Gene Ther Mol Biol Vol 8, 475-486, 2004

An erythroid-specific chromatin opening element increases β-globin gene expression from integrated retroviral gene transfer vectors

Research Article

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Key words: chromatin structure, β-globin, retrovirus, DNase I hypersensitive site, locus control region

Abbreviations: Fetal Bovine Serum, (FBS); green fluorescent protein, (GFP); hexamethylene bisacetamide, (HMBA); hypersensitive sites, (HS); locus control region, (LCR); multiple cloning site, (MCS); murine stem cell virus, (MSCV)

Received: 14 November 2004; Accepted: 29 November 2004; electronically published: December 2004

Summary

Gene therapy strategies requiring long-term high-level expression from integrated genes are currently limited by inconsistent levels of expression. This may be observed as variegated, silenced or position-dependent gene expression. Each of these phenomena involve suppressive chromatin structures. We hypothesized that by actively conferring an open chromatin structure on integrated vectors would increase transgene expression. To test this idea we used a 100bp element from the β-globin locus control region (LCR) which is able to independently open local chromatin structure in erythroid tissues. This element includes binding sites for GATA-1, NF-E2, EKLF and Sp-1 and is evolutionarily conserved. We constructed a series of MSCV-based vectors containing the β-globin gene driven by a minimal β-globin promoter with combinations of the HSFE and LCR derived enhancer elements. Pools of MEL clones containing integrated vectors were analyzed for chromatin structure and β-globin gene expression. The HSFE increased the extent of nuclease sensitive chromatin over the promoters of the constructs. The most effective vector included tandem copies of the HSFE and produced a 5-fold increase in expression compared to the promoter alone. These results indicate that the HSFE is able to augment the opening of β-globin promoter chromatin structure and significantly increase gene expression in the context of an integrated retroviral vector.

I. Introduction

Clinical applications of gene therapy that require long-term expression have been limited by an inability to achieve consistent, high-level expression from integrated gene therapy vectors such as retroviruses (Orkin and Motulsky, 1995). These vectors exhibit highly variable or position-dependent expression, which is proposed to be due in part to the formation of highly condensed, suppressive local chromatin structures at sites of integration. In this model, transgene expression can range from high to non-existent depending upon whether integration occurs into a region of transcriptionally active or inactive chromatin structure (Barklis et al, 1986). The wide range in expression is often due to position effect variegation, where local chromatin structure affects the probability that a given cell within a population will express the integrated gene (Karpen, 1994). Viral integration into transcriptionally favorable chromatin structure increases the probability of expression but some cells within a clonal population will still not express the transferred gene. Furthermore, integration could occur initially into a region that is transcriptionally favorable but becomes less permissive over time due to repressive alterations in local chromatin structure. The resultant transcriptional silencing is not due to a gradual decrease in expression of all cells but rather the complete loss of expression in an increasing proportion of cells (Hoeben et al, 1991) Changes in chromatin structure, specifically

increased DNA methylation and histone deacetylation, are often associated with transcriptional silencing (Jahner and Jaenisch, 1985; Hoeben et al, 1991; Challita and Kohn, 1994; Wang et al, 1998; Chen and Townes, 2000).

Chromatin structure can affect the ability of the integrated retroviral vector to achieve therapeutically relevant levels of gene expression. Overcoming this barrier is especially critical since retroviral vectors are the most frequently used vector in clinical and scientific applications where long-term gene expression is desired. Recent generation lenteviral vectors are still subject to these chromatin-related effects (Persons et al, 2003b). While strategies such as drug selection (Persons et al, 2003a) and methods to achieve improved rates of transduction (Imren et al, 2002) have been applied to overcome low levels of expression from retrovirally transduced globin genes, most approaches have focused on combining various fragments of the LCR and testing them to see which ones give optimal expression. Recently genetic insulators and scaffold attachment regions have been used to protect globin genes from the negative effects of surrounding chromatin and enhance expression (Emery et al, 2000,2003; Ramezani et al, 2003). Our approach has been to investigate development of gene transfer vectors that are able to autonomously open and maintain surrounding domains of active chromatin structure regardless of their site of integration within the genome (Iler et al, 1999; Nemeth et al, 2001). In this study, we have examined the strategy of incorporating a relatively small *cis*-acting element which is able to alter local chromatin structure in an erythroid-specific manner within a globin-expressing retroviral vector.

The HSFE is an erythroid-specific chromatin remodeling element derived from the human β-globin LCR. The LCR is comprised of five DNase I hypersensitive sites (HS) located 5 to 25 kb upstream of the β-globin locus and is necessary for high-level expression of the β-globin genes (Tuan et al, 1985; Grosveld et al, 1987; Epner et al, 1998; Reik et al, 1998). Originally, the HSFE was derived as a 101 bp element from the core of HS4 and was found to be both necessary and sufficient for the formation of a DNase I HS typical of the LCR HS core structures (Lowrey et al, 1992). The HSFE contains binding sites for the erythroid-specific factors NF-E2, GATA-1, and EKLF and the ubiquitous factor Sp-1, all of which are necessary to establish a hypersensitive chromatin domain (Pruzina et al, 1991; Lowrey et al, 1992; Stamatoyannopoulos et al, 1995; Goodwin et al, 2001). Similar clusters of binding sites are found within the other erythroid-specific LCR HS cores where they are also required for HS formation and are evolutionarily conserved (Philipsen et al, 1990; Talbot et al, 1990; Philipsen et al, 1993; Hardison et al, 1997; Pomerantz et al, 1998). Previously, we have demonstrated that the HSFE can mediate functional tissue-specific "opening" of a minimal human β-globin promoter and increase expression of a linked human β-globin gene in both MEL cell clones and in transgenic mice (Iler et al, 1999; Nemeth et al, 2001). We hypothesized that incorporation of the HSFE into a β-globin retroviral vector would result in a similar remodeling of human β-globin promoter chromatin structure and a subsequent increase in expression.

II. Materials and methods

A. β-globin retroviral vectors

All retroviral β-globin constructs were generated using a parent murine stem cell virus (MSCV) vector (Hawley et al, 1994). A 1.3 kb EcoR I-Hind III fragment was removed and replaced with a multiple cloning site (MCS) that contained 5' - EcoR I/Sal I/Xho I/Hind III-3'. A 1.3 kb Xho I-Sal I fragment containing an IRES-GFP sequence was inserted into the Sal I site of MSCV-MCS to create MSCV-GFP.

To construct rβG, a human β-globin gene vector (p141) containing a 372 bp deletion within the second intern was provided by Dr. Phillipe Leboulch (Harvard University, Boston, MA). A BamH I-Eco R I fragment from p141 containing the intern deletion (Genbank #U01317.1; bp 62718-63092) was then subcloned into a wild-type human β-globin sequence between the BamH I and EcoR I sites. The modified human β-globin gene and minimal 110 bp human b-globin promoter was then inserted into MSCV-GFP as an intact Xho I – Sal I fragment in an anti-sense orientation with regards to viral transcription.

To construct rHβG, a 190 bp PCR fragment containing the HSFE was synthesized (Genbank #U01317.1; bp 1060-1222) and inserted into the Xho I site of rβG. This fragment also serves as the 5' HSFE in the r2HbG construct. Both rEHβG and r2HβG were constructed by inserting Xho I-Bgl II fragments excised from the previously described pEHβG and p2HβG constructs that contain the *cis*-acting elements as well as 10 bp of the minimal promoter into the corresponding Xho I and Bgl II sites located within the minimal β-globin promoter in rβG (Nemeth et al, 2001). rHEβG was constructed by inserting a 220 bp Xho I-Bgl II fragment containing the 36 bp enhancer sequence upstream of the HSFE into the Xho I and Bgl II sites of rβG. rE'HβG was constructed by inserting a 385 bp PCR fragment from HS2 (Genbank #U01317.1; nucleotides 8480-8865) into rHβG at the Xho I site upstream of the HSFE.

B. Retroviral transduction

Briefly, 3 μg of each retroviral construct was transiently co-transfected along with 3 μg pVPack-GP vector and 3 μg pVPack-VSV-G vector (Stratagene, La Jolla, CA) into 2 x 10^6 293T cells by $CaPO_4$ transfection using the CellPhect transfection kit (Amersham Biosciences, Piscataway, NJ). The 293T cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen Gibco, Carlsbad, CA). Cells were incubated under cell culture conditions with the DNA precipitate for 8 hours. Media was then removed and the cells treated with 15% glycerol in isotonic HEPES (pH 7.5) for 3 minutes. The glycerol/HEPES solution was then removed and the cells washed once with media before being replenished with media and returned to culture conditions. Twenty-four hours later, the media was removed from the 293T cells and replaced with pre-warmed media and collection of viral particles begun. Media containing viral particles was collected 48 hours later and added to 1 x 10^5 MEL cells. Pre-warmed media was then added to the packaging cells, collected 24 hours later, and added to the MEL cells. Viral transduction was facilitated through the addition of 6 ng/ml hexamethedrine bromide (Polybrene®; Sigma, St. Louis, MO) to MEL cells co-cultured with viral supernatant. MEL cells were then cultured for 48 hours before FACS analysis.

Transduced MEL cells were collected and assayed for GFP expression. Approximately 1.5 x 10^4 GFP^+ cells per experimental vector were sorted in 1 ml FBS. The cells were then centrifuged and resuspended in 10 ml Improved MEM Zinc Option media

(Invitrogen Gibco) supplemented with 10% FBS and maintained at 37°C. To determine intact transfer of the β-globin and associated LCR sequence to the MEL cell, 10 μg of genomic DNA from each pool was digested with Xho I and Sal I. Digestion products were detected by Southern blotting as described. The bamboo-EcoR I region of the β-globin gene was used as the probe. To determine the multiplicity of infection, 10 μg genomic DNA from pools containing the rβG and rHβG vectors were digested with EcoR I. Digestion products were detected by Southern blotting using the same β-globin probe. Copy number for each pool was determined by slot-blot analysis.

C. Nuclease sensitivity assays

DNase I hypersensitivity assays were performed on nuclei isolated from transduced pools as previously described (Iler et al, 1999). Pools were maintained in culture conditions until they reached log phase growth (8 x 10^5 - 1 x 10^6 cells/ml). Nuclei corresponding to approximately 200 μg per reaction were incubated with DNase I (Worthington Biochemical, Lakewood, NJ) at concentrations ranging from 0 to 4.0 mg/ml DNase I for 10 minutes at 37°C. Regions of DNase I hypersensitivity were mapped by plotting the migration distance of the molecular weight markers versus the logarithm of their size in base pairs for each blot. These data points were then fitted to the equation:

$$\text{fragment size (bp)} = m\left(\text{migration distance in cm}\right) + i$$

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.

Restriction endonuclease sensitivity assays using Bln I were performed on intact nuclei as described (Iler et al, 1999). For initial experiments, nuclei (200 μg DNA/reaction) were digested with Bln I at amounts of 0, 10, 20, 40, 80 and 160 units at 37°C for 20 minutes. In subsequent experiments Bln I amounts of 0 and 100 units were used because complete cutting was consistently obtained above 80 units per reaction. Relative band intensities were determined by densitometry performed on images captured on a Phosphor Screen and resolved with the PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA). The percentage of restriction enzyme digestion was determined by dividing the intensity of the sub-band by the sum of the intensities of the sub-band and the parental band (S/(P+S)). Statistical analysis was performed using Student's t-test.

D. Human β-globin RNA analysis

For all pools, globin expression was induced by 3mM HMBA for 4 days. RNA was isolated with Trizol (Invitrogen) Human β-globin and mouse α-globin expression were quantified using ribonuclease protection analysis using the RPA III kit (Ambion, Austin, TX). RNA probes were synthesized using the T7 MaxiScript kit (Ambion). pT7Mα and pT7β^M were used to generate probes for mouse α-globin and human β-globin respectively and were a kind gift from Dr. Qiliang Li (University of Washington, Seattle, WA). pT7Mα protects a 128 bp fragment and pT7β^M protects a 206 bp fragment. Each hybridization reaction consisted of approximately 1 μg of RNA and 1 x 10^6 cpm of both probes (the specific activity of each probe generally ranged from 1-2 x 10^6 cpm/ng). Hybridization products were electrophoresed on an 8.0% acrylamide/6 M urea gel and relative expression levels were quantified by PhosphorImager analysis. Human β-globin expression was corrected for both copy number and the different specific activities of the probes and normalized to mouse α-globin. Statistical analysis was performed using Student's t-test.

III. Results

We subcloned the HSFE element upstream of a minimal human β-globin promoter and gene in the context of a MSCV vector (**Figure 1a**). This vector also contains the enhanced green fluorescent protein (GFP) gene, which is transcribed from the viral 5' LTR. In order to prevent removal of the β-globin introns, which are necessary for high-level expression, the LCR, promoter, and gene sequences are oriented in an antisense direction with respect to viral transcription (Karlsson et al, 1987). A 372 bp region from the second intern of the β-globin gene was also removed, which has been shown that this deletion improves both viral titer and the genetic stability of the vector without adversely affecting β-globin gene expression (Leboulch et al, 1994). Altogether, six β-globin vectors were made (**Figure 1b**). Briefly, rβG contains the 110 bp minimal human β-globin promoter alone, rHβG contains the HSFE upstream of the promoter, rEHβG contains a 36 bp erythroid-specific enhancer derived from HS2 located at the 5' end of the HSFE (Chang et al, 1992), r2HβG contains tandem HSFE elements separated by approximately 150 bp, rHEβG contains the 36 bp enhancer and the HSFE in reverse order, and rE'HβG contains a 374 bp fragment from HS2 (which contains the 36 bp enhancer) upstream of the HSFE.

These constructs were transduced into MEL cells using a transient VSV-G packaging cell line. FACS analysis was then used to select GFP^+ MEL cells (**Figure 2**). After transduction of MEL cells with either rβG or rHβG, approximately 1-3% of MEL cells were positive for GFP expression. Similar percentages were observed for the other constructs (data not shown). GFP^+ cells were sorted and separated into three to four pools per construct.

To ensure the intact transfer of the human β-globin gene and any associated regulatory elements, genomic DNA was isolated from the transduced pools and analyzed for copy number and the integrity of the β-globin gene sequence (**Figure 3a**). The β-globin gene, promoter, and any associated LCR elements were integrated intact into the MEL genome for all constructs except rE'HβG. This vector, which contains a 374 bp HS2 enhancer, was genetically rearranged as indicated by loss of the β-globin gene.

To determine whether our retroviral pools contained multiple integration sites, genomic DNA was digested with EcoR I, which cuts the human β-globin gene at a single site in all vectors. A representative analysis is shown in **Figure 3b** for the rβG and rHβG vectors. For both constructs, each pool contained multiple integration sites, as indicated by " smearing" of the Southern blot signal over a wide range.

A. Chromatin structure of the integrated human β-globin promoter

To determine the extent of the hypersensitive domain in our retroviral constructs, we performed DNase I sensitivity assays on the retroviral pools and mapped the size and location of detected HSs (**Figure 4**). Representative Southern blot analyses depicting formation

of the hypersensitive sites are shown in **Figure 4b**. The integrated rβG vector, which contains the minimal promoter by itself, contained a hypersensitive site approximately 110 bp long and included the first 20 bp of the promoter itself (**Figure 4c**). The addition of the HSFE approximately doubled the region of hypersensitive chromatin from 110 bp to 230 bp. In both the presence and absence of the HSFE, only approximately 20bp of the distal promoter was hypersensitive. However, while the incorporation of tandem HSFE elements created a similarly sized 190 bp HS, this HS was shifted to include most of the minimal β-globin promoter.

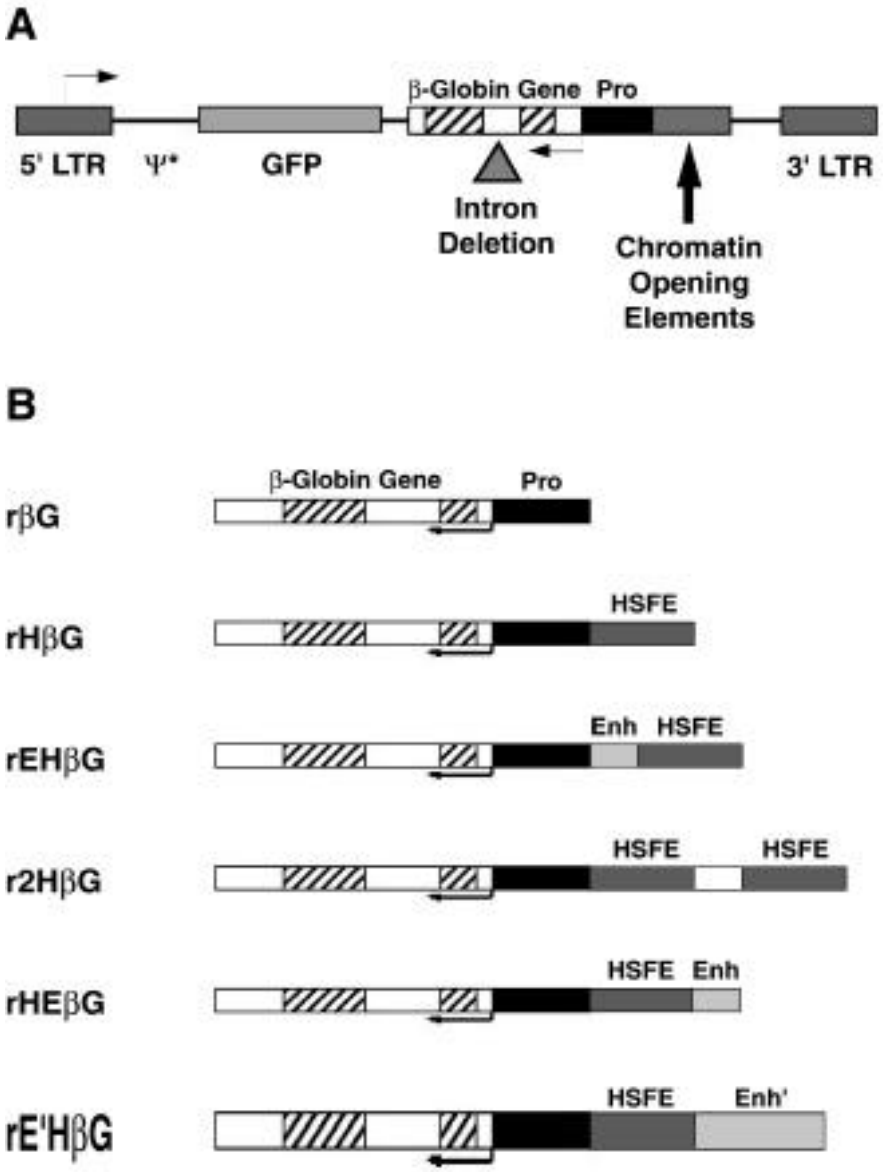

Figure 1. Retroviral β-globin vectors used to evaluate HSFE activity. (**a**) Design of the β-globin retroviral vector. The parent vector is the murine stem cell retrovirus (MSCV). The vector contains a GFP gene transcribed from the 5' LTR and the human β-globin gene transcribed from the minimal human β-globin promoter in an anti-sense orientation. A 372 bp region of the second β-globin intron has been deleted. The chromatin opening elements are subcloned 3' of the promoter in an anti-sense orientation. (**b**) β-globin retroviral vectors. Construction of vectors is described in Methods. Elements used to construct the vectors include the 110 bp minimal human β-globin promoter (Pro), HSFE, the 36 bp erythroid-specific HS2 enhancer (Enh), and a 385 bp fragment from HS2, containing which the 36 bp enhancer (Enh').

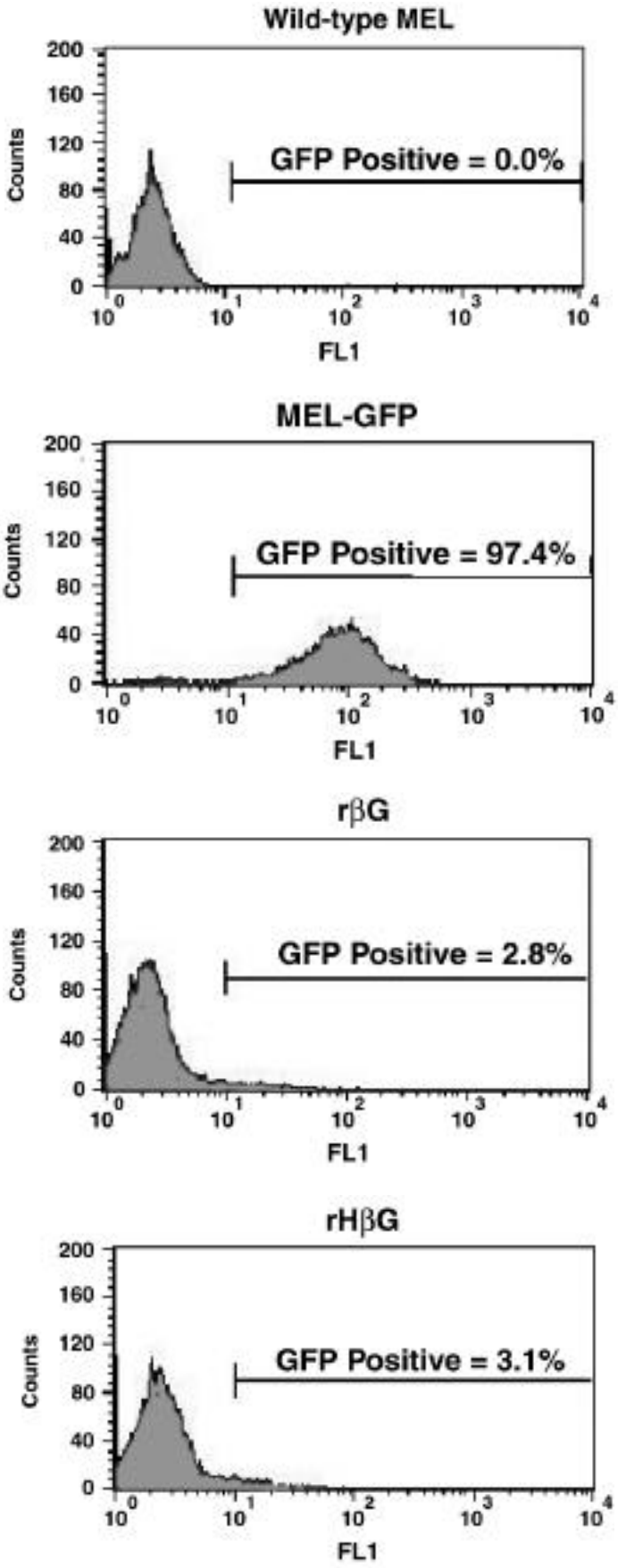

Figure 2. Generation of β-globin retroviral vector pools. GFP-FACS analysis of transduced MEL cells. Representative histograms displaying the percentage of GFP expressing MEL cells after transduction with rβG and rHβG vectors. Wild-type MEL cells and a MEL clone that expresses GFP served as negative and positive controls respectively.

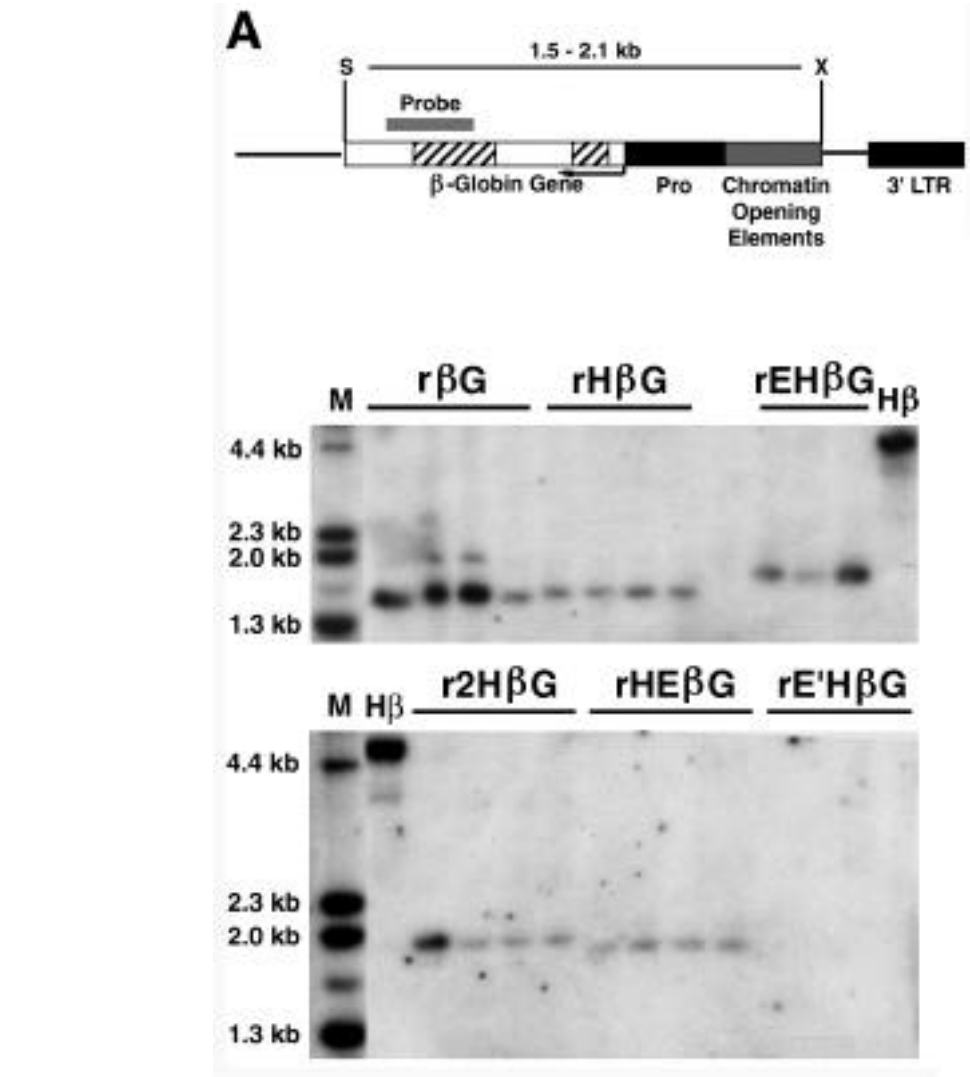

Figure 3. Analysis of integrated β-globin vector DNA. (**a**) Top: Vector schematic displaying Xho I (X) and Sal I (S) restriction sites and the range of sizes of the digestion products. Bottom: Integrity of retroviral vectors. Southern blot displaying intact β-globin sequences for 5 out of 6 vectors. Human bone marrow DNA (Hβ) was digested with Pst I/Bgl II as a positive control. Genomic DNA from each pool was isolated and digested with Xho I/Sal I. (**b**) Multiple integration sites of retroviral pools. Genomic DNA isolated from rβG and rHβG pools was digested with Eco RI which cuts once within the vector.

To quantify the proportion of β-globin promoters accessible to restriction endonuclease digestion, and therefore in an open chromatin configuration, we performed restriction endonuclease assays on all pools generated from selected constructs. Intact nuclei were performed restriction endonuclease assays on all pools generated from selected constructs. Intact nuclei were digested with Bln I, which uniquely digests at a single site within the β-globin promoter (**Figure 5a**).

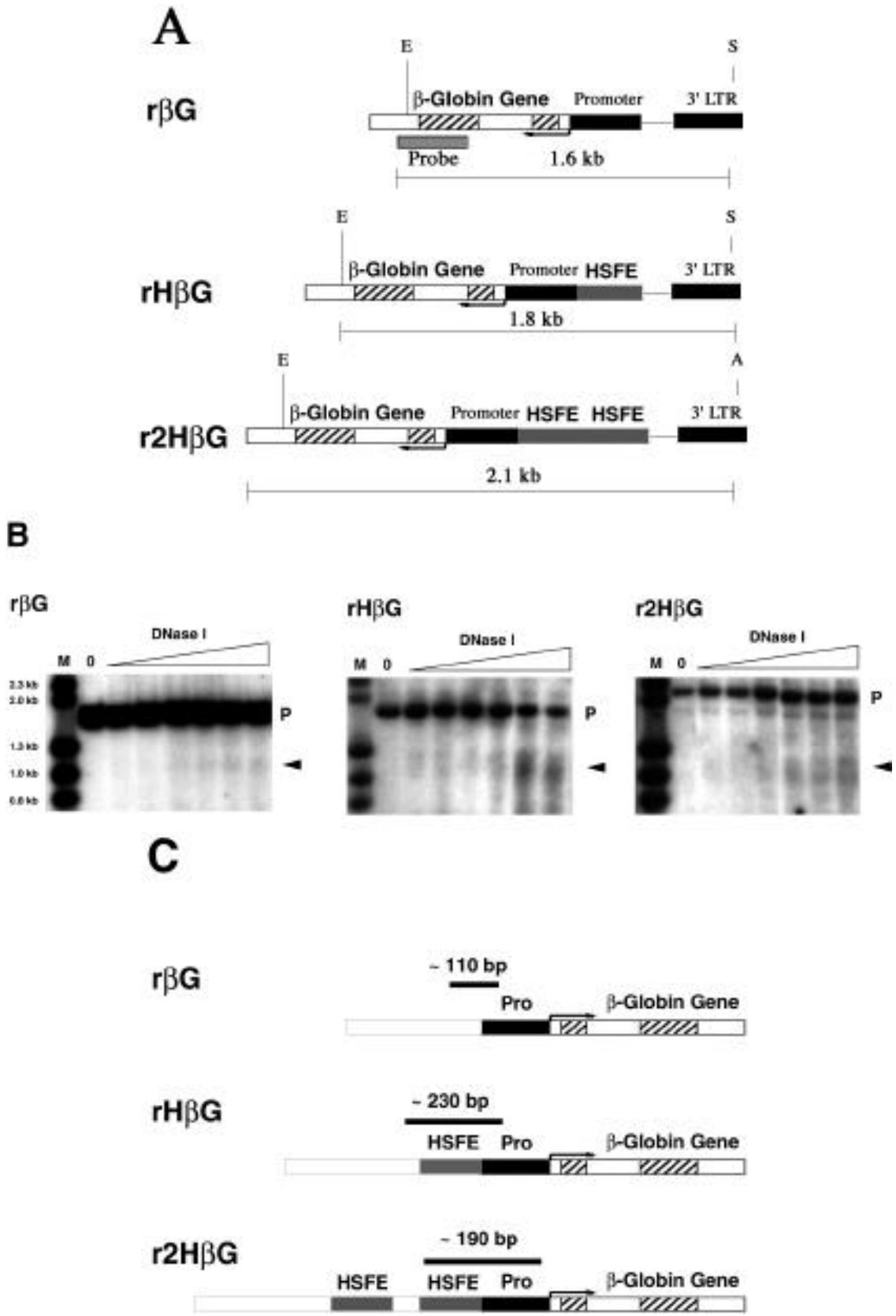

Figure 4. Effect of the HSFE on retroviral β-globin promoter DNase I sensitivity. (**a**) rβG, rHβG, and r2HβG construct maps showing EcoR I (E), Asc I (A), and Sst I (S), and restriction sites, location of Southern blot probe, and size of parental band. (**b**) DNase I assays of rβG, rHβG, and r2HβG pools. Intact nuclei were incubated with increasing concentrations of DNase I. Genomic DNA was digested with the appropriate restriction enzymes. Parental bands are indicated by P, DNase I hypersensitive sites by arrows. (**c**) Locations of the DNase I HSs for rβG, rHβG, and r2HβG. An approximately 110 bp HS maps over 20% of the promoter of integrated rβG constructs. In the rHβG pool, the HS is approximately 230 bp in size and maps to the HSFE and the first 20 bp of the promoter. The HS in the r2HβG pool is approximately 190 bp in size and maps to the promoter and 3' HSFE.

The Bln I concentration at which maximum promoter digestion was achieved was in excess of 80 units per reaction (data not shown). Pools were digested with 100 units of Bln I and representative Southern blot analyses are shown in **Figure 5b**. In pools containing the rβG vector, 45% of the promoters were digested by Bln I (**Figure 5c**). When a single HSFE or the enhancer plus an HSFE were added, the proportion of accessible promoters increased by 5%. Tandem copies of the HSFE were able to increase the percentage of open promoters to 56% ($p < .01$).

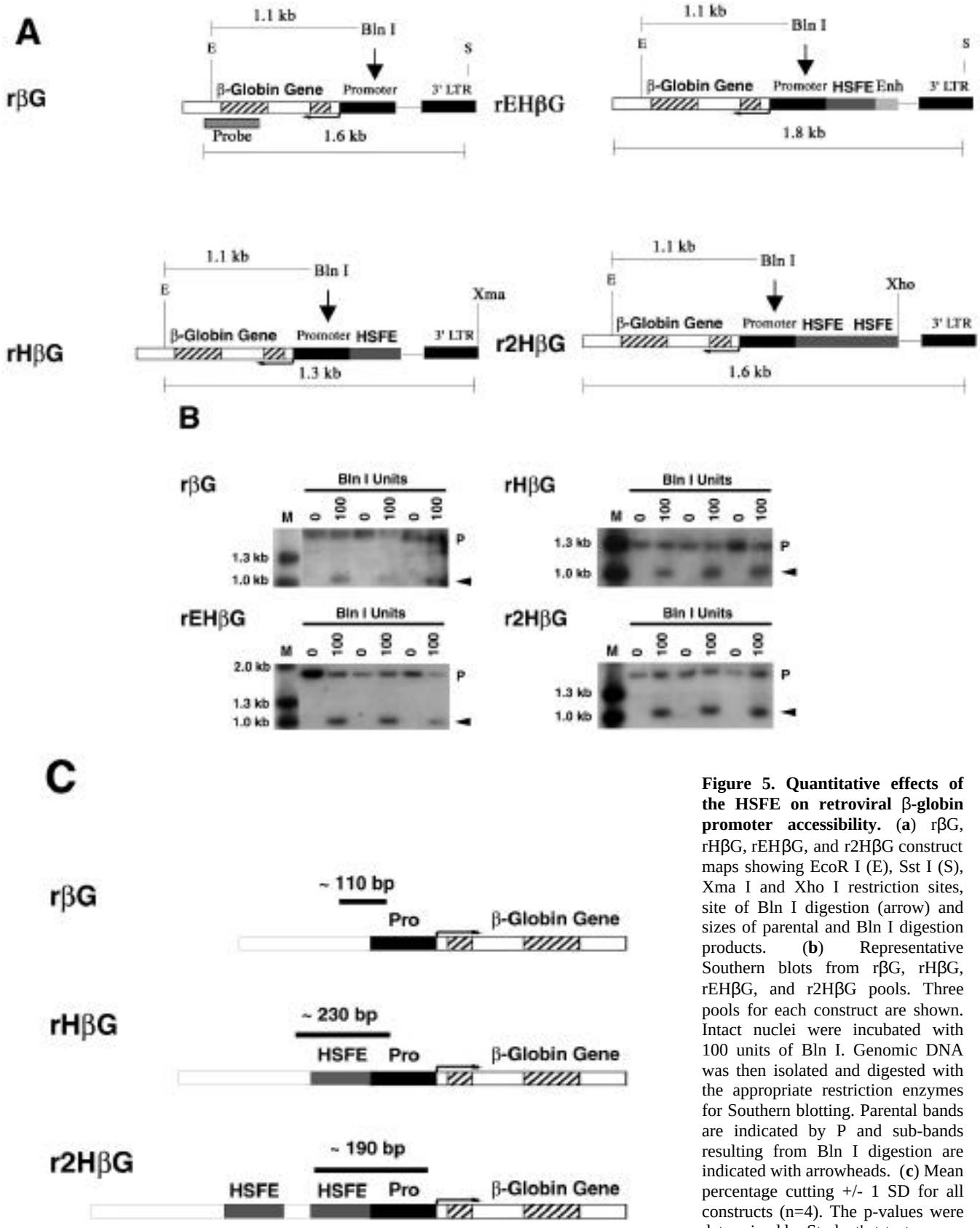

Figure 5. Quantitative effects of the HSFE on retroviral β-globin promoter accessibility. (**a**) rβG, rHβG, rEHβG, and r2HβG construct maps showing EcoR I (E), Sst I (S), Xma I and Xho I restriction sites, site of Bln I digestion (arrow) and sizes of parental and Bln I digestion products. (**b**) Representative Southern blots from rβG, rHβG, rEHβG, and r2HβG pools. Three pools for each construct are shown. Intact nuclei were incubated with 100 units of Bln I. Genomic DNA was then isolated and digested with the appropriate restriction enzymes for Southern blotting. Parental bands are indicated by P and sub-bands resulting from Bln I digestion are indicated with arrowheads. (**c**) Mean percentage cutting +/- 1 SD for all constructs (n=4). The p-values were determined by Student's t-test.

B. The effect of the HSFE on human β-globin gene expression

To address the effects of the HSFE on gene expression, we chemically induced globin gene expression with hexamethylene bisacetamide (HMBA) and performed ribonuclease protection assays on the isolated RNA (**Figure 6a**). Human β-globin expression was normalized to mouse α-globin expression and corrected for both the average copy number of the pool and the different specific activities of the probes. In pools containing the rβG vector, human β-globin expression was 2.6% +/- 0.8% of mouse β-globin (**Figure 6b**). Upon incorporation of the HSFE, β-globin expression increased to 9.6%, a significant increase of nearly 4-fold ($p < .01$) and incorporation of tandem copies of the HSFE resulted in a 5-fold increase in β-globin expression ($p < .001$). With the addition of the 36 bp HS2 enhancer 5' to the HSFE, human β-globin expression also increased 4-fold compared to the promoter alone ($p = .01$). When the positions of the HSFE and enhancer were exchanged, β-globin expression was increased only 3-fold ($p = .01$). There was no observable difference between placing the enhancer element 5' to the HSFE compared to 3'.

IV. Discussion

We have demonstrated that the HSFE is able to form its characteristic structure in the context of a retroviral vector and that tandem HSFEs increased the extent of DNase I accessible promoter chromatin structure. Furthermore, the HSFE, when present as single or tandem copies, is able to increase retroviral β-globin expression up to 5-fold compared to the promoter alone. These results indicate a tissue-specific chromatin-opening element such as the HSFE is able to significantly increase gene expression in the context of a retroviral vector.

Additionally, an advantage of using the 101 bp HSFE with a retroviral vector is that the probability of genetic rearrangement and other technical barriers associated with the use of larger LCR fragments vectors is reduced

By itself, the integrated human β-globin promoter can form a weak hypersensitive site. The formation of this site is consistent with previous reports describing the formation of a weak hypersensitive site by the globin promoters alone (Tuan et al, 1985; Forrester et al, 1986; Dhar et al, 1990; Iler et al, 1999). The inclusion of the HSFE doubled the region of hypersensitive chromatin in the neighborhood of the β-globin promoter to approximately 230 bp. However, the larger HS is almost entirely localized to the HSFE sequence, encompassing approximately 20% of the β-globin promoter. Although the majority of promoter region is not hypersensitive for either construct, the two critical CACCC boxes, which bind EKLF, do reside within the HS (Miller and Bieker, 1993). However, when tandem copies of the HSFE were used, the detected HS mapped to a region that included the entire minimal promoter. This HS is formed by the 3' HSFE. We observed a similar localization of the 3' HS when we stably transfected the tandem HSFE cassette into MEL cells. We were unable to observe the HS formed by the proximal HSFE, although in earlier studies we have shown that both HSFE elements can establish distinct HSs. Overall, the structural characteristics of the HSFE are still intact in the context of a retroviral vector.

The incorporation of the HSFE did not increase the percentage of open promoters. This was a somewhat surprising result, as we had previously observed that the addition of the HSFE resulted in a 20% increase in the

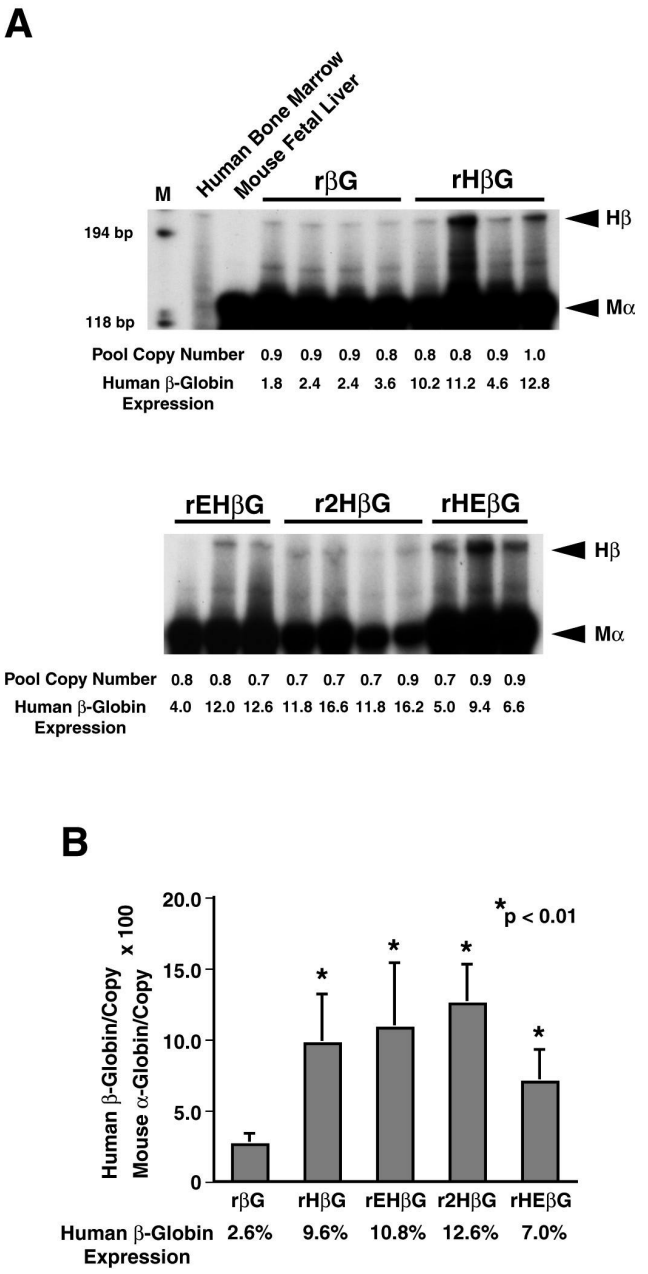

Figure 6. Effect of the HSFE on retroviral β-globin expression. (**a**) Representative ribonuclease protection assays for each set of pools for all constructs. Human bone marrow and mouse fetal liver controls are indicated. Experimental samples are underneath the black bar. Protected human β-globin (Hβ) and mouse α-globin (Mα) mRNAs are indicated by arrows. Copy numbers for each pool is shown in the top row of numbers beneath each assay. Human β-globin gene expression for each pool is shown in the second row of numbers. Expression was quantified using densitometry. (**b**) Mean human b-globin expression of each construct (n = 4). P-values were determined by t-test.

percentage of open promoters (Iler et al, 1999). However, tandem HSFEs (r2HβG) were able to significantly increase the number of accessible promoters by 10%. The question remains whether such an increase is physiologically meaningful. Thus, it appears that our elements have the capability to increase the size of the region of hypersensitive chromatin but not the proportion of promoters in an open configuration.

Even though inclusion of the HSFE did not cause formation of hypersensitive chromatin along the entire promoter, its presence resulted in a significant four-fold increase in human β-globin expression compared to the promoter alone. This increase was comparable to that observed when we stably transfected the HSFE into MEL cells (Nemeth et al, 2001). Novak et al, demonstrated a similar 6-fold increase in clones containing a β-globin retroviral vector incorporating the entire HS4 (Novak et al, 1990). Overall, we observed significant increases in gene expression with all combinations tested. Combining the HS2 enhancer element with the HSFE did not increase gene expression compared to a single HSFE. Since the 36 bp enhancer has been shown to double expression in a β-globin retroviral vector and the HSFE alone leads to a 4-fold increase, the addition of the enhancer may be redundant as the HSFE has already augmented expression in all the permissive cells in the pool population (Liu et al, 1992).

The mechanism by which the HSFE augments gene expression is still not clear. Our original hypothesis was that the HSFE would increase the opportunity for critical transcription factors to interact with the minimal β-globin promoter resulting in increased transcription regardless of the chromatin structure in which the vector was integrated. However, our results, combined with other studies, indicate that expression levels do not always correlate with chromatin accessibility (Milot et al, 1996; Pikaart et al, 1998; Nemeth et al, 2001).

A simple model of increased transcription factor accessibility does not explain the increased expression observed with the HSFE. HS4, where the HSFE was first mapped, has been shown to contain no classical enhancer activity when studied in transient assays (Tuan et al, 1989). The HSFE may be inducing more subtle changes in chromatin structure such as alterations in promoter nucleosome acetylation or methylation patterns by bringing important factors in these processes in proximity to the promoter. For example, NF-E2, which binds to the HSFE, has been shown to play a role in histone hyperacetylation (Kiekhaefer et al, 2002). HSFE-bound proteins may also recruit factors, such as CBP and p300, which have endogenous histone acetyltransferase activity and have been implicated in hematopoietic transcription (reviewed in Blobel et al, 2000).

In order to meet the minimum level of in vivo expression (roughly 15 to 20% of endogenous globin expression) that could be therapeutically beneficial, *cis*-elements in addition to the HSFE will have to be considered. One candidate is the 1.2 kb fragment from HS4 of the chicken β-globin LCR that has been shown to act as a chromatin insulator in several *in vitro* and *in vivo* systems (Chung et al, 1993; Pikaart et al, 1998). In retroviral vectors, it has been shown that the insulator increases gene expression by increasing the probability of transcription (Rivella et al, 1998; Emery et al, 2000). Another example is the inclusion of scaffold attachment regions in retroviral vectors to achieve increased expression (Murray et al, 2000). Potentially, the use of different chromatin remodeling elements to achieve specific molecular effects will be a useful strategy in the development of vectors capable of long-term, high-level therapeutic gene expression.

Acknowledgments

The authors wish to thank Drs. Brian Sorrentino, Elio Vanin, Steve Fiering, Phillipe Leboulch and Jane McInerney for reagents and helpful discussion. This research was supported by grants HL52243 and HL73442 (CHL). MN was the recipient of Ryan Foundation and Rosalind Borison Memorial Pre-Doctoral Fellowships.

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