γ Gene Promoter and Enhancer Structure in Seattle Variant of Hereditary Persistence of Fetal Hemoglobin

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A variant of hereditary persistence of fetal hemoglobin (HPFH), first described in a patient from Seattle, was studied by structural analysis of the γ-globin genes. A family study suggested that the determinant for this form of HPFH, in which the HbF contains both β- and γ-globin chains, segregated with the β+ gene. No deletions or other abnormalities were detected in the fetal to adult globin gene region by genomic mapping studies. All four γ-globin genes were isolated from a cosmid library, and allelic pairs of γ-globin genes were distinguished by linkage to either the β- or β+ globin gene. Nucleotide sequence analysis of the four γ-globin gene promoters revealed a total of three discrepancies compared with a reference sequence, but these were judged unlikely to be the underlying determinants. Sequence analysis of the enhancer region located 3' to the γ-globin gene from the putative HPFH chromosome revealed three base substitutions, whereas this region was normal in the β-globin gene linked to the β+ gene. These data raise the possibility that an alteration of enhancer function rather than promoter function could be the basis for this condition.

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MATERIALS AND METHODS

Molecular cloning of globin gene loci. High-molecular weight DNA was purified from the peripheral blood lymphocytes of individual II-1 (Fig 1) and subjected to molecular cloning as previously described. DNA was restricted with KpnI and fractionated by velocity sedimentation through a gradient of sodium chloride, and the portion of the β-globin gene cluster containing the fetal and adult globin genes was isolated by cosmid cloning from DNA in the 40-kb size class (Fig 2). Six positive colonies that carried the β+ through β- gene region were recovered from 60,000 recombinants. Structural analysis of the β-globin cluster was carried out by blot hybridizations as previously described except that nylon membranes (Amerham Corp, Arlington Heights, IL) were used in place of nitrocellulose and probes were labeled by random primer extension. Preliminary studies of the strength of promoters from certain of these HPFH genes after transfer to erythroid or nonerythroid cells tend to support the conclusion that promoter mutations are the underlying basis for these conditions, but the ultimate mechanism(s) remain unclear.

Here we present a structural analysis of the γ-globin genes in Seattle HPFH in which both the γ- and γ+ genes contribute to the HbF, which is heterogeneously distributed among the red cells. Genomic hybridization analysis showed that this condition is unlikely to have arisen from a deletion or other rearrangement. Because a family study showed that the γ+ gene is segregating with the β+ gene, a detailed structural analysis of the fetal genes was possible. Analysis of the γ-globin gene sequences from a Seattle HPFH heterozygote suggest that the molecular basis for this condition is distinct from the previously described examples of nondeletion HPFH. The data suggest that mutations other than those affecting the promoters of the γ genes may be responsible for the nondeletion (γ+ + γ- HPFH) phenotype.

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RESULTS

We studied the structure of the γ globin genes of a carrier of the Seattle (γγ+, γγ+) HPFH determinant who is also an AS heterozygote (individual II-1 of Fig 1). We have previously reported that the γγ+, γγ+ HPFH determinant cosegregates with the βγ-globin gene in this family.15 Genomic hybridization analysis of the Seattle HPFH determinant who is heterozygous for the βγ gene and for the Seattle HPFH determinant. His hemoglobin composition included 33.1% HbS, 2.5% Hba2, 7.8% Hba, and by difference, 56% Hba2. The fetal hemoglobin was composed of γγ and γγ chains in equal proportions and was heterogeneously distributed among the red cells, as was documented for other individuals with the high Hbf determinant in this family. For more detailed hematologic findings, see Stamatoyannopoulos et al.18

Fig 1. Pedigree of the Seattle HPFH family.19 The proband of this study is individual II-1, a middle-aged male who is heterozygous for the βγ gene and for the Seattle HPFH determinant. His hemoglobin composition included 33.1% HbS, 2.5% Hba2, 7.8% Hba, and by difference, 56% Hba2. The fetal hemoglobin was composed of γγ and γγ chains in equal proportions and was heterogeneously distributed among the red cells, as was documented for other individuals with the high Hbf determinant in this family. For more detailed hematologic findings, see Stamatoyannopoulos et al.18

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With the hope of identifying a polymorphic difference between the two \(^\gamma\) genes, the sequences of the 3' untranslated regions of both \(^\gamma\) genes were determined from position +1,413 through the 3' untranslated region beyond the termination codons. A sequence difference in the transcribed region of these genes would allow transcripts from the two \(^\gamma\) alleles to be distinguished. Such data would indicate whether the determinant for Seattle HPFH acted in \textit{cis} or \textit{trans}.

Both \(^\gamma\) sequences were identical to the normal B-type reference gene in this region (data not shown and Fig 4). The 0.753-kb \textit{HindIII} fragments located just 3' to the \(^\gamma\) genes were also sequenced on both strands as we continued our search for mutational differences between the two \(^\gamma\) genes. This fragment, when tested as part of a larger \textit{EcoRI} fragment, has enhancer activity in one in vitro assay system, although no evidence is yet available to suggest that this fragment acts as an enhancer in vivo. The sequence of this fragment derived from the /\beta^\alpha cosmid was identical to the reference B-type chromosome.

The sequence from the fragment 3' to the \(^\gamma\) gene derived from the \beta^\delta gene was also a B-type sequence but showed three base substitution mutations: T to C at position +2,285, C to A at +2,460, and A to G at +2,676. These results are summarized in Fig 4. Three regions of the \(^\gamma\) sequence that are similar to the sequences found at the same relative positions ("homologies on the diagonal") of other \beta-globin 3' enhancer regions are enclosed in boxes. Each of these regions has a counterpart at or near the same position in the chicken \beta-globin 3' enhancer (\(^\gamma\) element from +2,037 to +2,060; 18/24 matches), the human \beta-globin 3' enhancer (\(^\gamma\) element from +2,251 to +2,258; 65% homology to the human \beta-globin gene), or the human, rabbit, and mouse \beta-globin genes (\(^\gamma\) element from +2,284 to +2,300; 70% homology to the human \beta-globin gene). Interestingly, the C-to-T base substitution at position +2,285 increases the similarity of this region to the corresponding \beta-globin element, which makes this region of the putative \gamma enhancer more \beta-like, which would be consistent with the HPFH phenotype.

**Fig 4.** Flanking sequences. The sequence of the \(^\gamma\) gene linked to the \beta^\delta gene from the termination codon (+1,491) to residue +2,712 is presented. Base substitution mutations found in the \(^\gamma\) gene linked to the \beta^\delta gene are indicated below the line at residues +2,285, +2,460, and +2,676. The \textit{HindIII} sites that define the 0.753-kb fragment are underlined. The AA dinucleotide (residues +2,366, +2,367) that is characteristic of a B-type chromosome is marked with diamonds. Regions of similarity to other \beta-like–\globin gene enhancer regions are boxed. For more details see the text.
GENE STRUCTURE IN SEATTLE HPFH

DISCUSSION

Molecular studies of the nondeletion HPFH mutants, the $^\gamma$ and $^\delta$ HPFHs, have revealed that the $^\gamma$ gene promoters play a role in the developmental control of $^\gamma$-globin gene expression. Thus, the sequence of the $^\delta$ HPFH has disclosed a C-to-T base substitution at -202 of the $^\gamma$ gene promoter. The $^\gamma$ HPFH mutants proved to be genetically heterogeneous. A G-to-A substitution at -117 was found to underlie the Greek $^\gamma$ HPFH. A C-to-T substitution at -196 was detected in Italian and Chinese forms of $^\gamma$ HPFH. Collectively these data showed that at least two regions of the $^\gamma$ gene promoter, around -200 and the distal CCAAT box, are involved in the developmental regulation of $^\gamma$ genes.

The form of HPFH we studied is characterized by continued $^\delta$ and $^\gamma$ gene expression in heterozygous carriers. No crossover was found between the HPFH determinant and the $^\beta$ gene in the family members we studied. Gene mapping failed to disclose any deletion. It is clear that the $^\beta$ genes in cis to the determinant are active because the carriers of this form of HPFHH express both $^\beta$ and $^\delta$ genes on the $^\delta$ chromosome. Cellular studies have shown that in this form of HPFH the $^\delta$ gene is heterogeneously distributed among the red cells. This fact is consistent with a promoter mutation because the British HPFH, which has a base substitution in the $^\gamma$ promoter, is typically heterocellular and in Chinese HPFH, where the $^\gamma$ gene promoter is normal, 10% to 15% of the cells of the carriers have low levels of Hbf. Future plans include testing the putative synty of the Seattle HPFH determinant with the $^\beta$ gene by fusion of lymphocytes from a heterozygous carrier to murine erythroblasts in vitro assay. Surprisingly, three base substitution mutations were identified on the $^\beta$ chromosome in a region that contains several sequence motifs quite similar to sequences in the chick $^\beta$-globin enhancer or the human $^\beta$-globin 3' enhancer region. Three such motifs (boxed in Fig 4) are found at the same relative positions 3' to their respective genes. It is noteworthy that one of the base substitution mutations is included in a sequence element previously identified by Kollias et al as having a counterpart 3' to the $^\delta$-globin genes of the human, mouse, rabbit, and chicken.

The sequence data revealed no other allelic differences that might have been clues to the molecular basis of the HPFH phenotype. For example, no differences could be found in the portions of the $^\delta$ or $^\gamma$ genes corporated into RNA. Such differences might have been used to test whether only the $^\gamma$ and $^\delta$ genes on the $^\beta$ chromosome are active together or whether all four $^\gamma$ genes are active in vivo.

Finally, we sequenced the region 3' to the $^\gamma$ gene, which has been implicated as having enhancer activity in one in vitro assay. Surprisingly, three base substitution mutations were identified on the $^\beta$ chromosome in a region that contains several sequence motifs quite similar to sequences in the chick $^\beta$-globin enhancer or the human $^\beta$-globin 3' enhancer region. Three such motifs (boxed in Fig 4) are found at the same relative positions 3' to their respective genes. It is noteworthy that one of the base substitution mutations is included in a sequence element previously identified by Kollias et al as having a counterpart 3' to the $^\delta$-globin genes of the human, mouse, rabbit, and chicken. We are aware of no functional evidence that this region of the $^\gamma$ gene functions as an enhancer in vivo, nor is it entirely clear what phenotype would necessarily be expected for mutations to an enhancer. Our data do not establish whether these base substitutions are merely silent polymorphisms or whether they may be casually related to the HPFH phenotype. We do not exclude the possibility that a cis-acting determinant may lie outside the region we have sequenced.

The absence of these three mutations on the $^\beta$ chromosome is responsible for the presence of this residue in the promoter of an $^\gamma$ gene. Short gene conversion events have been repeatedly postulated to account for the similar sequence patterns present in different fetal globin loci.

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We thank M. Bender for discussions and C. Lotshaw for technical assistance.

ACKNOWLEDGMENT

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RE Gelinas, M Rixon, W Magis and G Stamatoyannopoulos