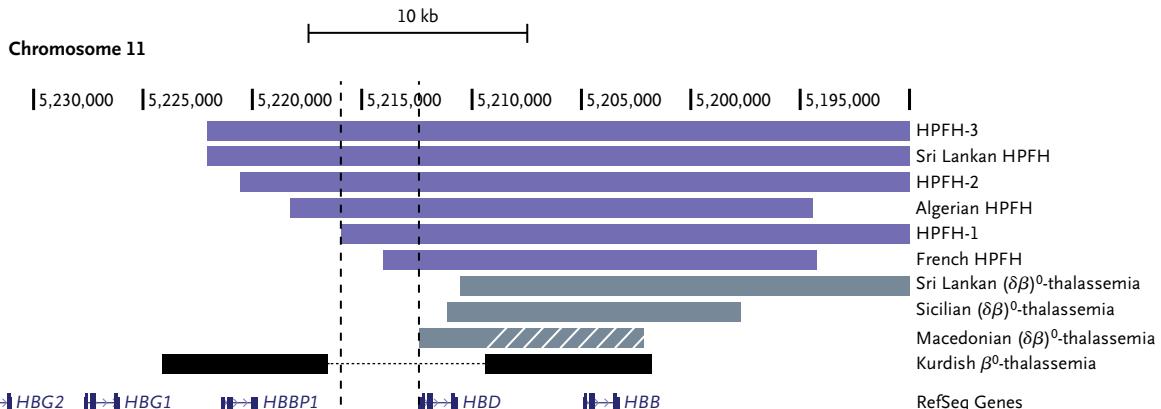
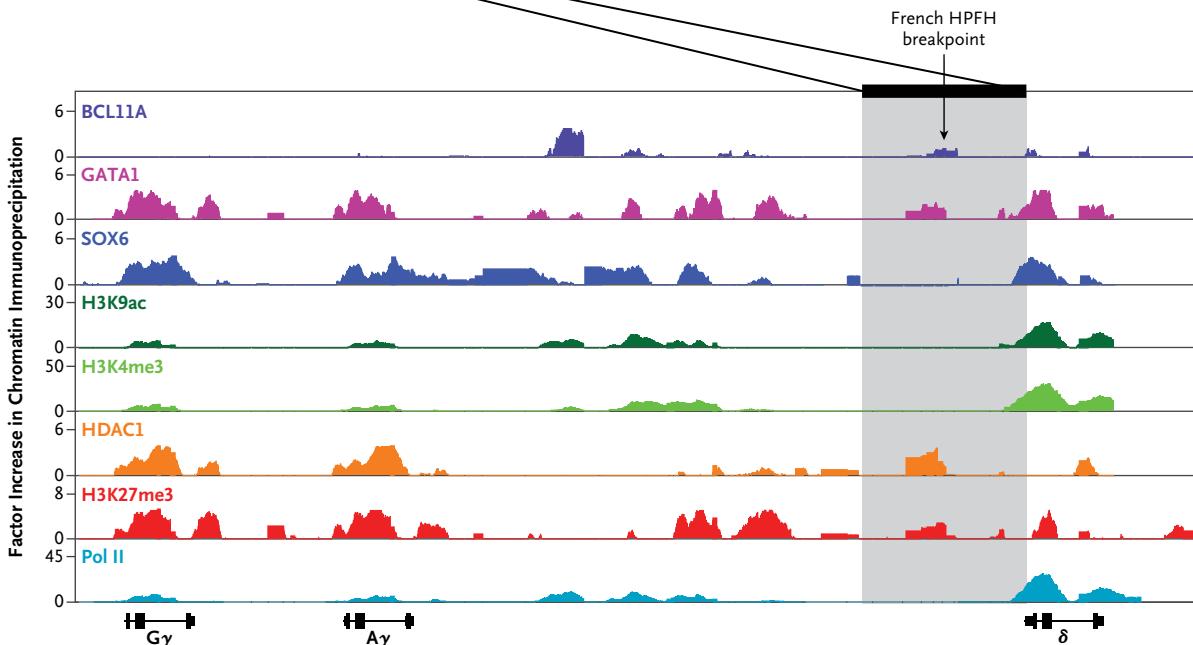
These deletions, along with previously sequenced deletions (Tables 1 and 2 in the Supplementary Appendix),<sup>18</sup> implicate a 3.5-kb region in

the silencing of the  $\gamma$ -globin gene (Fig. 2A). The upstream boundary of this region was determined with the use of all precisely mapped HPFH deletions, with the exception of the recently described French HPFH deletion<sup>21</sup> (discussed in detail below). This region is also removed by the Corfu deletion, which has independently been implicated in  $\gamma$ -globin gene silencing.<sup>23</sup> We assessed the chromatin occupancy of the transcription factor BCL11A, a critical silencer of the  $\gamma$ -globin genes in humans, by using chromatin immunoprecipitation,<sup>24</sup> and observed that the 3.5-kb region contains BCL11A as well as its associated repression partners, the genes encoding GATA protein 1 (GATA1) and histone deacetylase 1 (HDAC1) (Fig. 2B) in primary adult erythroid cells.<sup>22,25,26</sup> This region is devoid of activating histone marks (histone 3 lysine 4 trimethylation and histone 3 lysine 9 acetylation) but does reveal the repressive mark histone 3 lysine 27 trimethylation (H3K27me3),<sup>24</sup> which is also present at the silenced  $\gamma$ -globin genes in the primary adult erythroblasts we analyzed (Fig. 2B). We found that the only HPFH deletion that has a breakpoint within this 3.5-kb region, the French form of HPFH,<sup>21</sup> disrupts a portion of



the BCL11A occupancy site by means of its upstream breakpoint (Fig. 2B), an observation that provides strong support for a functional role of the region. The French HPFH is characterized by nor-

mocytic red-cell indexes with high levels of fetal hemoglobin expressed in heterozygotes,<sup>21</sup> features that are consistent with its classification as a bona fide form of HPFH.

**A Mutational Mapping****B Chromatin Immunoprecipitation Experiments****Figure 2. Results of Comparative Mapping of  $\beta$ -Globin Locus Deletions.**

Panel A shows the deletions that were mapped in this and prior studies, along with corresponding base-pair locations on chromosome 11: various hereditary persistence of fetal hemoglobin (HPFH) deletions, the  $(\delta\beta)$ <sup>0</sup>-thalassemia deletions, and the Kurdish  $\beta^0$ -thalassemia allele (with two deletions in *cis*). The corresponding genes, according to the Reference Sequence (RefSeq) database, are shown below the  $\beta$ -globin genes:  $\text{HBG}2$  ( $\text{G}\gamma$ -globin),  $\text{HBG}1$  ( $\text{A}\gamma$ -globin),  $\text{HBBP}1$  (the globin pseudogene),  $\text{HBD}$  ( $\delta$ -globin), and  $\text{HBB}$  ( $\beta$ -globin). A 3.5-kb region was delineated (indicated within the dashed vertical lines) by the upstream breakpoint of HPFH1 at one end and the 5'-untranslated region of the  $\delta$ -globin at the other end. The HPFH1 breakpoint represents one of the best-documented and most precisely mapped HPFH deletions with one of the most 3' ends of the upstream deletions (with the exception of the French HPFH breakpoint). The upstream breakpoint of the Macedonian (Macedonian-Turkish)  $(\delta\beta)$ <sup>0</sup>-thalassemia deletion<sup>2,14</sup> has been mapped and was used to define the downstream breakpoint, though the downstream end of the deletion remains imprecisely mapped (as indicated with hatch marks). Panel B shows the results of chromatin immunoprecipitation experiments performed on a microarray for several transcription factors and histone modifications. These data were analyzed in a manner similar to work described previously.<sup>22</sup> The y axis represents the relative enrichment at any particular chromosomal coordinate.

We obtained further support for a functional role of the intergenic region by assessing the chromatin occupancy of various factors in primary

adult erythroid cells in which BCL11A expression was reduced.<sup>25</sup> By decreasing BCL11A expression by 95% at the protein level with the use of a len-

tiviral short hairpin RNA construct,  $\gamma$ -globin levels could be increased to 35%.<sup>22,25</sup> We could then use these knockdown cells to assess the chromatin occupancy of various factors. We noted that the presence of the binding partner of BCL11A, HDAC1, was specifically reduced at the  $A\gamma-\delta$ -globin intergenic sites, as was H3K27me3 in these regions, as well as at other sites throughout the  $\beta$ -globin locus (Fig. 3 in the Supplementary Appendix). The chromatin occupancy of GATA1 in this region was unchanged by BCL11A knockdown, showing the specificity of this effect.

## DISCUSSION

Our mapping of HPFH and  $(\delta\beta)^0$ -thalassemia deletions in persons from Sri Lanka provided a unique opportunity to define the genomic region responsible for the phenotypic differences between these two molecularly similar conditions. The phenotypic differences among the HPFH and  $(\delta\beta)^0$ -thalassemias are remarkable; coinheritance of a  $\beta^0$ -thalassemia allele along with an HPFH deletion can result in an asymptomatic condition involving mild anemia, as exemplified by the proband in Family B, whereas coinheritance of  $(\delta\beta)^0$ -thalassemia with another form of thalassemia results in transfusion-dependent disease.<sup>14</sup> The reasons for these differences have been difficult to discern, given the heterogeneity and rarity of such mutations. Various theories have been put forth, but it has not been possible to test them in human cases, and testing in animal models is not suitable, given critical interspecies differences in globin gene expression.<sup>26</sup>

In identifying these two deletions with markedly different phenotypes, we had an opportunity to delineate a region immediately upstream from the  $\delta$ -globin gene that is likely to mediate silencing of the  $\gamma$ -globin genes. The Kurdish  $\beta^0$ -thalassemia deletion retains this intergenic site and is associated with a phenotype involving robust fetal hemoglobin silencing. Together, these phenotypes provide strong genetic evidence of a functional role of this intergenic region in fetal hemoglobin silencing. Although the region appears to be necessary for  $\gamma$ -globin silencing, it alone cannot be sufficient; other elements in the locus may contribute. The level of  $\gamma$ -globin expression may be influenced by intergenic enhancer (or repressor) elements and by the juxtaposition of enhancer elements at the downstream breakpoint.<sup>14</sup> Such complexity in regulation is illustrated by the presence of a few larger deletions that remove the

3.5-kb silencing region we describe here but nonetheless result in mild thalassemic phenotypes, possibly owing to effects of other *cis*-regulatory elements (Table 1 in the Supplementary Appendix).

Genetic and functional studies have identified BCL11A as a critical mediator of the fetal-to-adult hemoglobin switch in humans and as a silencing factor for the  $\gamma$ -globin genes in adults.<sup>1,25,26</sup> We found that BCL11A occupies a site within the 3.5-kb intergenic region in adult erythroid chromatin along with two associated proteins, GATA1 and HDAC1,<sup>25</sup> that participate in transcriptional repression. Recent work suggests that inhibitors of histone deacetylases may be potent fetal hemoglobin inducers.<sup>27</sup> We found repressive chromatin marks at the silent  $\gamma$ -globin genes of the adult cells, as well as within the 3.5-kb region, and a lack of activating chromatin marks. A portion of the region containing BCL11A is disrupted by the upstream breakpoint of a French HPFH deletion, a finding that provides strong support for the functional relevance of the region. Finally, we found that the knockdown of BCL11A in primary human cells, which results in robust induction of fetal hemoglobin,<sup>25</sup> is accompanied by decreased chromatin occupancy of its partner, HDAC1, and the repressive H3K27me3 mark found in the region. These data suggest that BCL11A facilitates recruitment of HDAC1 and establishment of repressive chromatin modifications at these sites and provide clues to how the 3.5-kb region may mediate  $\gamma$ -globin silencing. Although BCL11A is critical for  $\gamma$ -globin silencing, we cannot rule out the possibility of contributions by other regulatory factors that may bind to this region, such as IKZF1 (Ikaros family zinc finger 1), which could act independently or in concert with BCL11A to silence fetal hemoglobin.<sup>28</sup> Our findings highlight the role of this  $A\gamma-\delta$ -globin intergenic site, which is a possible hub for  $\gamma$ -globin silencing, and also underscore the potential importance of long-range interactions mediated by BCL11A.

Future studies should yield further information regarding functional elements within the  $\beta$ -globin locus that are necessary for various aspects of globin-gene regulation. For example, there are likely to be functional roles of other elements in the  $A\gamma-\delta$ -globin intergenic region, such as the other BCL11A binding site found downstream of  $A\gamma$ -globin in the intergenic region, which contains a distinct combination of binding factors and histone modifications (Fig. 2B). Common single-nucleotide polymorphisms associated with elevated

fetal hemoglobin levels in humans are located in a region approximately 5 kb upstream of the BCL11A binding site,<sup>8</sup> suggesting that other critical regulatory *cis* elements may exist. Our study highlights the value of genetic mapping and phenotypic comparisons in humans to gain insight into clinically relevant biologic problems that can be difficult to study with other approaches.<sup>29</sup>

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