

Targeted Remodeling of Human β -Globin Promoter Chromatin Structure Produces Increased Expression and Decreased Silencing

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ABSTRACT: The chromatin structure of the human β -globin gene locus assumes a transcriptionally-active conformation in erythroid cells. One feature of this chromatin reorganization is the formation of DNase 1 hypersensitive sites in the regions of active globin gene promoters. This reorganization requires the globin locus control region and is associated with normal expression of the β -like globin genes. To determine whether it is possible to artificially enhance the opening of the chromatin structure of a minimal β -globin promoter, we placed a 101bp, erythroid-specific DNase 1 hypersensitive site-forming element (HSFE) immediately upstream of the β -globin promoter and gene. This element includes binding sites for NF-E2, AP-1, GATA-1 and Sp-1. Constructs were stably transfected into murine erythroleukemia cells and promoter chromatin structure and gene expression were analyzed. The HSFE induced an area of enhanced DNase 1 hypersensitivity extending from the transcriptional start site to -300bp of the artificial promoter and significantly increased the proportion of β -globin promoters in an open chromatin configuration. This remodeling of promoter chromatin structure resulted in 3-fold increases in β -globin gene transcription and induction, and inhibited long-term β -globin gene silencing. These results indicate that a relatively small *cis*-acting element is able to enhance remodeling of promoter chromatin structure resulting in increased β -globin gene expression. © 1999 Academic Press

INTRODUCTION

The human β -globin gene locus contains five expressed genes which are transcribed in a tissue-specific and developmentally-regulated fashion (1). The upstream locus control region, or LCR, is critical to the proper expression of these genes. This regulatory element is composed of five DNase I hypersensitive sites (5' HS1-5) located from 5 to 23 kb 5' of the expressed globin genes (Fig. 1)(2-4). Natural mutations of the human β -globin locus which eliminate portions of the LCR, but leave structural genes intact, result in complete

silencing of β -globin gene expression (5-7). The LCR is able to confer high-level, position-independent expression of stably integrated β -globin genes in transgenic mice (3). Despite intense investigation, the precise mechanisms of LCR function remain unknown.

One of the major actions of the LCR is to create an erythroid-specific chromatin structure throughout the β -globin locus. This "open" or "active" chromatin structure has been characterized as an increased sensitivity to DNase

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I digestion (8-10). While in non-erythroid cells the entire locus is resistant to DNase I, at least

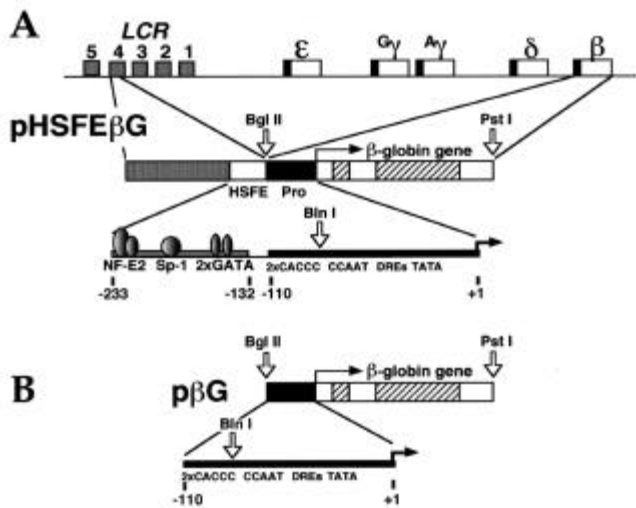


Figure 1. DNA constructs used to evaluate the HSFE. (A) Top: The human β -globin locus. Middle: Plasmid pHSFE β G contains the HS4 HSFE directly upstream of the minimal promoter. Bottom: Enlargement of the HSFE and minimal promoter with regulatory elements shown. (B) The p β G construct lacking the HSFE serves as a control. Horizontal arrows indicate the transcriptional start site.

established in erythroid cells. These include an overall increased sensitivity to DNase I throughout the entire locus (9,10), specific DNase I HSs associated with the promoters of expressed globin genes (11) and DNase I “major” or “super” HSs associated with the active elements of the LCR (2-4,12). Evidence that the LCR is required for the formation of these domains of altered chromatin structure comes from investigations of the naturally occurring Hispanic $\gamma\delta\beta$ -thalassemia mutation in which the globin structural genes are intact but a major portion of the LCR has been deleted (5). In chromosomes carrying this deletion none of the β -globin genes are expressed and none of the erythroid-specific chromatin structures of the locus are formed (10). Transgenic mouse experiments have also demonstrated that the formation of globin gene promoter HSs is dependent on the presence of a linked LCR (13,14). Artificial mutants, in which the hygromycin resistance gene was inserted between HS1 and HS2 of the LCR, exhibited loss

three tissue-specific chromatin structures are

of promoter-associated DNase I HSs downstream of the inserted gene (15). While the mechanisms by which the LCR is able to remodel the chromatin structure of the β -globin gene locus remain to be defined, it has been proposed that one of the key components of LCR function is the interaction of individual β -globin LCR elements to form a holocomplex which, in turn, confers an open chromatin structure on distant promoter elements (16-18). It has been further hypothesized that these LCR-promoter interactions create domains in which nucleosomes are displaced or disrupted, allowing increased access and recruitment of necessary transcription factors to promoter *cis*-acting elements (13,16).

To determine whether we could enhance the opening of promoter chromatin structure of a stably transfected human β -globin gene in an erythroid environment, we placed a previously described, erythroid-specific, DNase I HS-forming element (HSFE) immediately upstream of a minimal human β -globin promoter (19,20). This 101 bp element, which is derived from LCR HS4, is necessary and sufficient to reorganize local chromatin structure in erythroid cells (19). This HS-forming ability is dependent on the presence of NF-E2 and tandem, inverted GATA binding sites located approximately 50bp 3' of the NF-E2 site (20). Within the core of LCR HS4 the HSFE remodels local nucleosomal structure by repositioning nucleosomes over a several hundred bp region (20). Similarly arrayed NF-E2 and GATA elements are found in each of the human LCR HS cores, are conserved in other species and are required for the formation of human LCR HS2 and HS3 (20,21). Our goal in these experiments was to determine whether this tissue-specific, *cis*-acting element can be used to enhance remodeling of local promoter chromatin structure and to determine the effects of this remodeling on gene expression. These experiments are relevant to understanding the role of local chromatin structure in β -globin gene regulation and to the

development of effective erythroid-specific gene therapy vectors.

MATERIALS AND METHODS

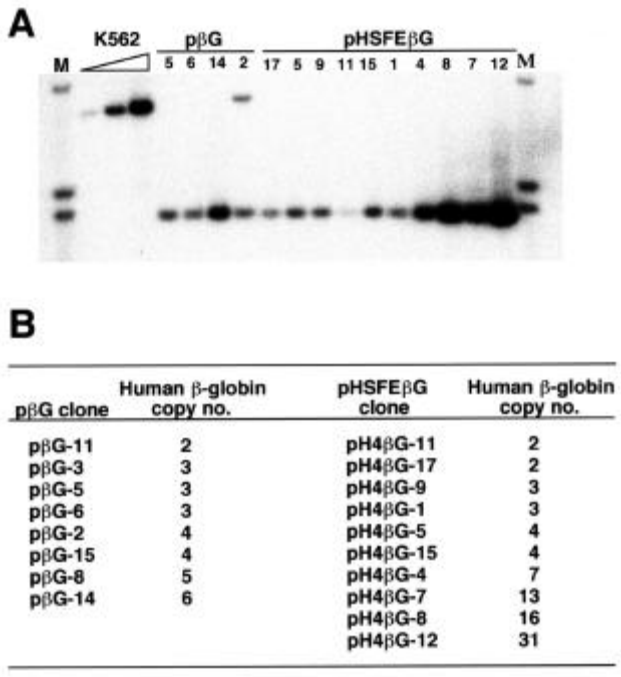


Figure 2. Determination of human β-globin copy number. (A) Four pβG and ten pHSFEβG clones were assayed in the Southern blot shown. Genomic DNA was digested with Bgl II and Pst I, which cut within the construct. The probe was a 900 bp Bam HI/Eco RI fragment of the human β-globin gene. Copy numbers were quantitated by PhosphorImager analysis and based on K562 genomic DNA standards (1, 6 and 12 copies). Clone numbers are indicated above each lane. Markers (M) correspond to the 4.4, 2.3 and 2.0 kb fragments of lambda DNA HindIII digest. (B) Human β-globin copy numbers for the eight pβG and ten pHSFEβG clones.

on a pUC-derived plasmid containing the human β-globin gene and 280bp promoter. To prepare the pβG construct, an Xho I/Bam HI fragment from -280bp of the promoter to +477 of the coding sequence was replaced by a PCR-generated fragment from -110 of the promoter to the +477bp Bam HI site. To prepare the pHSFEβG construct, a 920 bp fragment containing the HS4 HSFE at its 3' border was isolated from the HS4-containing plasmid HS4 TR-4 (19). This fragment was then ligated into the

Globin expression constructs

pβG construct directly upstream of the β-globin minimal promoter. pβG and pHSFEβG constructs and a separate plasmid containing the neomycin resistance gene were co-electroporated into MEL cells and stable transfectants selected with G418 as previously described (20).

Individual clones were isolated and then analyzed for the presence of the human β-globin gene by DNA dot blotting. Human β-globin gene copy number was determined by Southern blot using a 900bp Bam HI/Eco RI β-globin gene fragment as a probe for a 2kb Bgl II/Pst I internal fragment contained within both constructs (Fig 3). Eight pβG and ten pHSFEβG-containing clones were selected for further analysis.

Nuclease Sensitivity assays

In vivo DNase I sensitivity assays were performed on nuclei isolated from stable transfectants as previously described (19,20). Nuclei (200 μg DNA/reaction) were digested in a volume of 200 μl with DNase I at concentrations from 0 to 4.0 μg/ml. Following DNase I treatment, genomic DNA was digested to completion with either Afl III and Sal I for pβG clones or Xho I and Sal I for pHSFEβG clones and analyzed by Southern blotting (Fig 3).

Regions of DNase I sensitivity were mapped by plotting the migration distance of the molecular weight markers vs. the logarithm of their size in base pairs for each blot. These data points were then fitted to the equation:

$$\text{fragment size (bp)} = m (e^{i(\text{migration distance in cm})}).$$

This produces a straight line where "m" is the slope of the line and "i" is the y-intercept. By measuring the migration distances of the upper and lower limits of each DNase I HS and applying the above formula, the size, and therefore location, of the HS boundaries within the parental fragment was determined.

In vivo restriction endonuclease sensitivity assays using Bln I were performed on intact nuclei resuspended in 1X restriction enzyme buffer. For initial experiments, nuclei (200µg DNA/reaction) were digested in a volume of 200 µl with Bln I at amounts of 0, 10, 20, 40, 80 and 160 units at 37°C for 20 minutes. In later experiments Bln amounts of 0 and 100 units were used because complete cutting was consistently

containing clones includes the entire promoter. *Cis*-acting elements of the β-globin promoter are shown.

obtained above 80 units per reaction. Following Bln I treatment genomic DNA was analyzed by Southern blot and relative band intensities were determined by PhosphorImager analysis.

RNA Analysis

MEL cells were maintained in Improved MEM Zinc Option (Gibco BRL) supplemented with 10% fetal bovine serum. Globin expression was induced by 3mM hexamethylene bisacetamide (HMBA) for 4 days. RNA was isolated with RNAzol B (Tel-test, Inc.). For primer extension analysis, radiolabeled oligonucleotides were hybridized to 15 µg total RNA (22). 21-mers used for detection of mouse α-globin and human β-globin transcripts were: 5'-CAGGCAGCCTTGATGTTGCTT-3' and 5'-CCACAGGGCAGTAACGGCAGA-3', respectively (23). Oligonucleotides (10 pmol) were radiolabeled by T4 kinase in the presence of [³²P]ATP for 45 minutes at 37°C. Products were then electrophoresed on a 12% polyacrylamide gel and eluted in 500mM ammonium acetate, 1mM EDTA. Radiolabeled human β-globin and mouse α-globin-specific oligonucleotides were hybridized to 15 µg total RNA in 30 µl of 150mM KCl, 10mM Tris-HCl pH 8.3, 1mM EDTA for 90 minutes at 65°C. After cooling to ambient temperature, primers were then extended with the addition of avian reverse transcriptase (10 units) in the presence of 20mM Tris-HCl pH 8.3, 10mM MgCl₂, 5mM DTT, Actinomycin D (150 µg/ml), 1mM dNTP, 20 units RNasin (22). Extension products were electrophoresed on an 8.0% acrylamide/6M urea gel and relative expression levels were quantified by PhosphorImager analysis.

Solution hybridization was performed as previously described (24). Briefly, 5 µg of total RNA was hybridized with ~20 fmol radiolabeled oligonucleotide in 0.75M NaCl, 0.2% SDS, 4mM EDTA, 20mM Tris-HCl pH7.5 for 18 hours at

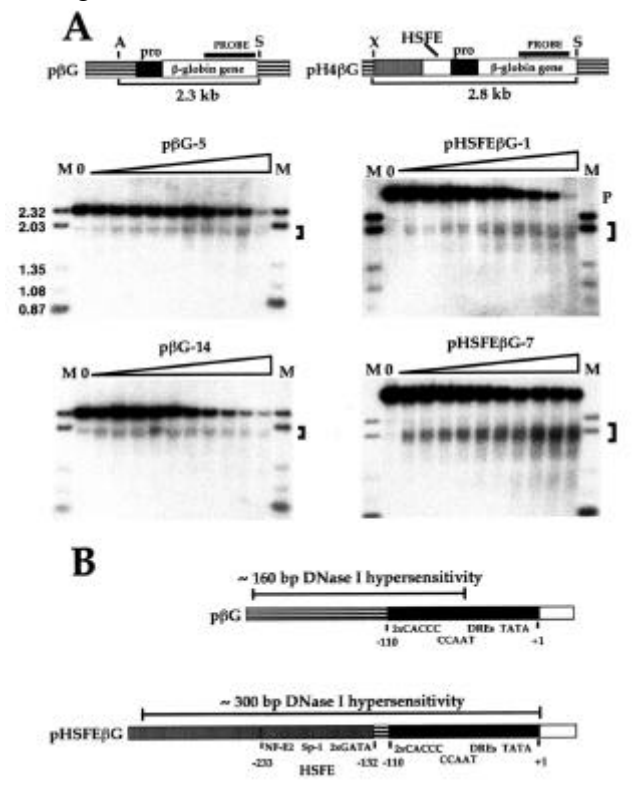


Figure 3. Effect of the HSFE on β-globin promoter DNase I sensitivity. (A) Top: pβG and pHSFEβG construct maps with Afl III (A), Sal I (S), and Xho I (X) restriction sites shown. Black boxes indicate minimal promoter (pro). The probe is the 900 bp Bam HI/Eco RI fragment of the human β-globin gene. Bottom: DNase I assays of pβG and pHSFEβG clones. Intact nuclei were isolated from stable transfectants and treated with DNase I at increasing concentrations. Genomic DNA was digested with either Afl III and Sal I to release a 2.3 kb parental band in pβG clones or Xho I and Sal I to release a 2.8 kb parental band in pHSFEβG clones. Parental bands are indicated by arrows DNase I HSs are indicated by brackets. (B) Mapping of the respective DNase I hypersensitive regions within the pβG- and pHSFEβG-containing clones. An approximately 160 bp HS maps to the promoter region of pβG-containing clones. A 300 bp HS in pHSFEβG-

55°C. Following hybridization, non-hybridized nucleic acids were digested in the presence of 8 units S1 nuclease for exactly 1 hour at 37°C and DNA-RNA hybrids were then precipitated in the presence of 10% trichloroacetic acid for at least 45 minutes at 4°C. Hybrids were then collected on glass filters (Schleicher & Schuell, #30) using a

dot-blot apparatus and filters assayed by scintillation counting. 24-mers used for detection of mouse α -globin and human β -globin transcripts were: 5'AGTAGGTCTTGGTGGTGGGGAAGC-3' and 5'AGCTTGTCACAGTGCAGCTCACTC-3', respectively.

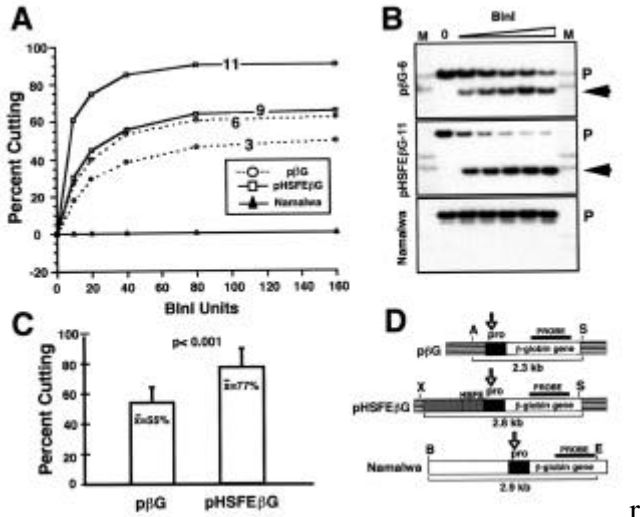


Figure 4. Quantitative analysis of β -globin promoter chromatin accessibility. (A) p β G-3 and -6 and pHSFE β G-9 and -11 clones were selected for restriction endonuclease analysis. Intact nuclei were digested with increasing amounts of Bln I. The percent of promoters within each clone that are cut by Bln I at each enzyme concentration is graphed. (B) Representative blots from p β G-3, pHSFE β G-11 and the human lymphoid cell line, Namalwa are shown. P, parental bands. Sub-bands resulting from Bln I digestion are indicated by arrows. (C) Mean percentage cutting, \pm 1 standard deviation, for each set of constructs is shown. P-value determined by t-test. (D) Diagrams of constructs and native β -globin gene with expected sizes of the parental bands shown. Vertical arrow indicates the Bln I site. A=Afl III, S=Sal I, X=Xho I, B=Bgl II, E= Eco RI.

Assays were performed in quadruplicate for each clone. Average MEL cell murine α -globin copy number was estimated to be four by comparing the α -globin Southern blot signal for the parental MEL cell line to mouse genomic DNA. Statistical analyses were performed using the t-test.

RESULTS

To evaluate the effect of the HS4 HSFE on β -globin promoter chromatin structure and β -globin gene expression we positioned the HSFE immediately 5' of the β -globin minimal promoter and gene (pHSFE β G). As shown in Fig. 1 this promoter, which was previously defined by its ability to yield maximal activation by the LCR (25), contains two CACCC boxes, a CCAAT box, three direct repeat elements (DREs) and the TATA box sequence (25,26). A construct containing the promoter, but lacking the HSFE, served as a control (p β G). These two constructs were individually co-transfected with a plasmid containing the neomycin resistance gene into MEL cells. Clones were first selected for G418 resistance. Dot blotting was then used to identify clones with stably integrated human β -globin gene constructs. Eight independent clones containing p β G and ten clones containing pHSFE β G were selected for further analysis. As shown in Fig. 2, copy numbers ranged from two to six in the minimal β -globin promoter construct-containing cells, and from two to 31 in the cells containing the HSFE construct.

Chromatin structure of stably integrated constructs

The major goal of our experiments was to determine whether the HS4-derived HSFE could enhance "opening" of the chromatin structure of a linked β -globin promoter. To characterize the effect of the HSFE on the chromatin structure of the promoter in stably transfected constructs, we performed two different nuclease sensitivity assays

on individual MEL cell clones. These were DNase I and quantitative restriction endonuclease sensitivity assays. DNase I assays detect regions of displaced or disrupted nucleosomal structure and allow identification of regions of DNA which are potentially accessible to *trans*-acting regulatory factors (27). We analyzed three clones containing each test construct with this assay. The results of two of these assays for each construct are shown in Fig. 3. Clones with similar copy numbers and experiments with similar exposures are compared. Clones containing the minimal β -globin gene

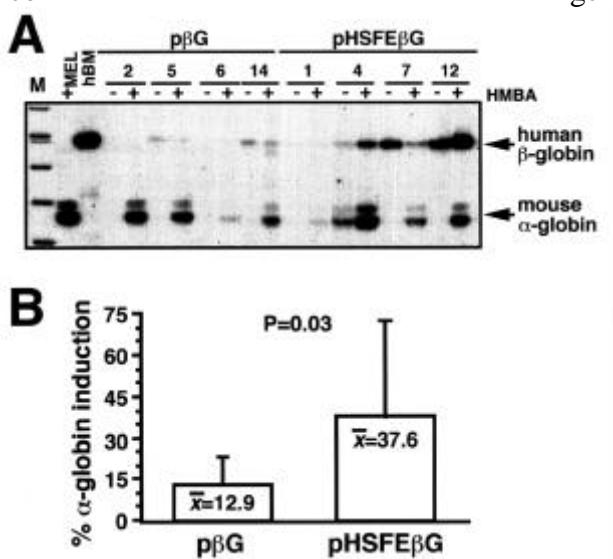


Figure 5. Effect of the HSFE on human β -globin gene transcriptional initiation and induction. (A) Primer extension analysis of MEL clones. RNA from HMBA-induced (+) and uninduced (-) MEL clones was analyzed. Human bone marrow RNA (hBM) and HMBA-induced wild-type MEL RNA are positive controls. The human β -globin primer extension product is 98 bp, and the mouse α -globin product is 77 bp. Markers are a 10 bp DNA ladder. All clones produce appropriately initiated human β -globin transcripts. (B) Comparison of HMBA induction of human β -globin expression between p β G and pHSFE β G clones. All p β G and pHSFE β G clones were analyzed. Induction levels for each clone are normalized to endogenous mouse α -globin induction and are graphed as mean \pm one standard deviation. P-value determined by t-test.

promoter (p β G) demonstrated a weak DNase I hypersensitive site adjacent to the 2.03kb molecular weight marker (Fig. 3A). A smaller

band was consistently present in the control lane without DNase I of p β G experiments and is likely due to endogenous nuclease cutting. In contrast, clones containing the β -globin gene linked to the HSFE (pHSFE β G) formed a broader region of DNase I sensitivity. The fact that the HSFE-containing construct directs the formation of a larger area of DNase I sensitivity can be appreciated by comparing the width of the HS bands to the adjacent 2.03kb molecular weight markers on each blot. A more precise mapping of the positions of these DNase I sensitive regions was performed as described in *Materials and Methods*. As shown in Fig. 3B, p β G clones exhibited a DNase I hypersensitive region of approximately 160 base pairs. This is consistent with findings that globin genes possess weak hypersensitive sites associated with their promoters (2,12,28). This region extended from approximately the CCAAT box at -50bp to approximately -210bp in relation to the transcriptional start site of the β -globin gene. In clones containing the HSFE, the region hypersensitive to DNase I was extended in both the 3' and 5' directions to encompass the entire minimal promoter from the transcriptional start site to -300 bp. While it is estimated that the mapping of the limits of the areas of DNase I sensitivity are accurate to approximately \pm 25bp (29), the boundaries in the six clones evaluated consistently mapped to the above regions. These changes in local chromatin structure result in the exposure of the direct repeat elements and the TATA region of the β -globin promoter to potential DNA-protein interactions.

While the above results indicate that the HSFE is able to direct the formation of a larger area of nuclease sensitivity in the region of the β -globin promoter, another question is whether the HSFE is able to increase the proportion of promoters within a given clonal population of cells that are in an open nucleosomal configuration. Work from the Felsenfeld laboratory has recently emphasized that the formation of nuclease HSs appears to be an "all or none" phenomenon, and that an

equilibrium between open and closed conformations is likely to exist (30). For the HSFE to be an effective opener of chromatin structure it should not only increase the region of nuclease sensitivity but should also increase the proportion of promoters in an open conformation. To address this question we examined the local chromatin structure of the promoters using the

quantitative restriction endonuclease assay. Restriction enzymes, unlike DNase I, do not digest DNA to completion at high concentrations. Therefore, an estimate of the percentage of promoters in an open (restriction nuclease sensitive) chromatin configuration in a given cell the enzyme Bln I which cuts at a unique site

bars indicate 1 standard deviation. P-values determined by t-test. (B) Relationship between human β -globin gene expression and transgene copy number. (C) Relationship between human β -globin gene expression and restriction endonuclease accessibility of the β -globin gene promoter.

population can be determined (31,32). We used within the CACCC box of the human β -globin promoter (see Figs. 1 and 4D). Fig. 4D also shows the restriction enzyme sites used in this analysis for MEL clones containing the $\beta\beta$ G and pHSFE β G constructs and the native human β -globin gene. Fig. 4A shows that cutting within the β -globin promoter of each construct plateaus at a given percentage allowing the proportion of promoters in an open configuration within each clonal population to be determined. $\beta\beta$ G clones 3 and 6 have 49% and 62% of their β -globin promoters in an open configuration respectively. pHSFE β G clones 9 and 11 showed 65% and 90% accessibility to Bln I cutting. Blots for experiments with clones $\beta\beta$ G-6 and pHSFE β G-11 are shown in Fig. 4B. Also shown is an analysis of the endogenous β -globin promoter from the Namalwa human lymphoid cell line. This cell line serves as a negative control demonstrating complete resistance to Bln I digestion. Because this initial experiment indicated that there might be a significant difference in the proportion of promoters in an open configuration depending on the presence of the HSFE, we analyzed the remainder of our clones. As shown in Fig. 4C, the mean accessibility of the $\beta\beta$ G clones was 55%, and was 77% for the pHSFE β G clones ($p < 0.001$). These results demonstrate that the HSFE is able to significantly increase the proportion of β -globin promoters in an open chromatin configuration.

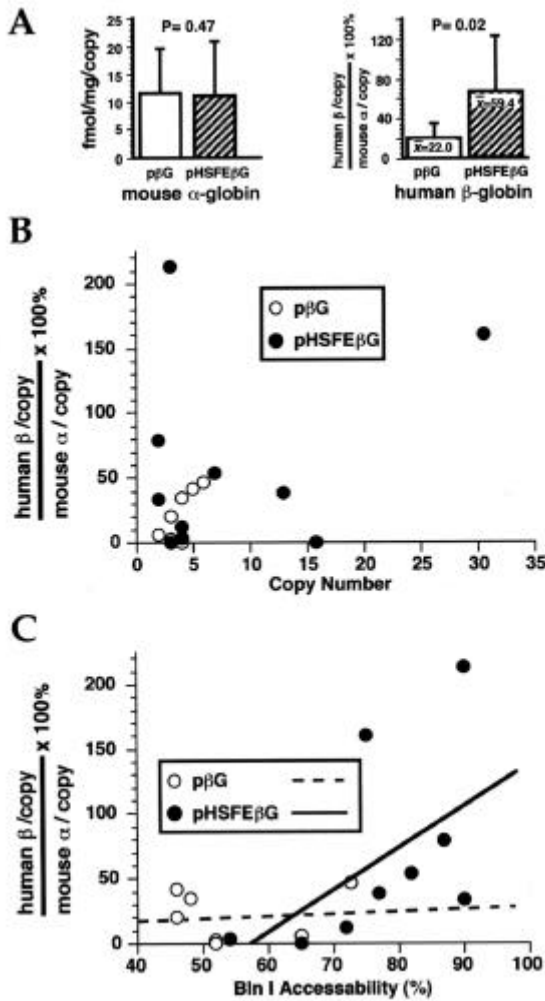


Figure 6. Effect of HSFE on human β -globin gene expression. Solution hybridization with probes for mouse α -globin and human β -globin mRNA was performed on total RNA isolated from HMBA-induced $\beta\beta$ G and pHSFE β G clones. (A) Comparison of mean expression levels for mouse α -globin and human β -globin genes between $\beta\beta$ G and pHSFE β G clones. α -globin mRNA levels expressed as fmol per mg total RNA per gene copy number. Human β -globin expression normalized to transgene copy number and endogenous mouse α -globin expression. Error

It would also be of interest to compare the restriction endonuclease accessibility of our test constructs to that of the endogenous β -globin promoter in human adult erythroid cells where the gene is normally expressed. We have analyzed the accessibility of the β -globin gene promoter in MEL cell clones containing a 230kb human β -globin yeast artificial chromosome (33). However, because Bln I accessibility of the human

β -globin promoter in these cells was low (approximately 5-10%) and expression of the β -globin gene was subject to silencing (data not shown), these results are not likely to be representative of native β -globin promoter chromatin structure. Unfortunately, no appropriate human erythroid tissue culture cell line with an adult globin phenotype is available

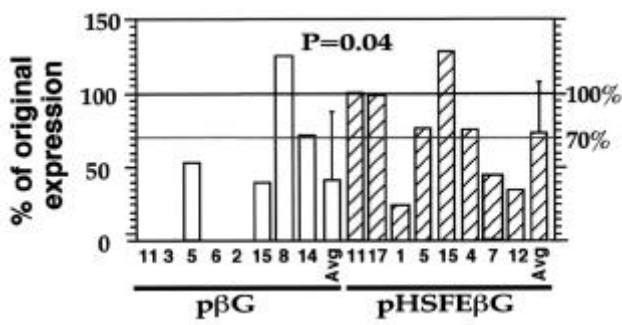


Figure 7. Effect of the HSFE on clonal extinction. pβG and pHSFEβG clones were maintained in culture for three months. RNA was then isolated from HMBA-induced cells and analyzed by solution hybridization. Human β -globin expression levels normalized to copy number and α -globin expression are graphed as a percentage of original expression. Mean values and standard deviations for pβG and pHSFEβG clones shown. Only clones with originally detectable human β -globin expression were analyzed.

for these experiments and a large number of highly purified human adult bone marrow-derived erythroid cells would be required to perform these experiments in primary tissue.

Effect of the HSFE on β -globin transcriptional initiation and induction

Our ultimate goal in manipulating the chromatin structure of the β -globin promoter is to attain increased transcription in a chromatin context. It is possible, however, that by altering the chromatin structure of the promoter, transcriptional initiation and normal gene regulation might be adversely effected. To determine the effect of the HSFE on β -globin gene transcriptional initiation we performed primer

extension analysis on all clones. Extension products from MEL clones were compared to normal human bone marrow. As shown in Fig. 5A, while expression varied widely, all clones exhibited the appropriate size human β -globin extension product indicating that the HSFE did not interfere with normal transcriptional initiation.

To determine whether the presence of the HSFE would alter β -globin gene regulation in MEL cells, we also used the primer extension assay to study HMBA induction of the stably transfected constructs. These results are also shown in Fig. 5A and are summarized in Fig. 5B. For each clone the level of human β -globin gene induction was normalized to the induction of the endogenous α -globin gene. While there was a wide variation in the induction of both the endogenous mouse α -globin genes and the transfected human β -globin genes, an approximately three-fold increase in average induction of the pHSFEβG clones was observed compared to pβG clones (Fig. 5B; 37.6% versus 12.9% of alpha-globin induction; p=0.03). These results indicate that the HSFE, on average, increases inducibility of the linked human β -globin gene in response to HMBA treatment.

Effect of the HSFE on β -globin gene expression level

While primer extension analysis is useful for verifying correct transcriptional initiation and determining induction ratios, it is less appropriate for quantifying steady-state levels of specific mRNA species. We therefore used solution hybridization to quantify β -globin mRNA levels

and to normalize these to endogenous α -globin mRNA levels (23,34). Analyses of each clone were performed in quadruplicate. Expression results for all clones except p β G-6 are included. Analysis of this clone produced inconsistent results on multiple assays. The results of experiments comparing p β G- to pHSFE β G-containing clones are shown in Fig. 6. When steady-state β -globin mRNA levels were analyzed, as shown in Fig. 6A, the mean values of α -globin expression were equivalent for both sets of clones. When human β -globin expression is normalized to both copy number and α -globin expression, a statistically significant 3-fold increase is seen (59.4% versus 22.0% of endogenous mouse α -globin per copy; $p=0.02$). These results demonstrate that while expression varies between clones, inclusion of the HSFE increases the average expression per copy of the stably transfected human β -globin gene.

To determine whether the HSFE was able to confer copy number-dependent (position-independent) expression on integrated constructs in MEL cell clones we plotted human β -globin gene expression normalized to β -globin gene copy number and endogenous mouse α -globin expression vs. the copy number of each clone. If copy number-dependence is conferred, then normalized β -globin expression should be within a tight range. As shown in Fig. 6B, β -globin gene expression ranges from undetectable to 47% of endogenous mouse α -globin for clones containing p β G and from undetectable to 213% for clones containing pHSFE β G. Clearly, neither of the two constructs direct copy-number dependent expression in MEL cell clones.

We next examined the hypothesis that the percentage of promoters in an open chromatin conformation within each clonal population would correlate with β -globin gene expression for that clone. To assess this, we plotted normalized human β -globin gene expression vs. Bln I accessibility for each clone (Fig. 6C). Linear regression was used to draw best-fit lines for each set of clones. While there is no correlation

between accessibility and expression for p β G clones, there appears to be a trend toward higher expression of β -globin in pHSFE β G clones with higher levels of Bln I accessibility. A similar correlation was recently demonstrated in stably integrated constructs using the chicken β^A -globin promoter and enhancer (35).

Effect of the HSFE on clonal extinction

The final hypothesis we tested was that the HSFE could reduce the rate of silencing of the integrated human β -globin gene over time. This process is thought to involve the formation of an inactive chromatin conformation in the region of the transgene (36,37). To assess the role of the HSFE in preventing clonal extinction, individual clones were maintained in culture for a period of three months. Gene expression in HMBA-treated cells was then analyzed by solution hybridization. Results of these experiments are shown in Fig. 7, where human β -globin expression after three months of culture is shown as a percentage of original expression of each clone (expressed as percentage of mouse α -globin per copy). Only those clones which initially had detectable human β -globin expression are shown. No originally silent clone showed expression after three months in culture. Four of the eight p β G clones were completely silenced after the three-month period and only two of the clones exhibited expression equivalent to original levels. In contrast, none of the clones containing the HSFE showed complete extinction and five of the eight clones maintained expression of at least 70% the original value. On average, β -globin expression in the p β G clones was 36% of their original values, while average expression in the pHSFE β G clones was 73% of their original levels ($p=0.04$).

DISCUSSION

The HSFE was originally identified as the minimal functional element required for

establishing a DNase I hypersensitive region within β -globin LCR HS4 (19). The present studies were based on the hypothesis that positioning the HSFE directly upstream of the β -globin gene would enhance the formation of an active chromatin conformation over the promoter and increase the opportunities for interactions of key regulatory proteins with their respective *cis*-acting DNA sequences. To this end, we have used a combination of chromatin structure and expression analyses to assess the effects of the HSFE. Incorporation of the HSFE into a minimal β -globin gene promoter not only increased the area of the artificial promoter available for DNA-protein interactions, as demonstrated by DNase I digestion, but also increased the proportion of promoters in an open configuration. These changes are likely to be the result of both nucleosome repositioning within the promoter region and a shift in the equilibrium between accessible and inaccessible local chromatin conformations. The latter conclusion is based on the Boyes and Felsenfeld model of β -globin LCR HS formation (30).

Responsiveness of β -globin expression to HMBA induction was significantly increased as, on average, HSFE-containing clones exhibited a three-fold increase in induction. Human β -globin expression per gene copy was also increased as HSFE-containing clones expressed human β -globin at a mean level of nearly 60% the endogenous mouse α -globin per copy while β -globin expression of control clones averaged only 22% of the endogenous mouse α -globin expression per copy. In addition, the HSFE also acts to decrease the likelihood of silencing of stably integrated globin gene constructs.

Chromatin Opening vs. Enhancer Activity

Our experimental model is based on the hypothesis that induction of an "open" chromatin conformation throughout the human β -globin gene promoter would yield increased expression of the linked β -globin gene. A possible alternative

interpretation of these results is that the HSFE possesses intrinsic enhancer-like activity that is separable from its chromatin opening activity and may account for the increased levels of β -globin expression. However, work from Tuan and colleagues has demonstrated that HS4, unlike LCR HS2, exhibits no enhancer activity (38). Other reports which demonstrated minimally increased expression of an HS4-linked globin gene have been performed in stably-integrated, chromatin contexts (39,40). These data support the conclusion that the increase in β -globin expression we have observed is not due to an enhancer-like function, but is the consequence of reorganization of the chromatin structure of the β -globin promoter.

Globin promoter chromatin structure and gene expression

Addition of the HSFE to the β -globin minimal promoter expression constructs resulted in an expansion of the *open* chromatin region so that a larger portion of the promoter was exposed to potential DNA-protein interactions. This included exposure of the direct-repeat elements of the β -globin promoter. These elements were first identified in the mouse β -major globin promoter on the basis of their contribution to transcriptional regulation of a linked heterologous gene (41). The direct repeat elements are conserved among mammalian adult β -globin promoters and contribute to maximal β -globin gene induction during MEL chemical differentiation (26). Deletion of the direct repeat elements in the mouse β -globin promoter resulted in a 2.5-fold decrease in the levels of linked metallothionein reporter gene expression and incorporation of the direct repeat elements into a heterologous promoter resulted in a three-fold increase in expression upon MEL differentiation (26). Inclusion of the HSFE in our constructs resulted in similar three-fold increases in both inducibility and expression from the human β -globin transgene. It is possible that these increases are a

result of the increased accessibility of the direct repeat elements to their associated trans-acting factors.

While the formation of an “open” or nuclease-sensitive chromatin structure in the regions of gene promoters is clearly associated with active gene expression (27,42), our results indicate that this nucleosome disruption or repositioning is not sufficient to yield consistent high-level expression. Clones containing constructs with similar degrees of restriction endonuclease accessibility demonstrated marked differences in normalized gene expression (Fig 5C). Similar results have been reported in a study from Milot, et. al., investigating the effects of β -globin LCR HS deletions in transgenic mice (43). In these experiments, some transgenic lines containing LCR HS deletions exhibited very low levels of globin gene expression. DNase I and restriction enzyme sensitivities of the β -globin genes in some of these lines were found to be equivalent to those of higher expressing lines. These low levels of expression, despite a seemingly active β -globin chromatin conformation, were attributed to a decrease in the rate of globin transcriptional activity in all cells rather than position effect variegation due to heterochromatinization. Similarly, the Felsenfeld laboratory has recently studied the effects of the chicken β -globin insulator on stably integrated constructs containing β -globin regulatory elements (35). Here too, there was variable correlation between restriction enzyme accessibility of the chicken β -globin enhancer and reporter gene expression. Together these findings support the concept that the establishment of a locally open chromatin structure within the regulatory elements of a gene may be necessary, but is not sufficient, for high-level, position independent globin gene expression.

A third point involving chromatin structure and β -globin gene expression relates to the use of MEL cells as a model. These cells appear to be an appropriate model for studying the ability of *cis*-acting sequences to form erythroid-specific local chromatin structures. In previous reports, we and

others have shown that when human LCR sequences are stably transfected into MEL cells in the context of either plasmids (19,44,45) or yeast artificial chromosomes (33) the appropriate DNase I HSs are formed. However, while MEL cells are a unique tissue culture model of adult erythropoiesis and are a standard model for studying the function of globin-expressing constructs including retroviruses, for example, they have been noted to exhibit substantial clonal variation of not only stably integrated human β -globin transgenes, but also of the endogenous murine globin genes (44,46). The inclusion of LCR-derived elements, which confer position-independence in transgenic mice, do not result in position-independent expression of linked globin genes in individual MEL cell clones (44,45,47). These findings indicate that while we have demonstrated the ability of the HSFE to reorganize local chromatin structure, increase average expression and inhibit globin gene silencing, rigorously testing the ability of this element to direct position-independent expression will eventually require a different model system, such as transgenic mice.

Artificial manipulation of chromatin structure

Effective gene replacement therapy for human hemoglobinopathies will require consistent, high-level expression of transferred globin genes. Although combinations of LCR elements are able to confer such expression in transgenic mouse models (3), inclusion of LCR elements within globin-containing retroviral vectors has resulted in low titer producer cell lines, genetic instability, and highly-variable expression levels of globin transgenes (48-51). Despite recent progress in the design of globin-expressing viruses, therapeutically-useful vectors have yet to be produced (52,53). As an alternative to the use of combinations of intact LCR HS cores to enhance β -globin gene expression, we have begun to test the hypothesis that specific LCR functions, such as the opening of promoter chromatin structure, can

be separated from other LCR functions. The use of elements such as the HS4 HSFE to alter chromatin structure offers an alternative strategy in the design of future gene transfer vectors.

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