DNA methylation in hereditary persistence of fetal hemoglobin (HPFH-2)

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Received March 27, 1987; Revised and Accepted June 6, 1987

ABSTRACT

Recent studies show that the region of DNA brought into close proximity to the fetal γ -globin genes in deletional forms of hereditary persistence of fetal hemoglobin (HPFH) is selectively hypomethylated (and presumably active) in normal erythroid tissue. This region is normally located approximately 100 kilobases (kb) 3' to the fetal genes. The continued expression of fetal hemoglobin in adult life in these forms of HPFH has been ascribed to the effect of this erythroid-specific region in altering local chromosomal structure and allowing transcription. Because transcriptional activity is often associated with hypomethylation, we have examined the methylation status of the γ -globin genes and the truncated $\psi\beta$ gene on the HPFH chromosome to determine whether juxtaposition of this erythroid-specific region results in a generalized hypomethylation of the globin gene region upstream of the deletion breakpoint. Genomic DNA purified from nucleated red blood cells (nRBC) from a patient with HPFH-2/ β^0 thalassemia was digested with Msp I or Hpa II, and the methylation pattern determined on the HPFH chromosome by using secondary cleavage with restriction enzymes which span the deletion breakpoint. These studies show that in nRBC the HPFH-2 chromosome is hypomethylated in the 3'-juxtaposed region (3'JR) and in the region of the γ -globin genes. In contrast, Msp I sites near the truncated $\psi\beta$ -globin gene remain methylated, suggesting that only a subset of CpG sites upstream from the 3'JR become hypomethylated in HPFH-2. This subset of sites corresponds to those which are normally hypomethylated when fetal genes are active. The continued high level of fetal globin expression is, therefore, not associated with a generalized hypomethylation upstream from the deletion junction.

INTRODUCTION

The switch from fetal to adult hemoglobin expression begins in the midtrimester of gestation (1). There has been interest in the molecular basis of this switch in part because continued expression of Hb F into adult life has been reported to ameliorate the clinical severity of sickle cell disease and β thalassemia (2,3). A better understanding of the regulation of this switch, which occurs during normal development, may help in designing future strategies for the treatment of human hemoglobinopathies.

Deletional and non-deletional forms of hereditary persistence of fetal hemoglobin (HPFH) have been described (4,5) and are characterized by continued expression of high levels of fetal hemoglobin into adult life. Two deletional forms of HPFH, HPFH-1 and HPFH-2, are associated in Blacks with large deletions of approximately 100 kilobases (kb) that remove the adult globin genes, δ and β , and result in expression of G_γ and A_γ chain synthesis (6-8). These deletions are virtually the same size, but begin at slightly different sites 3' to the

A γ -globin gene (6-9). The molecular basis of the continued high level of Hb F expression in these individuals is unknown. Two general hypotheses have been suggested: 1) The deletion removes a region of DNA within the β -like globin gene cluster that normally suppresses Hb F expression in adults (10), or 2) the deletion moves sequences near the fetal globin genes that potentiate continued Hb F expression (11). At present, a variety of deletions have been characterized within the β -like globin gene cluster (4,5), and it has not been possible to provide a single unifying model that explains the varying levels of Hb F expression in these patients.

A region of DNA normally located approximately 100 kb 3' to the fetal γ -globin genes is moved next to these genes as a result of the deletions associated with HPFH-1 and HPFH-2 (9,11). This 3'-juxtaposed region (3'JR) contains a sequence which shares homology with enhancer elements (12). The importance of this juxtaposed enhancer within the 3'JR to the HPFH phenotype is not clear.

Approximately 90% of the cytosine residues found in 5'-CpG-3' dinucleotides in mammalian DNA are methylated (13). The degree of methylation at specific cytosine residues has been inversely related with expression of a variety of genes, including the globin genes (14-17). These studies usually involve Southern blot analysis of restricted genomic DNA from appropriate tissues using the isoschizomers Msp I (which cleaves CCGG and C^mCGG) and Hpa II (which cleaves CCGG but not C^mCGG) (14). Recent studies show that the 3'JR is only hypomethylated in DNA from normal erythroid tissue (12). In deletional HPFH-1 and HPFH-2, the continued expression of the γ -globin genes may be facilitated by the juxtaposition of this hypomethylated, erythroid-specific domain which potentiates a chromosomal structural pattern consistent with continued transcriptional activation of the γ -globin genes. This alteration in the chromosomal structure encompassing the γ -globin genes may be associated with a regional hypomethylation of all or a subset of 5'-CpG-3' sites 5' to the 3'JR.

A negative role for DNA methylation in the regulation of globin gene expression has been proposed in regard to a Dutch patient with $\gamma\beta$ thalassemia. The DNA of this patient has a large deletion with a 3' endpoint 2.5 kb upstream of an intact but inactive β -globin gene (18). Methylation studies of nucleated red cells (nRBC) from an affected fetus revealed that the DNA region juxtaposed next to the β -globin gene as a result of the deletion is in an inactive transcriptional conformation. From these results, it was proposed that the downstream inactivation of the normal β gene was due to its transposition into an inactive methylated region as a result of the deletional event (18).

We have examined the degree of hypomethylation at sites near the γ -globin genes, the 3'JR and the truncated $\psi\beta$ -globin gene in nRBC from a patient with HPFH-2/ β^0 thalassemia to determine what affect transposition of the hypomethylated 3'JR has on the methylation pattern of sites 5' to the deletion breakpoint on the HPFH chromosome.

MATERIALS AND METHODS:**Patient and DNA preparation**

The Black propositus with HPFH-2/ β^0 thalassemia was evaluated at one year of age. His hemoglobin level was 9.0 g/dl, MCV 60 μ^3 and Hb F 98%, with no detectable Hb A. A Kleihauer-Betke stain revealed that all his red cells contained fetal hemoglobin. His father's hemoglobin level was 13.1 g/dl, MCV 57 μ^3 and Hb A₂ level 5%, consistent with β -thalassemia trait; his mother's hemoglobin level was 12.2 g/dl, MCV 83 μ^3 and Hb F 22%, consistent with HPFH trait. Southern blot analysis using a $\psi\beta$ probe revealed that the propositus and his mother each had an anomalous 10.5 kb Hinc II band consistent with heterozygosity for deletional HPFH-2 (data not shown). Approximately 5-10% of the patient's nucleated cells in the peripheral blood were nRBC. These cells were purified as described below.

In addition to obtaining DNA from the nRBC of the propositus, DNA was also obtained from the following sources: 1) purified nRBC from the peripheral blood of four splenectomized adolescents with Cooley's anemia and at least one β^{+} -thalassemia gene, 2) purified nRBC from the peripheral blood of a splenectomized adolescent with autoimmune anemia, and 3) white cells from peripheral blood of normal controls.

Nucleated RBC were purified from 10-30 ml of heparinized peripheral blood as follows: The blood was centrifuged at 100 x g for 20 minutes, and the platelet-rich plasma was removed. Granulocytes were removed by passing the cells through an α -cellulose: microcrystalline cellulose column (Sigma, St. Louis, MO), and the column was washed with isotonic saline until the red cells (including the nRBC) were eluted. Mononuclear cells were removed using a Ficoll-Hypaque gradient and centrifuging the cells for 20 minutes at 1,500 x g. The red cell pellet was examined by Wright-Giemsa staining to determine degree of nRBC purification. Only samples of greater than 90% purity were processed further. Approximately 20-30% of the nRBC were recovered from each sample by this procedure.

DNA was prepared from peripheral blood white cells and purified nRBC as previously described (19), except the nRBC samples were resuspended and digested overnight with Proteinase K in 10 ml of buffer.

Methylation studies of genomic DNA

Southern blot analyses were performed on genomic DNA samples as previously described (20) with minor modifications. Parallel samples of 10 μ g of DNA were digested overnight with an excess (5-10 units/ μ g of DNA) of either Msp I or Hpa II (Bethesda Research Laboratory (BRL), Gaithersburg, MA). Completeness of digestion was monitored by removing 10% of each digest, adding 0.5 μ g of full-length lambda phage DNA (BRL) and analyzing for digestion of the lambda DNA the next day. The remaining digested genomic DNA samples were precipitated with two volumes of ethanol. Appropriate secondary digests were done, and the resulting DNA samples were subjected to electrophoresis on 0.8% (w/v) agarose gels and transferred to nitrocellulose filters. Prehybridization of these filters, annealing to

^{32}P -labeled probes and washings were done as previously described (21). Autoradiography was done at -70°C for periods of up to 2 weeks, and relative band intensities were determined by scanning autoradiographs using a Quick Scan Jr TLC densitometer (Helena Laboratories, Beaumont, TX).

DNA probes

Three probes were used in these studies. A 0.9 kb Bam HI-Eco RI γ -IVS2 fragment subcloned into PBR322 (Figure 1) (kindly provided by O. Smithies (22)) was used to study the γ -globin gene region, a 1.1 kb Sst I-Bam HI fragment isolated from 1.08 θ IS β (Figure 2) (kindly supplied by O. Smithies (9)) was used to study the 3'JR, and a 1.7 kb Bgl II-Xba I fragment of the $\psi\beta$ -globin gene from pP3.9 (Figure 3) (kindly supplied by T. Maniatis (23)) was used to study the $\psi\beta$ -globin gene region. Probes were radiolabeled with ^{32}P , as previously described (21).

RESULTS

γ -globin gene region

Digestion of DNA with Eco RI and probing with a γ IVS2 probe results in a 7.0 kb G_{γ} - and a 2.6 kb A_{γ} -gene bands (Figure 1, lane 1 and (23)). Additional digestion with Msp I (Figure 1, lane 5) yields a band of 1.5 kb (resulting from cleavage at sites M_2 and M_4), a 2.6 kb band and a 4.9 kb band (resulting from cleavage at M_1 , but not M_2 and M_4). The 2.6 and 4.9 kb bands are present due to the previously reported partial Msp I resistance at M_2 and M_4 (24).

Double digestion of DNA derived from white cells with Eco RI and Hpa II, gives only the 2.6 and 7.0 kb band pattern, indicating complete methylation at the M_1 , M_2 and M_4 sites (Figure 1, lane 3). When nRBC DNA from a non-thalassemic control (Figure 1, lane 4) or from patients with β thalassemia (data not shown) are digested with Eco RI and Hpa II, major bands are seen at 2.6 and 7.0 kb. A light 1.5 kb band (5% of the total band density) is present in lane 4, reflecting partial susceptibility to Hpa II digestion at M_2 and M_4 . In a patient with β thalassemia, the 1.5 kb band represents approximately 10% of the total density. These results are consistent with a previous study of adult erythroid precursors obtained from patients with β thalassemia which showed from 0-40% hypomethylation at M_2 and M_4 (16). A faint band is also discernable at 4.9 kb in nRBC from the non-thalassemic control and the patient with β thalassemia, with 6% and 20%, respectively of the total density in the 4.9 kb and 7.0 kb bands. These findings are again consistent with the published range of 0-30% (16).

Similar digests of nRBC DNA from the patient with HPFH-2/ β^0 thalassemia indicate less methylation at M_2 and M_4 , since the 1.5 kb band contains 40% of the optical density of the bands in Figure 1, lane 2 and 30% in a duplicate study involving a different preparation of nRBC DNA from the same patient (data not shown). This degree of hypomethylation at M_2 and M_4 is near the upper limit of the range of hypomethylation previously described for nRBC

Figure 1. Methylation pattern in the γ -globin gene region

(A) Southern blot analysis of genomic DNA samples hybridized to A_{γ} IVS2 (22). Lane 1 = Eco RI digest of DNA from normal white cells. Lanes 2, 3 and 4 = Eco RI Hpa II digest of DNA from nRBC of the patient with HPFH-2/ β^0 thalassemia, normal white cells and nRBC from a normal control, respectively. Lane 5 = Eco RI/ Msp I digest of DNA from nRBC of a normal control. (B) Diagrammatic representation of the restriction sites of interest in the region studied (23). (E = Eco RI, M = Msp I.)

from patients with β thalassemia and is consistent with the previously-shown relationship between Hb F expression and hypomethylation at M_2 and M_4 (16). In lane 2, the density of the 2.6 kb band is greater than that of the 7.0 kb band. However, in the repeat study, the 2.6 kb and the 7.0 kb bands were of equal density. The basis of this discrepancy is unclear. In nRBC from patients with β thalassemia, the M_2 and M_4 sites have previously been shown to be equally sensitive to digestion with Hpa II.

3' erythroid-specific domain (3'JR)

Methylation sites near the 3'JR were studied using single digestion with Msp I or Hpa II (Figure 2). Msp I digestion of white cell DNA gives a major 4.2 kb band (Figure 2, lane 1). Hpa II digestion reveals a high degree of methylation at Msp I sites in this region, since only high molecular DNA is present (Figure 2, lane 4). Hpa II digestion of nRBC DNA from either a non-thalassemic control (Figure 2, lane 2) or from the patient with HPFH-2/ β^0

Figure 2. Methylation pattern in the 3'JR

(A) Southern blot analysis of genomic DNA samples hybridized to the 1.1 kb Sst I-Bam HI 3'JR probe (9). Lane 1 = Msp I digest of normal white cell DNA. Lanes 2, 3 and 4 = Hpa II digest of DNA from nRBC of a normal control, nRBC of the patient with HPFH-2/ β^0 thalassemia and normal white cells, respectively. Lanes 5, 6 and 8 = Xba I/Hpa II digest of DNA from nRBC of a normal control, nRBC of the patient with HPFH-2/ β^0 thalassemia and normal white cells, respectively. Lane 7 = Xba I/Msp I digest of normal white cell DNA. (B) Diagrammatic representation of the restriction sites of interest in the region. All restriction sites other than M_b were from previous mapping studies (9,11,12,27). M_b was derived from the determined size of the M_b to M_c band on our blots. B = Bam HI, S = Sst I, M = Msp I and X = Xba I. The extra bands above 4.0 kb seen in lanes 5,6 and 8 are thought to be due to partial Xba I digestion as they are not present following Xba I/Msp I digestion and are not seen in repeat experiments (data not shown).

thalassemia (Figure 2, lane 3) gives the 4.2 kb M_b - M_c band as well as several intermediate molecular weight bands, consistent with hypomethylation at M_b and M_c and other nearby Msp I sites.

The specific degree of hypomethylation at M_c was directly tested by Xba I/Msp I and Xba I/Hpa II double digests. An Xba I/Msp I digest of normal white cell DNA resulted in a 1.1 kb band (Figure 2, lane 7), while an Xba I/Hpa II digest of DNA from the same patient did not generate the 1.1 kb band, but did contain a 4.0 kb band, indicating methylation at M_c (Figure 2, lane 8). The same region is hypomethylated in nRBC since Xba I/Hpa II digestion of nRBC DNA from the normal control and from the patient with HPFH-2/ β^0 thalassemia gave the 1.1

Figure 3. Methylation pattern in the $\psi\beta$ -globin gene region

(A) and (B) Southern blot analysis of genomic DNA samples hybridized to the Bgl II-Xba I genomic $\psi\beta$ -globin gene probe (23). Lanes 1, 2 and 3 = DNA from nRBC of a patient with β^+ thalassemia digested with Pst I/Sst I, Pst I/Sst I/Msp I and Pst I/Sst I/Hpa II, respectively. Lanes 4 and 5 = DNA from normal white cells digested with Pst I/Sst I/Msp I and Pst I/Sst I/Hpa II, respectively. Lanes 6 and 7 = DNA from nRBC of the HPFH-2/ β^0 -thalassemia patient digested with Pst I/Sst I/Msp I and Pst I/Sst I/Hpa II, respectively. Lane M = end-labeled Hind III digest of lambda phage DNA. Lane 8 = Pst I/Sst I/Hpa II digest of DNA from total peripheral nucleated cells (95% white blood cells) from the HPFH-2/ β^0 -thalassemia patient. Lane 9 contains the same DNA digest as lane 7. Lanes 10 and 11 = nRBC DNA from a normal control digested with Pst I/Sst I/Hpa II and Pst I/Sst I/Msp I, respectively. (C) Diagrammatic representation of the restriction sites of interest in the normal chromosome (top) and in the chromosome containing the HPFH-2 deletion (bottom). These sites were previously described (12,14) except for 2 sites: M_b (see legend to Figure 2) and M_a which was derived from the 2.6 kb band of the Pst I/Sst I/Msp I digest of the HPFH-2 chromosome (lane 6). (P = Pst I, M = Msp I and S = Sst I).

kb band (Figure 2, lanes 5 and 6, respectively), with approximately 50% of the total density in the 1.1 kb band. nRBC DNA preparations from patients with β thalassemia also showed significant hypomethylation at M_c (data not shown).

$\psi\beta$ -globin gene region

The studies described so far do not distinguish between methylation sites on the chromosome containing the HPFH-2 deletion and the one containing the β^0 -thalassemia gene. To directly distinguish between methylation sites on the two different chromosomes, we used Msp I and Hpa II digests of DNA which were also digested with restriction enzymes which span the deletion breakpoint in HPFH-2. The approach results in differently-sized bands on the two chromosomes, when the blots are hybridized to the $\psi\beta$ probe (Figure 3). This strategy allows definitive probing of M_7 , M_9 and M_{10} within and 5' to $\psi\beta$ as well as M_a and M_b within the 3'JR on the HPFH-2 chromosome.

The $\psi\beta$ -globin gene region was studied using Pst I and Sst I double digests of genomic DNA samples which had also been digested with either Msp I or Hpa II. After Pst I/Sst I digestion and hybridization with the $\psi\beta$ probe, a 3.9 kb band is generated from normal genomic DNA (Figure 3, lane 1). A 6.5 kb band is generated from the patient's HPFH-2 chromosome using either Pst I/Sst I (data not shown) or Pst I/Sst I/Hpa II digests of white cell DNA (Figure 3, lane 8). Additional digestion with Msp I generates a 1.8 kb band (M_{10} to P) on non-deletional chromosomes (Figure 3, lanes 2, 4, 6 and 11) and a 2.6 kb band (M_{10} and M_a) from the HPFH-2 chromosome (Figure 3, lane 6). Therefore, in the $\psi\beta$ -globin gene region, Msp I sites on the HPFH-2 chromosome can be distinguished from those on the non-deletional chromosome.

Hpa II/Pst I/Sst I digests reveal that M_7 is hypomethylated and that the degree of hypomethylation is further increased in adult erythroid-specific cells. In DNA from white cells, approximately 10% of the M_7 site is hypomethylated as seen by the generation of a 3.3 kb band (Figure 3, lane 5), while in DNA from nRBC derived from a non-thalassemic patient (Figure 3, lane 10) and a patient with β thalassemia (Figure 3, lane 3), M_7 is approximately 80% hypomethylated. In all cases, M_9 and M_{10} are fully methylated.

Hpa II/Pst I/Sst I digestion of total peripheral blood nucleated cells from the patient with HPFH-2/ β^0 thalassemia (approximately 5% nRBC) reveals that M_7 , M_9 , M_{10} , M_a and M_b are fully methylated in a non-erythroid tissue (Figure 3, lane 8). A similar digestion of nRBC DNA from this patient reveals that M_7 and M_b are both appreciably hypomethylated, resulting in a family of bands between 5.1 and 6.5 kb (Figure 3, lanes 7 and 9). The 2.6 kb band (M_{10} to M_a) seen in lane 6) is not present in lanes 7 and 9 nor are there any intermediate bands consistent with hypomethylation on the HPFH chromosome at M_9 , M_{10} and M_a .

DISCUSSION

Previous studies of DNA methylation using a 3'JR probe showed that DNA from this region is hypomethylated in erythroid cells lacking major deletions (12). The juxtaposed

erythroid-specific 3'JR region might influence γ -gene expression by altering the γ -gene chromosomal structure. This might be reflected in an alteration of the DNA methylation pattern, resulting in hypomethylation of all or a select critical set of sites upstream from the deletional breakpoint. Our results show that the 3'JR is indeed hypomethylated, but not in white blood cells from a patient heterozygous for deletional HPFH-2 and β^0 thalassemia. M_2 and M_4 in the immediate 5'-flanking region of the $G\gamma$ and $A\gamma$ genes, respectively, are also selectively hypomethylated in DNA from this patient's nRBC; however, intermediate sites in both the $\psi\beta$ -globin gene region (M_9 and M_{10}) and at the 5' end of the 3'JR (M_a) remain fully methylated. The fact that these sites remain methylated in erythroid cells containing the deletion argues against the hypothesis that transposition of the erythroid-specific hypomethylated 3'JR results in a regional non-specific hypomethylation of all CpG sites in the γ -gene region upstream of the deletion breakpoint. The methylation pattern of all Msp I sites studied in nRBC derived from the patient with HPFH-2/ β^0 thalassemia was qualitatively similar to that seen in nRBC from both a patient without a hemoglobinopathy and from patients with β^+ thalassemia in this and other studies (15,16). These results suggest that if the transposed hypomethylated 3'JR exerts a positional effect on chromatin structure, only certain select CpG sites are affected, the same as those associated with expression of the globin region in erythroid cells lacking the HPFH-2 deletion.

Our results show that the erythroid-specific hypomethylated region of DNA normally located approximately 100 kb 3' to the fetal γ -globin genes remains selectively hypomethylated in erythroid tissues in HPFH-2. These results and those of Elder *et al.* (25), showing retention of DNase I hypersensitive sites in the 3'JR after transposition, are consistent with the hypothesis that this region exerts a positional effect associated with activation of the fetal genes in deletional HPFH. This positional effect on the upstream fetal-globin genes is associated with hypomethylation of the same subset of sites which are normally methylated in erythroid tissues from normal controls.

Analysis of methylation and DNase I sensitivity patterns in erythroid tissue in relation to the location of 3' breakpoints in other forms of HPFH and $\delta\beta$ thalassemia with deletions may increase our understanding of the role of positional effects in potentiating fetal globin gene expression. In the Spanish deletional type of $\delta\beta$ thalassemia (26), the deletion removes the 3'JR, and these patients do not express high levels of fetal hemoglobin. Further studies using erythroid cells from these patients are needed to show whether the region juxtaposed next to the γ -globin genes in the Spanish $\delta\beta$ thalassemia is transcriptionally inert. In a Chinese form of $\delta\beta$ thalassemia (27), the 3' breakpoint of the deletion occurs just upstream of a retrovirus-like element which is positioned between the 3'JR and the fetal globin genes, possibly providing a barrier to a positive chromatin positional effect (12). Further studies concerning the chromatin organization in these cases as well as improved knowledge of the molecular mechanisms responsible for formation of transcriptionally-active

chromatin domains will further increase our understanding of the 3'JR's role in continued fetal globin gene expression in these deletional forms of HPFH and $\delta\beta$ thalassemia.

ACKNOWLEDGEMENTS

We thank Dr. Eric F. Rappaport for his invaluable assistance with the photography, and Dr. Paula S. Henthorn for her suggestions and critical reading of the manuscript. This work was supported in part by a Basil O'Connor Starter Research Grant from the March of Dimes and by grants from the National Institutes of Health (AM16691 and HL28157).

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