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Translocation of an Erythroid-Specific Hypersensitive Site in Deletion-Type Hereditary Persistence of Fetal Hemoglobin

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Hereditary persistence of fetal hemoglobin (HPFH) can involve large deletions which eliminate the 3′ end of the β-like globin gene cluster and more than 70 kilobases (kb) of flanking DNA. Blot hybridization revealed a DNase I-hypersensitive site extending from 1.1 to 1.4 kb downstream of the HPFH-1 3′ deletion endpoint. The site was found in normal fetal and adult nucleated erythroid cells and in two erythroleukemia cell lines but not in nonerythroid cells and tissues. Simian virus 40 core enhancer-like sequences were found nonrandomly distributed within the boundaries of the site, which is contained in a fragment of known enhancer activity (E. A. Feingold and B. G. Forget, Blood, in press). A second hypersensitive site was found 0.5 kb upstream of the HPFH-1 3′ deletion endpoint but was not erythroid specific. A third site, most prominent in fetal liver-derived erythroid cells, was found 1 kb upstream of the HPFH-2 deletion endpoint. As predicted by the locations of the deletion endpoints, the first two sites were translocated to within 12 kb of the $^{A}\gamma$ gene in erythroid colonies derived from an HPFH-2 heterozygote and in hybrid mouse-human erythroid cells carrying the HPFH-2 deletion chromosome. Further analysis of this region showed that it was DNase I sensitive in erythroid and myeloid cells, indicating that it resides in an open chromatin domain. These observations suggest that alterations of chromatin structure flanking the fetal globin genes may contribute to abnormal gene regulation in deletion-type HPFH.

The human β-like globin gene cluster contains embryonic (ε), fetal ($^{G}\gamma$ and $^{A}\gamma$) and adult (δ and β) globin genes which are expressed differentially during development (35). Analysis of the chromatin structure of this locus in fetal and adult erythroid cells has revealed that all of the β-like globin genes are in a DNase I-sensitive domain in erythroid cells throughout development regardless of transcriptional activity (15, 18). For example, the embryonic ε globin gene displays an intermediate level of DNase I sensitivity in fetal erythroid cells, even though it is transcriptionally inactive in this environment (15). In contrast, DNase I-hypersensitive sites located in the promoter regions 5′ to the $^{G}\gamma$ and $^{A}\gamma$ globin genes are specifically associated with the expression of these genes in fetal erythroblasts. These hypersensitive sites are not present in adult bone marrow erythroid cells, in which the genes are transcriptionally silent (18) despite retaining DNase I sensitivity (2). The notion that chromatin structure may be important in the regulation of gene expression has been reviewed extensively (10, 44).

The potential contribution of long-range chromatin structure to human globin gene expression is suggested by disorders involving the inactivation of specific globin genes or the maintenance of their activities at inappropriate developmental times. For example, in the $(\gamma\beta)^{\circ}$ thalassemias, there is no expression of any of the β-like globin genes. This class of thalassemias involves large (20- to 100-kilobase [kb]) genomic deletions of the 5′ end of the β-like globin locus, leaving some or all of the β-like globin genes intact (11, 12, 36). In Dutch $(\gamma\beta)^{\circ}$ thalassemia, the deletion translocates DNA normally found 100 kb 5′ to the beta globin locus to within 2.5 kb of the β-globin gene, thus removing all other genes in the β-globin cluster (36). In English $(\gamma\beta)^{\circ}$ thalassemia, a 100-kb deletion terminates within the $^{G}\gamma$ globin gene, 25 kb 5′ to the β-globin gene, leaving the $^{A}\gamma$-, δ-, and β-globin genes intact but unexpressed (11). In a third example, a 20- to 40-kb deletion terminates within 10 kb 5′ to the ε-globin gene (12).

Interestingly, each of these deletions eliminates a region containing several erythroid-specific DNase I-hypersensitive sites 5′ to the ε gene (15, 39). This region appears to enhance the transcriptional competence of the globin gene cluster in erythroid cells (14, 17). In addition, the Dutch $(\gamma\beta)^{\circ}$ thalassemia deletion translocates DNase I-resistant flanking sequences to the vicinity of the β-globin gene and confers a DNase-resistant, transcriptionally inactive phenotype to an otherwise transcriptionally competent β-globin allele (21). The deletional $(\gamma\beta)^{\circ}$ thalassemias, therefore, appear to involve elimination of a chromatin structure necessary for the activation of the β-like globin locus and/or the juxtaposition of a chromatin structure inhibitory to β-like globin gene expression.

These $(\gamma\beta)^{\circ}$ thalassemia deletions, which effectively silence the remaining globin genes without eliminating them, have interesting counterparts in deletions which appear to

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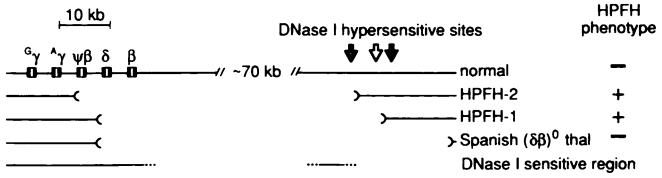

FIG. 1. Characteristics of 3′ deletional syndromes. Deletion endpoints are indicated (≺ ≻). Arrows indicate DNase I-hypersensitive sites discussed in the text.

facilitate the prolonged expression of the fetal globin genes into adult life (43). Hereditary persistence of fetal hemoglobin (HPFH) can be of two types, nondeletional and deletional (38). Nondeletional HPFH alleles display point mutations in promoter regions 5′ to either the $^{G}\gamma$ (8) or $^{A}\gamma$ (16) gene, resulting in persistent expression of that gene in adult erythroid cells. In contrast, persistent expression of both the $^{G}\gamma$ and $^{A}\gamma$ genes is characteristic of the deletional forms of HPFH, such as HPFH-1 and HPFH-2 (33). This phenotype is associated with the removal of sequences from the 3′ end of the β-like globin gene locus, including the δ- and β-globin genes, thereby introducing DNA normally very far (≥100 kb) 3′ to the β-globin gene into the globin cluster (7). Shorter deletions which remove the δ and β genes but end within the β-like gene cluster produce lower but significant amounts of fetal hemoglobin in adult life and are called (δβ)° thalassemias (28). By analogy to the (γβ)° thalassemias, the mechanism of prolonged expression of the fetal globin genes could be either the elimination of sequences involved in the transcriptional shutoff of the γ-globin genes during appropriate globin gene switching or the introduction of sequences capable of enhancing the expression of the adjacent γ-globin genes on the deletion chromosome. The Spanish (δβ)° thalassemia deletion is helpful in distinguishing between these possibilities. This deletion is nearly coterminal at its 5′ end with that of HPFH-1, but its 3′ endpoint lies 10 kb distal to the HPFH-1 3′ endpoint (6; E. A. Feingold and B. G. Forget, Blood, in press) (Fig. 1). This deletion is characterized by lower levels of fetal hemoglobin in adult life than is HPFH (28) and suggests that deletion of inhibitory sequences is not the only explanation for the phenotype of deletion-type HPFH.

In search of clues to the molecular basis of the deletional HPFH phenotype, we have analyzed the chromatin structure of the 3′ deletion breakpoint region in erythroid and nonerythroid cells derived from normal and HPFH-2 deletion chromosomes. Our results indicate that DNA sequences in the region just distal to the HPFH-1 3′ deletion endpoint specify a chromatin structure with erythroid-specific but not developmentally specific features. When translocated to the vicinity of the fetal hemoglobin genes, this chromatin structure may be important in determining the phenotype of deletional HPFH.

MATERIALS AND METHODS

Cell populations. All cell populations were maintained at 4°C between harvest and DNase I digestion of nuclei to minimize endogenous nuclease activity.

Nucleated erythroid cells. Normal adult human bone marrow aspirates (ca. 2×10^{10} nucleated cells) were sedimented through discontinuous Ficoll-Hypaque density gradients as part of a bone marrow transplantation procedure (32). The most dense ($\rho > 1.077$) fraction of these gradients contained erythrocytes, nucleated erythroid cells, and immature polymorphonuclear leukocytes (band forms) and was the starting material for further purification. Mature erythrocytes were selectively agglutinated with 1.5% high-molecular-weight dextran in RPMI 1640 medium with 10% fetal calf serum and removed by unit gravity sedimentation for 45 min. The supernatant, generally containing 1×10^9 to 2×10^9 cells, was washed twice with RPMI 1640 with 10% fetal calf serum and then subjected to elutriation as described previously (40). Fractions enriched for nucleated erythroid cells were pooled, suspended in medium, and used immediately. Cell purity was determined by examination of Wright-Giemsa-stained cytocentrifuge preparations.

Single-cell suspensions enriched for fetal erythroid cells were prepared by two or three cycles of unit gravity sedimentation in RPMI 1640 plus 10% fetal calf serum after disaggregation of finely minced human fetal liver tissue by gentle pipetting. Under these conditions, clumps of fetal hepatocytes settled on the bottom of the tube, while erythroblasts remained suspended.

Fetal brain cells. After removal of the meninges, fetal brain tissue fragments were washed once in reticulocyte standard buffer (RSB) (10 mM Tris hydrochloride, 10 mM NaCl, 3 mM $MgCl_2$, pH 7.4) and then homogenized in 10 volumes of RSB with a loose-fitting Dounce homogenizer. The homogenate was layered over a cushion of 0.88 M sucrose in RSB–0.25% Nonidet P-40 and centrifuged at $400 \times g$ for 10 min (3). The pellet was washed once in RSB–0.25% Nonidet P-40, yielding a homogeneous nuclear preparation which was used immediately.

Cultured cells. K562 (22), HEL (24), Manca (27), Jurkat (41), and HL-60 (9) cells and Epstein-Barr virus (EBV)-transformed human lymphoblastoid cells (14) were propagated in suspension culture in RPMI 1640 with 2 mM L-glutamine, 10% fetal calf serum, and antibiotics in a humidified 5% CO_2 atmosphere at 37°C. Cultures of peripheral blood erythroid burst-forming units (BFU-e) were prepared and harvested as described previously (29).

Isolation of nuclei and DNase I digestions. Cells (1×10^8 to 2×10^9) were collected by centrifugation at $400 \times g$ for 5 to 10 min and washed once in phosphate-buffered saline, and the pellet was suspended by tapping to a final cell density of 2×10^7 to 4×10^7 cells per ml in ice-cold RSB. Cell membranes were disrupted by dropwise addition of a 10% solution of Nonidet P-40 to a final concentration of 0.25% with gentle vortexing for 10 s. The resulting nuclei were pelleted at $400 \times g$ for 5 to 10 min, suspended in RSB at a DNA concentration of 1 mg/ml, and digested with DNase I as previously described (5). Nuclei were lysed with 0.1% sodium dodecyl sulfate–5 mM EDTA–0.3 M NaCl and prepared for restriction endonuclease digestion either as previously described (34) or as described below. In some cases, optimal DNase I concentrations were predetermined by preparing and digesting nuclei from small samples of cells followed by electrophoresis of nuclear lysates on a 1% agarose minigel.

In some cases, DNA was purified from nuclear lysates by the addition of 1 volume of 4 M ammonium acetate with vigorous vortex mixing followed by precipitation with two volumes of isopropanol at room temperature for 30 min. After centrifugation at $10,000 \times g$ for 20 min, the pellet was washed twice with 70% ethanol–30% 10 mM Tris hydrochloride, pH 7.4–1 mM EDTA–50 mM NaCl and redissolved in 10 mM Tris hydrochloride, pH 7.4–1 mM EDTA (TE buffer). A second cycle of ethanol precipitation from 0.2 M sodium acetate in TE buffer was occasionally required. This method

was substantially less laborious than repeated phenol-chloroform extraction.

Restriction endonuclease digestion, blot hybridization, nick translation of probe fragments, and nuclear runoff transcription were all carried out as described previously (5, 19, 34). The restriction mapping of the HPFH breakpoint region and the molecular cloning of the probes used in these experiments have been described previously (19, 23, 26, 31, 37).

RESULTS

Characterization of adult nucleated erythrocytes. Photomicrographs depicting successive steps in the enrichment protocol are shown in Fig. 2A. Routinely, 75 to 90% of the purified nucleated cells were of erythroid origin and were in late stages of maturation as judged by extensive chromatin condensation and cytoplasmic hemoglobinization. Despite the maturity of these cells, nuclear runoff transcription showed strong hybridization to α- and β-globin probes, demonstrating that these nuclei remain transcriptionally active as isolated (data not shown). The developmental specificity of this preparation was demonstrated by the presence of DNase I-hypersensitive sites 5′ to the β-globin gene but not to the γ-globin gene (Fig. 2B) and by the absence of nuclear runoff transcripts homologous to the γ genes (data not shown).

Chromatin structure of the HPFH-1 3′ deletion endpoint region. A panel of DNase I digestion series representing fetal and adult erythroid, erythroleukemic, and nonerythroid cell populations was prepared and digested with *Hin*dIII. Blot hybridization was performed by using a 1.4-kb *Bam*HI-*Eco*RI probe fragment which maps 2 kb 3′ to the HPFH-1 3′ deletion breakpoint (23, 37). This analysis revealed a parent fragment of 12.5 kb and two major subbands of 6.5 and 4.5 kb generated by DNase I digestion (Fig. 3A). In addition, several minor subbands were detectable in some series. The 4.5-kb subband (solid arrow) was present only in DNase I digests of nuclei derived from erythroid cells, including fetal and adult nucleated erythroid cells and K562 and HEL erythroleukemia cells. This subband, which is also marked by a solid arrow in Fig. 1, was undetectable in nuclei derived from human fetal brain and Manca B lymphoblastoid cells (Fig. 3A), Jurkat T lymphoblastoid cells, HL-60 myelomonocytic cell lines, and secondary cultures of diploid human fibroblasts (data not shown). Each of the nonerythroid DNase I digestion series yielded strong hypersensitive sites when hybridized to a β-actin probe (data not shown). The subband marked by the open arrows in Fig. 1 and 3 was seen faintly in the nonerythroid cell types in addition to being clearly present in all four erythroid cell populations. The HPFH-1 3′ deletion endpoint is located between these two sites (Fig. 3). A third DNase I-hypersensitive site was found 1.1 kb upstream of the HPFH-2 3′ deletion endpoint. A 1-kb *Sac*I-*Pvu*II fragment derived from the plasmid p4.3O1BgS (23) was hybridized to various *Sac*I-digested DNase I digestion series (Fig. 3B). This site was also detectable but was not as prominent in K562, HEL, and adult nucleated erythroid cells (data not shown). It was not detectable in nuclei prepared from Manca B lymphoblastoid cells (Fig. 3B) or from fetal brain (data not shown). This site is depicted by the striped arrow in Fig. 1.

The position and size of the erythroid-specific site 3′ to the HPFH-1 breakpoint was mapped at a higher resolution in HEL erythroblastoid cells (Fig. 4A). The 1.08-kb *Sac*I-*Bam*HI fragment used as the probe detected a parent band of

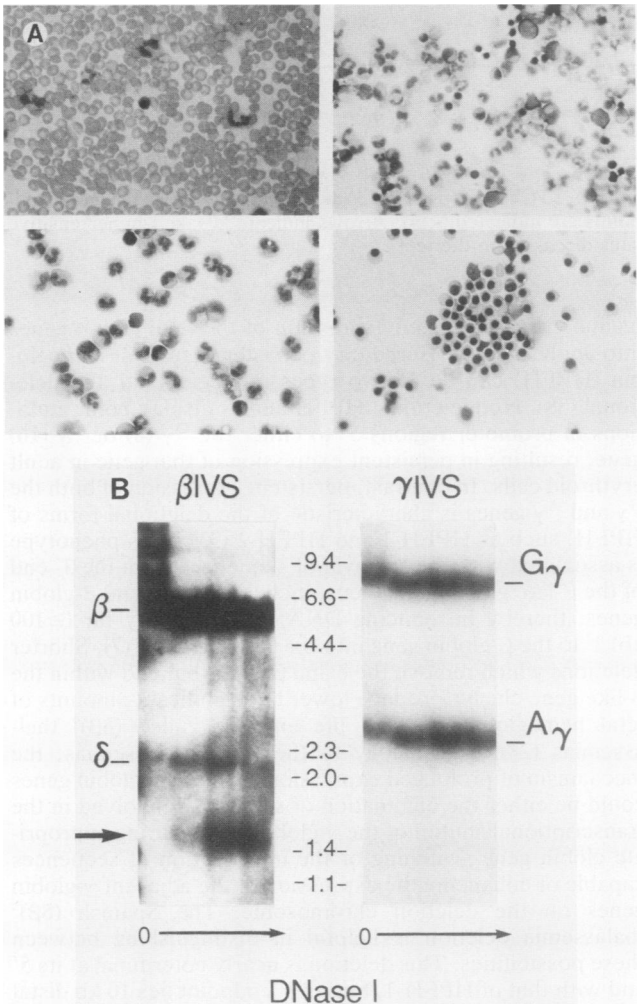

FIG. 2. Characterization of adult nucleated red blood cells. (A) Photomicrographs of cytospin preparations representing successive steps in the purification procedure. Upper left, starting material (see text). Upper right, nucleated cells remaining after dextran sedimentation of RBCs. Lower left, myeloid fraction after elutriation. Lower right, erythroid fraction after elutriation. Magnification, ×400. (B) DNase I-hypersensitive sites of adult nucleated erythroid cells. A DNA blot of an *Eco*RI-digested DNase series prepared from the cells shown in Fig. 2A, lower right, was hybridized with human β (left panel) or γ (right panel) intervening sequence (IVS) probes as described previously (2). The arrow on the left indicates the 1.6-kb subband generated by the hypersensitive site present in the 5′-flanking region of the β-globin gene (2). In the left lanes, DNase I was omitted. Increasing digestion is from left to right.

10 kb and a single subband of 1.4 ± 0.15 kb. We analyzed the DNA sequence of the hypersensitive site region (kindly provided by E. A. Feingold and B. Forget), and within the boundaries of the site we found five copies of a sequence, 5′-TGTGG-3′, which is contained in the simian virus 40 core enhancer (20) (Fig. 4B).

Characterization of hypersensitive sites on the HPFH-2 deletion chromosome. Approximately 10^4 BFU-e were propagated and harvested from the peripheral blood of an individual heterozygous for the HPFH-2 deletion (1). DNase I digestion series were prepared from these cells, from EBV-transformed lymphocytes from the same patient, and from a mouse-human hybrid erythroid cell line containing the dele-

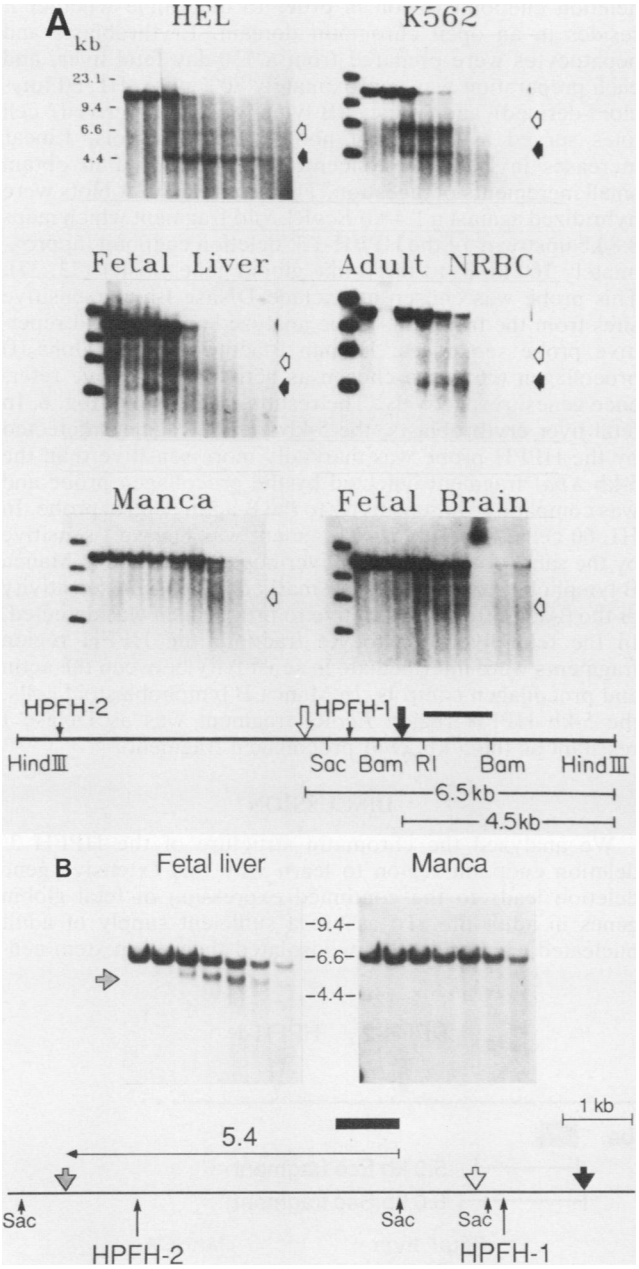

FIG. 3. Erythroid-specific hypersensitive site 3′ to the HPFH-1 3′ deletion breakpoint. (A) Hypersensitive sites downstream of the HPFH-2 breakpoint. Sizes of *Hin*dIII-digested λ DNA fragments are given in upper left panel. Marker fragments are displayed in the left-hand lanes of other panels. The probe fragment used is displayed above the restriction map as a horizontal bar. Subbands generated by DNase I-hypersensitive sites are indicated by arrows. In each panel, DNase I was omitted in first two lanes (first lane, 0°C control; second lane, 37°C control). Increasing digestion time in remaining lanes is from left to right. (B) Hypersensitive site upstream of the HPFH-2 breakpoint. Sizes of molecular weight markers (in kilobases) are indicated. The probe fragment used is displayed above the restriction map as a horizontal bar. Subband generated by hypersensitive site is indicated (⇨). DNase-omitted controls are as described for panel A.

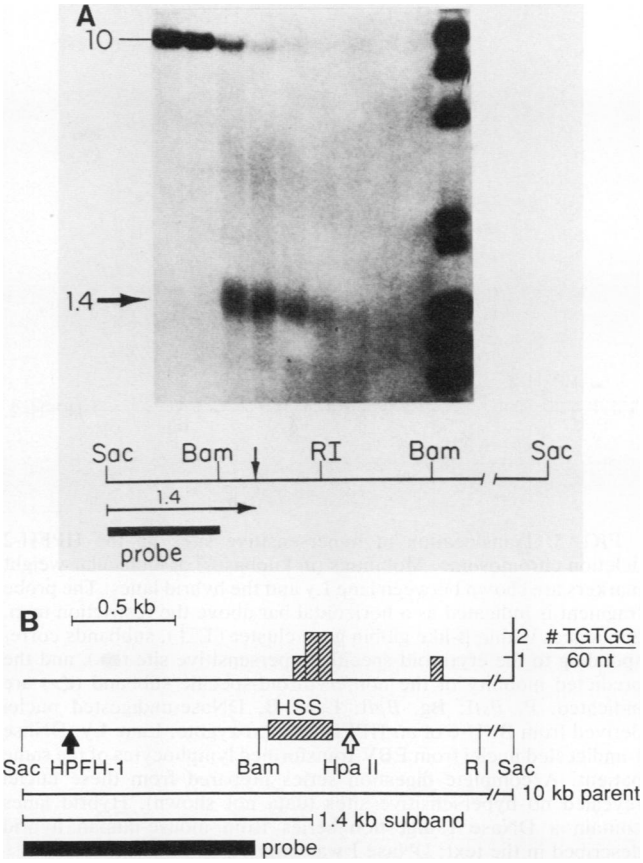

FIG. 4. Fine mapping of erythroid-specific site 3′ to the HPFH-1 breakpoint. (A) Blot hybridization analysis. Size markers (in kilobases) are shown to the left. The probe fragment is displayed below the restriction map as a horizontal bar. The subband generated by the hypersensitive is indicated by the arrow. DNase I-omitted controls are as described for Fig. 3A. (B) Localization of simian virus 40 core enhancer-like sequences. The number of times the sequence 5′-TGTGG-3′ is encountered per 60 nucleotides is shown at the top, and boundaries of the hypersensitive site shown in Fig. 4A are shown below. The locations of hypomethylated *Hpa*II site (Feingold and Forget, in press) (⇩) and of the HPFH-1 3′ breakpoint (◆) are indicated.

tion chromosome. This line was prepared by fusion of the patient's EBV-transformed lymphoblasts and MEL mouse erythroleukemia cells followed by selection for retention of human chromosome 11 as described previously (14, 30). An *Eco*RI-*Acc*I probe fragment mapping just upstream of the pseudo-β gene was hybridized against *Pst*I-*Bgl*I digests of these series such that the deletion chromosome generated a larger hybridizing restriction fragment than did the intact chromosome. The subband corresponding to the erythroid-specific hypersensitive site was detectable in the DNase-undigested control lane of the BFU-e-derived series (Fig. 5, lane B), and after DNase digestion of the hybrid-derived nuclei (Fig. 5, hybrid lanes). Translocation of the non-erythroid-specific site was also detectable in the BFU-e-derived series (Fig. 5, open arrows). These sites were not detectable in EBV-transformed lymphocytes from the same patient (Fig. 5, lane Ly), whereas hypersensitive sites for the β-actin gene were clearly detectable (data not shown). The presence of subbands in the DNase I-undigested nuclei obtained from the BFU-e is probably due to endogenous nuclease activity during the extended time (2 days) required

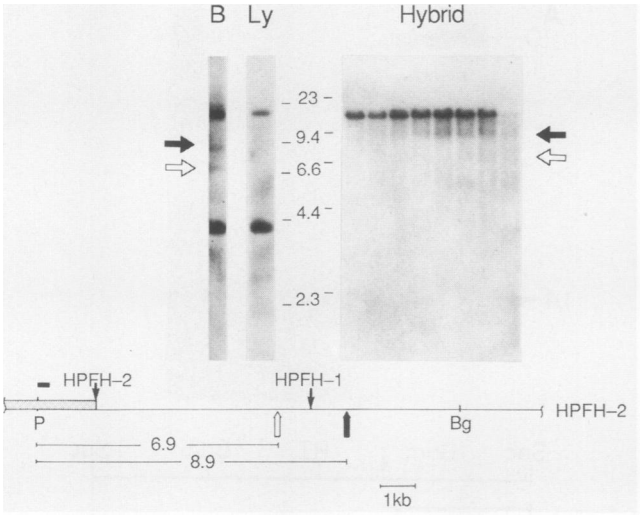

FIG. 5. Translocation of hypersensitive sites on the HPFH-2 deletion chromosome. Mobilities (in kilobases) of molecular weight markers are shown between lane Ly and the hybrid lanes. The probe fragment is indicated as a horizontal bar above the restriction map. Sequences within β-like globin gene cluster (▩), subbands corresponding to the erythroid-specific hypersensitive site (➡), and the predicted mobility of the non-erythroid-specific subband (⇨) are indicated. P, *Pst*I; Bg, *Bgl*I. Lane B, DNase-undigested nuclei derived from BFU-e of an HPFH-2 heterozygote. Lane Ly, DNase I-undigested nuclei from EBV-transformed lymphocytes of the same patient. A complete digestion series prepared from these nuclei revealed no hypersensitive sites (data not shown). Hybrid lanes contain a DNase I digestion series from mouse-human hybrid described in the text; DNase I was omitted in the left lane.

to harvest these nuclei. There is substantial evidence supporting the concept that endogenous nucleases and DNase I cleave chromatin at similar if not identical sites (42).

The HPFH-1 3′ deletion endpoint lies within a DNase I-sensitive chromatin domain of the nondeletion chromosome. We next measured the DNase I sensitivity of the HPFH-1 3′ deletion endpoint region in order to determine whether it resides in an open chromatin domain. Erythroblasts and hepatocytes were prepared from a 130-day fetal liver, and each preparation was approximately 80% pure. HL-60 (myeloid-derived) and Manca (B-lymphoblastoid-derived) cell lines served as additional nonerythroid controls. Linear increases in DNase I concentration were used to obtain small increments of digestion. The resulting DNA blots were hybridized against a 1.4-kb *Eco*RI-*Stu*I fragment which maps 8.8 kb upstream of the HPFH-1 3′ deletion endpoint, approximately 100 kb 3′ to the β-like globin gene cluster (23, 37). This probe was chosen to exclude DNase I-hypersensitive sites from the fragments to be analyzed and to avoid repetitive probe sequences. Human β-actin (31) and alpha$_2$(I) procollagen (26) were chosen as active and inactive reference genes, respectively. The results are shown in Fig. 6. In fetal liver erythroblasts, the 5-kb *Eco*RI fragment detected by the HPFH probe was markedly more sensitive than the 5-kb *Xba*I fragment detected by the procollagen probe and was comparable in sensitivity to the β-actin control probe. In HL-60 cells, the 6-kb *Sac*I fragment was DNase I sensitive by the same criteria. In fetal liver hepatocytes and in Manca B lymphoblastoid cells, a less marked increase in sensitivity of the β-actin fragment relative to procollagen was detected. In the fetal liver hepatocyte fraction, the HPFH region fragments were intermediate in sensitivity between the actin and procollagen controls. In Manca B lymphoblastoid cells, the 5-kb HPFH region *Eco*RI fragment was as DNase I resistant as the 5-kb *Xba*I procollagen fragment.

DISCUSSION

We analyzed the chromatin structure of the HPFH 3′ deletion endpoint region to learn why this extensive gene deletion leads to the continued expression of fetal globin genes in adult life. To assure a sufficient supply of adult nucleated erythroid cells, we isolated them from stem cell-

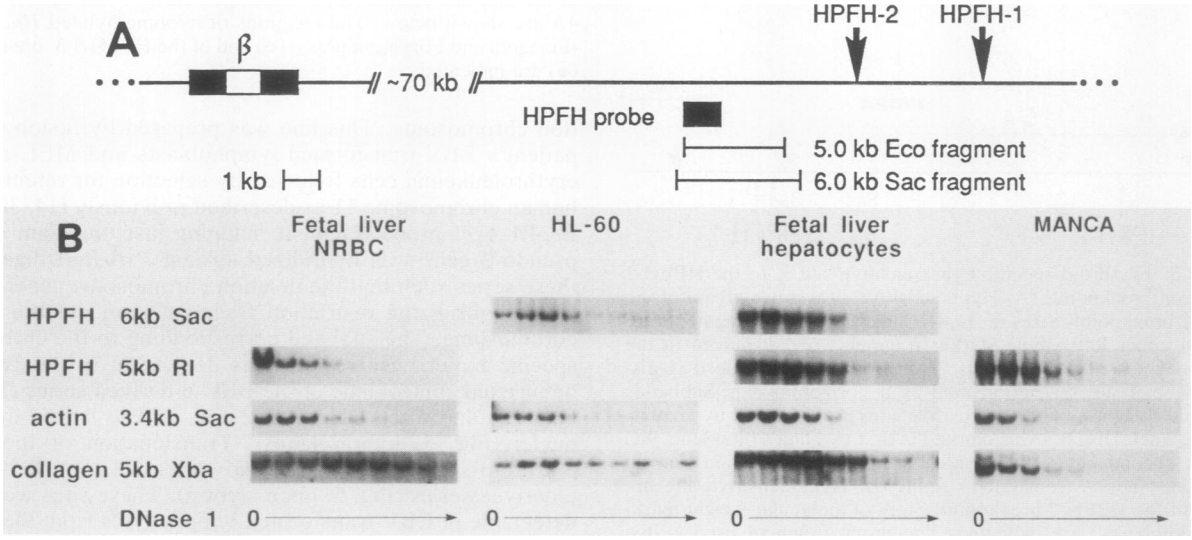

FIG. 6. DNase sensitivity of HPFH 3′ breakpoint region. (A) Map of the non-deletion chromosome region selected for analysis of DNase I sensitivity. The probe fragment used is shown as a horizontal bar beneath the restriction map. Locations of the HPFH-1 and -2 3′ breakpoints are shown. The β-globin gene is depicted on the left. (B) DNase I sensitivity profiles. Only the relevant hybridizing bands are shown. Sizes of fragments detected, restriction enzyme used, and hybridization probe used are shown on the left. DNase I-omitted controls are as described for Fig. 3A. Increasing digestion time is from left to right.

depleted bone marrow transplantation specimens by elutriation (Fig. 2A). Nuclei prepared from these cells maintained transcriptional activity and adult chromatin structure and transcriptional specificity (Fig. 2B and data not shown). An erythroid-specific DNase I-hypersensitive site was identified approximately 1.2 kb downstream of the 3′ endpoint of the HPFH-1 deletion. This site is present in normal erythroid cells of both fetal and adult lineages (Fig. 3A and 4A). Analysis of the DNA sequence within the boundaries of the site revealed a clustering of a pentanucleotide sequence, 5′-TGTGG-3′, found in the simian virus 40 core enhancer (20) (Fig. 4B). Another major DNAse I-hypersensitive site was found 0.5 kb 5′ to the HPFH-1 3′ breakpoint (Fig. 3B), and several minor sites were also sometimes observed. However, the latter sites were detected in cells of erythroid and nonerythroid origins. A third hypersensitive site, most prominent in fetal liver erythroblasts, was mapped approximately 1 kb upstream of the HPFH-2 3′ deletion endpoint. The erythroid-specific hypersensitive site mapping downstream of the HPFH-1 3′ breakpoint was retained on the deletion chromosome in BFU-e from an HPFH-2 heterozygote and in a human-murine erythroid hybrid cell line bearing the HPFH-2 deletion chromosome. This site is therefore transposed to within 12 kb of the $^{A}\gamma$ globin gene in HPFH-2. The locations of these sites are summarized in Fig. 1.

The erythroid-specific hypersensitive site downstream of the HPFH-1 breakpoint is eliminated in Spanish δβ°-thalassemia, which is characterized by smaller elevations of fetal hemoglobin in adult life than are HPFH-1 and -2 (10% in Spanish thalassemia versus 26 and 44% in HPFH-1 and -2, respectively [28]). The Spanish δβ°-thalassemia deletion extends approximately 10 kb farther downstream than does HPFH-1 (6; Feingold and Forget, in press). We found no hypersensitive sites other than those reported here in the interval between the HPFH-1 and Spanish δβ°-thalassemia endpoints (Fig. 4A). A strong correlation exists between the presence of DNase I-hypersensitive sites and binding sites for factors which regulate transcription (4, 10, 13, 44). Therefore, it seems likely that this site is involved in the up regulation of fetal hemoglobin characteristic of HPFH-1 and -2.

Feingold and Forget have shown that the 1.05-kb *Bam*HI-*Eco*RI fragment containing the erythroid-specific hypersensitive site (Fig. 4A) functions as an enhancer element in K562 cells (Feingold and Forget, in press). In addition, this region was specifically hypomethylated in normal erythroid cells of fetal and adult origin (Feingold and Forget, in press) and in mouse-human erythroid hybrids bearing the deletion chromosome (30). While this region contains an open reading frame (Feingold and Forget, in press), we have been unable to detect transcriptional activity by nuclear runoff assay within 5 kb upstream or downstream of this hypersensitive site (data not shown). As would be predicted for the action of an enhancer element, comparisons of somatic cell hybrids bearing either of the HPFH-1 or -2 deletion mutations to those bearing a normal chromosome 11 demonstrate that the γ genes are active only in *cis* to the deletion chromosome (30).

In a variety of deletional δβ° thalassemias, fetal hemoglobin is increased roughly 40-fold relative to normal adult levels in the heterozygous state, while heterozygotes for deletional HPFH display an additional two- to threefold increase (28). Our results, taken in conjunction with those of Feingold and Forget (in press), suggest that the incremental increase in fetal hemoglobin seen in HPFH relative to other δβ° thalassemias reflects the translocation of a segment of chromatin capable of binding erythroid-specific (but not developmentally specific) regulatory factors to the vicinity of the globin gene cluster on chromosome 11. The mechanism by which the loss of intervening DNA in the deletional δβ° thalassemias results in a 40-fold increase in fetal hemoglobin relative to normals remains unresolved. However, the diminished fetal hemoglobin characteristic of Spanish δβ° thalassemia relative to HPFH (28) suggests that deletion of intervening DNA alone is insufficient to account for the phenotype of deletional HPFH.

Forrester et al. have described DNase I-hypersensitive sites mapping 5 to 10 kb upstream of the ε globin gene (15) and have shown that these sites are also preserved in mouse-human erythroid hybrids (14). These sites may be involved in conferring a chromatin conformation permissive to transcriptional activity to the entire β-like globin gene cluster (14, 17). Chromosomal alterations of flanking regions may be important in determining the phenotype of increased γ gene expression which is common to HPFH and δβ° thalassemia. However, no 3′-flanking regions which inhibit globin gene expression at a distance (and which would be eliminated by these deletions) have yet been described. Even relatively short deletions which eliminate the δ and β genes display increased fetal hemoglobin in adult heterozygotes (6, 28). One hypothesis is that *trans*-acting factors normally interact with the regulatory regions of the adult genes to activate adult gene transcription and inactivate fetal gene transcription. However, in the deletional syndromes, these factors serve instead to suboptimally activate fetal gene transcription.

Analysis of the DNase I sensitivity of the HPFH 3′ breakpoint region was complicated by the presence of hypersensitive sites and repetitive sequences. We selected an area just upstream of the HPFH-2 breakpoint for analysis in order to avoid hypersensitive sites. Since the myeloid and erythroid lineages are thought to arise from a common precursor distinct from the lymphoblastoid lineage (25), it is interesting that the HPFH region is DNase I sensitive not only in erythroid cells but also in HL-60 cells representing the myeloid lineage. In contrast, the HPFH region was DNase I resistant in Manca B lymphoblastoid cells. The intermediate DNase sensitivity of the HPFH region in fetal hepatocytes may be explained by the presence of approximately 20% erythroid cells in the fetal hepatocyte preparation. Whether or not the increased DNase I sensitivity of this region is erythroid specific, it would be predicted that translocation of these sequences to the vicinity of the globin genes would maintain the remaining globin genes on the deletion chromosome in a DNase I-sensitive domain.

These findings and those of Feingold and Forget (in press) strongly suggest that the translocation of a distant, erythroid-specific chromatin structure characteristic of active genes contributes to the phenotypes of HPFH-1 and -2. More generally, these data provide support for the concept that the chromatin structure of flanking DNA sequences is of importance in the physiologic regulation of gene expression. Elucidation of the molecular mechanisms involved in this process remains highly relevant to our understanding of gene regulation and cell determination in development.

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