Title: Locus control region elements HS1 and HS4 enhance the therapeutic efficacy of globin gene transfer in β-thalassemic mice


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Abstract

Globin gene transfer in autologous hematopoietic stem cells is a promising therapeutic option for subjects with β-thalassemia major. In this approach, high level, erythroid-specific globin transgene expression should correct ineffective erythropoiesis and hemolytic anemia following the delivery of only 1-to-2 vector copies per cell. The generation of vectors that provide high-level globin expression and require low vector copy (VC) integration is therefore essential for both safety and efficacy. We show here the major roles played by two lesser-known locus control region elements, termed HS1 and HS4. Partial deletions within HS4 markedly reduce in vivo globin expression requiring multiple VC per cell to correct the anemia. Most strikingly, addition of HS1 to HS2-3-4 increases globin expression by 52%, yielding 9g Hb/VC in β-thalassemic mice. Thus, while vectors encoding HS2-3-4 provide curative levels of hemoglobin at 1-to-2 copies per cell, adding HS1 is a promising alternative strategy if upcoming clinical trials prove higher levels of expression to be necessary.
Introduction

The β-thalassemias are inherited autosomal recessive anemias caused by mutations that diminish or abolish expression of the β-globin gene. The only current means to cure the disease is allogeneic bone marrow transplantation (BMT). In the absence of a histocompatible donor, however, the genetic correction of autologous hematopoietic stem cells (HSCs) represents a highly attractive alternative treatment. Achieving therapeutic expression of the human β-globin transgene in hematopoietic chimeras has long posed major challenges in terms of transgene regulation, vector stability and transduction efficiency (reviewed in refs 5-7). A careful selection of proximal and distal β-globin transcription control elements and the use of a recombinant HIV-1 genome to generate a stable vector eventually enabled May et al. to cure β-thalassemia in mice. Their vector, termed TNS9, and all subsequent therapeutic globin vectors published to date, express the globin transgene under the control of the β-globin promoter and the HS2, 3 and 4 LCR elements. The HS2 and HS3 elements are the most powerful single elements within the LCR (reviewed in refs 16). The relative importance of HS1 and HS4 is less well defined.

In order to select a vector for clinical evaluation in patients with β-thalassemia, we have therefore undertaken a quantitative analysis of the contribution of the β-globin HS1 and HS4 elements to the specificity, inducibility, long-term in vivo expression and therapeutic potential of globin lentiviral vectors. It is indeed central to the success of safe stem cell engineering that therapeutic transgene expression be achieved with a low (ideally 1-2) vector copy number per cell. We show here that HS1 and HS4 are major contributors to achieving therapeutic globin expression in β-thalassemic mice.

Materials and Methods

All vectors used in this study were derived from the previously described TNS9. Detailed description of vector construction and production is available as Supplemental Materials. Vector copy number quantification was performed by Southern blot as previously described and by TaqMan analysis (primers and probes appear in Supplementary Materials and Methods). MEL cell transduction and differentiation were done as previously described. For β-globin transgene analyses, total RNA was extracted from MEL cells or total mouse peripheral blood (PB) using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative primer extension assays were performed as described previously. Hbbth3/+ bone marrow (BM) chimeras were generated as described. BM cells were prestimulated for 12hrs, transduced at 1x10⁶ cells/ml/well at MOI 20-35 for 8hrs and injected into lethally irradiated recipients (5x10⁵-1x10⁶ cells per recipient). A detailed description of the transduction procedure appears in Supplementary Materials and Methods. At several time points after bone marrow transplantation PB was collected and hemoglobin levels were measured on a Coulter A³T diff. instrument (Beckman Coulter, Brea, CA). Red cell lysates of freshly collected PB were analyzed by cellulose acetate electrophoresis (pH 8.5, Helena Laboratories) and quantified as described using ImageJ 1.38x software, http://rsb.info.nih.gov/ij/. Statistical analysis was done by the Student’s t-test using SigmaStat 2.03.0 software (Softek Inc.).
Results and Discussion

In order to evaluate the contribution of the 5′HS1 LCR element to lentivirus-encoded human β-globin expression, we created vectors encoding either HS2-3-4 or HS1-2-3-4 (Figure 1Ai). In MEL cells, addition of 5′HS1 had no effect on average transgene expression per vector copy in one vector (T9 vs. T10, p=0.669, Figure 1Aii), and even significantly decreased the average transgene expression per vector copy in another (S9 vs. S10, p<0.001). In stark contrast, addition of the 5′HS1 element significantly improved the vectors’ performance in vivo (S9 vs. S10 and T9 vs. T10, Figure 1C). Globin transgene expression, at the mRNA level and per vector copy, increased from 27±6% (S9 and T9) of endogenous β-globin mRNA to 41±9% (S10 and T10, p<0.001). The average copy number ranged from 0.42 for T10 to 0.71 for S9 and remained stable throughout the experiment (Figure 1B). The mRNA data were confirmed on the protein level. All animals (n=73) showed sustained amelioration of their anemia, without decrease over the period of observation. The T9 and S9 vectors conferred similar levels of chimeric hemoglobin (cHb, mα₂:hβ₂), on the order of 44-54% per vector copy and normalized to endogenous murine Hb, while vectors containing 5′HS1 element (S10 and T10) generated even higher levels of cHb (Figure 1D).

We next examined the contribution of the flanking regions of the HS4 element, which is a staple of TNS98 and other therapeutic globin vectors.9-11,13-15 The T12 vector, which harbors a truncation of the 5′ flanking region of HS4 (Figure 2Ai), showed significantly decreased globin expression both in vivo and in vitro in comparison to T9 (Figure 2Aii and 2C). The average vector copy number of vectors was stable throughout the course of the study (0.41 to 1.24) (Figure 2B). To further characterize the role of 5′ HS4, the 5′ flanking region of HS4 was replaced with different elements shown in Figure 2Ai. Only replacement of 5′ HS4 with the human IFN-β S/MAR20 sequence in forward orientation restored average expression to the level obtained with the T9PD vector in MEL cells (Figure 2Aii), showing that HS flanking regions play an important role in LCR function and do not function solely as spacers between HS cores.

Mice treated with vectors containing the 5′HS1 (T10, n=19 and S10, n=17) exhibited the highest Hb levels, 12.9±0.9g/dL and 12.7±1.1g/dL, respectively (Figure 2D). When normalized to vector copy, those vectors provide 9.5g/dL and 8.8g/dL of Hb per vector copy (Figure S1B). The total hemoglobin levels in mice treated with T9 (n=19) and S9 (n=18) were very similar (11.9±0.8 and 11.7±0.6 g/dL, respectively), and significantly lower than for T10 (n=19) and S10 (n=17) (p=0.002, Figure 2D). When normalized to vector copy, the T9 and S9 vectors provided about 6.4g/dL and 4.2g/dL of cHb per vector copy, respectively (Figure S1B). Addition of HS1 thus nearly doubled the hemoglobin output of S9. On the other hand, the T12 vector performed the least well, as mice treated with this vector exhibited significantly lower Hb levels (10±0.5; p≤0.001, compared to T9, Figure 2D), which translated into 0.9g cHb/dL per vector (Figure S1B). Protein expression was directly proportional to β-globin mRNA transcript levels for all vectors as shown in Figure S1A (R² = 0.98). Figure 2E and Figure S1B summarize the net hemoglobin increase in relation to vector copy number. These results indicate that 1-2 vector copies per cell achieve major therapeutic responses with vectors T9, S9, S10 and
T10. In contrast, 6 copies per cell of the T12 vector would be required to treat anemia to the same extent as with 1 copy of T9.

Our data clearly demonstrate that HS1 significantly improves LCR function in vivo (p<0.001) (Figure 1C and Figure S1B). This fits with results of Pasceri et al., who found that HS1 enhanced LCR-dependent globin expression in non-viral constructs tested in transgenic mice. These findings were not predicted by MEL cell studies (Figure 1A and ref 21), reinforcing the utmost importance of using in vivo disease models to assess complex regulatory elements and the design of therapeutic vectors.

Our studies also support an important role for the intact HS4 element. Truncation of 5' HS4 indeed significantly decreased β-globin transgene expression at the mRNA (Figure 2C) and protein levels (Figure 2D, E and Figure S1B). Intriguingly, addition of the human IFN-βS/MAR sequence in forward orientation (T12SAR2PD) restored LCR function to the level found in the parental T9 vector. This observation suggests that a functional S/MAR element may be present in the 5'flanking region of HS4, as previously suggested by Cunningham et al. Alternatively, the IFN-β S/MAR may rescue expression by anchoring proviral sequences to nuclear matrix and positioning them in transcriptional “hotspots.”

We therefore conclude that HS1 and HS4 are important contributors to achieving therapeutic expression from globin vectors, which directly affects the vector copy number that is required to treat the anemia. Indeed, more than 5 copies per cell are required when using the T12 vector, whereas 1 or 2 copies per cell are sufficient with T9, S9, S10 and T10. This dosage requirement is likely to bear on the safety of HSC transduction, which is thought to be increased by diminishing the vector copy number in single cells. The use tissue-restricted vectors, which should greatly diminish the probability of trans-activating neighboring oncogenes in HSC, progenitor cells and lymphocytes, is expected to further increase the safety of globin gene transfer.

Based on previous findings and those reported here, we are preparing a phase I clinical trial to investigate the safety and tolerability of globin gene transfer in subjects with β-thalassemia major who lack a matched donor. In this study, which was recently reviewed by the Recombinant DNA Advisory Committee, autologous CD34+ cells obtained after G-CSF mobilization will be transduced with the TNS9.3 vector and infused following non-myeloablative conditioning. Should higher levels of globin expression be eventually needed, the S10 and T10 vectors described here will prove to be very useful.

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LL designed and executed experiments, and co-wrote the manuscript;
MS designed experiments and co-wrote the manuscript.

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References:

Figure Legends

Figure 1. Effect of HS1 on transgene expression. (Ai) Schematic representation of vector pairs used to evaluate the effect of 5’HS1 element on transgene expression. The human β-globin transgene (gene, promoter and 3’enhancer) is represented in red. The locus control region (LCR) elements HS2 and HS3 are indicated in green and HS4 in blue. The orange box indicates the HS1 element. The triangle above the 3’LTR indicates deletion in the U3 region making it a self-inactivating (SIN) vector. Numbers between vector pairs indicate the size of promoter utilized in each vector. The letters “S” and “T” in vector names indicates 265bp and 615bp promoters, respectively. Number “9” in the vector name indicates LCR2-3-4, while “10” stands for LCR1-2-3-4. Through the course of the study, none of the vectors expressed transgene in mouse lymphoma (EL4) cells or undifferentiated MEL cells (data not shown) confirming tissue and differentiation stage specificity of the vectors. (Aii) Quantification of vector expression in independent MEL cell pools. Expression at the RNA level (Huβ / (Huβ+Muβ)) is normalized to vector copy number (VCN). p values were calculated using Student’s t-test. n values indicate the number of independent MEL cell pools. (B) Long term stability of vector copy number in vivo assayed by TaqMan analysis. Three sample mice for each vector are shown. (C) Human β-globin transgene mRNA expression in peripheral blood (PB) shown as fraction of total β-globin mRNA and normalized to vector copy [(Huβ / (Huβ+Muβ)) / VCN]. For each vector, bars indicate time point during the experiment – in order from left: Week 6, 12, 17, 23, 29, and 37. The number of mice ranged between 8 and 19 per group (total: 73 mice). p values were calculated using Student’s t-test. (D) Cellulose acetate gel electrophoresis shows chimeric Hb (cHb, mα2:hβ2) levels in vector-transduced bone marrow chimeras. Data shown for three representative animals from each group at week 23 post transplantation. Vertical lines have been inserted to indicate repositioned gel lanes. Results were very similar at different time points. Control lanes contain normal C57BL/6 (B6) or Hbb-Thβ/ (Th) blood samples. The fraction of chimeric Hb (%cHb) relative to total hemoglobin (cHb+cHb + mHb) and vector copy number (VCN) are indicated below each sample.

Figure 2. Evaluation of the effect of HS4 on transgene expression. (Ai) Vector constructs used to study the 5’flanking region of LCR HS4. The dark blue box indicates the core of 5’HS4. The 5’flanking region was truncated (dotted box in T12), replaced with spacer DNA of same size (brown box in T12Jseq), a fragment of HS3 flanking region of same size (green box in T12HS3), or the human IFN-β S/MAR element (light green box) in reverse (T12SAR1) or forward (T12SAR2) orientation. Throughout the course of the experiment, all vectors were tested for stability by Southern blot. The only vector found to undergo rearrangements was the T12HS3 vector (data not shown). Only three MEL pools showed no signs of rearrangements and were used in the experiment. In MEL cell experiments, hPGK-DHFR (PD) cassette was inserted between LCR HS4 and 3’LTR. (Aii) Quantification of β-globin mRNA expression (Huβ / (Huβ+Muβ)) normalized to vector copy number (VCN) in independent MEL cell pools. n values indicate the number of independent cell pools. p values were calculated using Student’s t-
test. \( p^* < 0.001; \ p^{**} = 0.716 \). (B) Long term stability of vector copy number \textit{in vivo} assayed by TaqMan analysis. Three sample mice for each group are shown. (C) Human \( \beta \)-globin transgene mRNA expression in peripheral blood (PB) shown as fraction of total \( \beta \)-globin mRNA and normalized to vector copy \([(\text{Hu} \beta) / (\text{Hu} \beta + \text{Mu} \beta) / \text{VCN}] \). For each vector, bars indicate time point during the experiment – in order from left: Week 6, 12, 17, 23, 29, and 37. The number of mice ranged between 8 and 19 per group (total: 37 mice). (D) Total Hb level [g/dL] in peripheral blood (PB) of chimeric mice. Representative data for week 23 is shown. Red line indicates the level of Hb in Th\(^{3/+}\) mice. \( n \) indicates number of animals in each group. (E) Correlation between delta(\( \Delta \))Hb and provector copy number. \( \Delta \)Hb level was obtained by subtracting Th\(^{3/+}\) Hb ((D) Th\(^{3/+}\) = 8.9g/dL)) from total Hb level for each animal and corroborated by acetate gel electrophoresis (data not shown). Each square represents a single animal. The larger dots represent the average for each group, plus or minus s.d. Data collected 23 weeks post bone marrow transplantation. See inset for color-coding of each vector.
Figure 2

A1

- Diagram showing genetic expression levels for different genes.

B

- Graph showing VCN over time with different lines for T9 and T12.

C

- Bar graph showing (mRNA (Mol) / VCN) with error bars.

D

- Bar graph showing total mRNA levels with a red line indicating a significant difference.

E

- Scatter plot showing VCN on the x-axis and ΔMb on the y-axis for different samples.
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